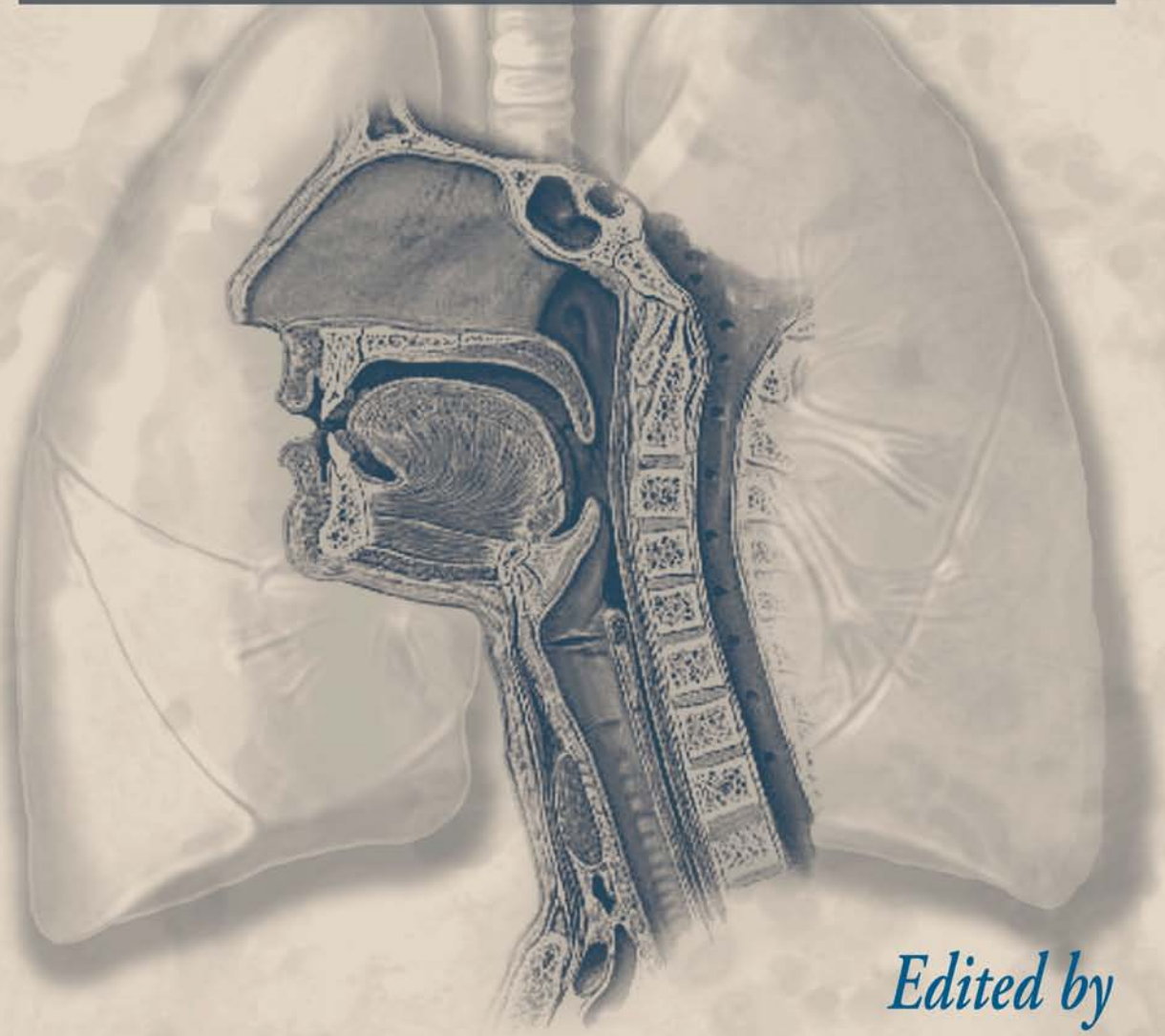
Inhalation Toxicology

Second Edition



Edited by

Harry Salem
Sidney A. Katz

CRC Taylor & Francis
Taylor & Francis Group

Inhalation Toxicology

Second Edition

Inhalation Toxicology

Second Edition

Edited by

Harry Salem
Sidney A. Katz

Taylor & Francis
Taylor & Francis Group

Boca Raton London New York

A CRC title, part of the Taylor & Francis imprint, a member of the Taylor & Francis Group, the academic division of T&F Informa plc.

Back cover illustrations are (top) Figure 10.1 on page 218 and (middle and bottom) Figure 27.2 on page 649.

Published in 2006 by
CRC Press
Taylor & Francis Group
6000 Broken Sound Parkway NW, Suite 300
Boca Raton, FL 33487-2742

Printed in the United States of America on acid-free paper
10 9 8 7 6 5 4 3 2 1

International Standard Book Number-10: 0-8493-4049-7 (Hardcover)
International Standard Book Number-13: 978-0-8493-4049-9 (Hardcover)
Library of Congress Card Number 2005051482

Library of Congress Cataloging-in-Publication Data

Inhalation toxicology / edited by Harry Salem, Sidney A. Katz--2nd ed.
p. cm.
Includes bibliographical references and index.
ISBN 0-8493-4049-7
1. Gases, Asphyxiating and poisonous--Toxicity testing. 2. Gases, Asphyxiating and poisonous--Physiological effect. I. Salem, Harry, 1929- II. Katz, Sidney A., 1935-

RA1245.I54 2005
615.9'1--dc22 2005051482

informa
Taylor & Francis Group
is the Academic Division of Informa plc.

Visit the Taylor & Francis Web site at
http://www.taylorandfrancis.com

and the CRC Press Web site at
http://www.crcpress.com

This book is dedicated to our colleagues whose contributions provided us with the material for *Inhalation Toxicology* as well as to our families, Flo, Jerry and Amy, Joel, Marshall and Abby Rose, and Sheila, Craig and Ji, Kevin and Hana, Jeff and Wendy and Little Syd and Daniel, whose patience, support, and encouragement enabled us to bring together the many aspects of *Inhalation Toxicology*.

Preface

The human body is subjected to numerous chemical exposures from the external environment. The atmosphere is the largest component of this environment, and the lungs are the largest surface exposed to this environment. Protecting the lungs from exposures to hazardous chemicals in the environment is often more difficult than protecting the human body from ingestion and dermal absorption hazards. Hazardous chemicals enter the body through the lungs in both the occupational and domestic environments. Among the substances frequently inhaled with air are an array of gases, aerosols, and particulates from natural and anthropogenic sources. In addition, some humans occasionally inhale therapeutic and/or abused drugs.

The objective of *Inhalation Toxicology* is to provide the practicing professional as well as the aspiring student with a pragmatic textbook. Included in *Inhalation Toxicology* are contributions from scientists in the academic, commercial/industrial, and governmental sectors focusing on regulatory aspects of exposure and testing, testing equipment and procedures, biomarkers and pathology of exposure, respiratory allergy and irritation of the respiratory tract, risk assessment, bioaerosols ranging from household molds to anthrax and botulinum toxin, low-level exposures, toxicology theory and toxicology modeling, and toxic effects of some individual toxicants ranging from tobacco smoke to botulinum toxins.

Inhalation Toxicology recognizes that the 100-m^2 surface of the lungs coupled with the 15-l/min respiration rate provide significant opportunity for the entry of toxic and therapeutic chemicals into the human body. Transfer across the alveolar–gas interface is rapid and often enhanced by large partition coefficients. Toxicokinetic models have been developed for the absorption, distribution, metabolism, and elimination of inhaled chemicals. Only recently has this approach been extended to biological materials such as molds and fungi. Proteomics and genomics are also among the more recent approaches included in *Inhalation Toxicology*.

Inhalation Toxicology includes chapters on updated testing procedures and testing equipment. Some recent changes in the European and North American guidelines for testing procedures receive special attention in *Inhalation Toxicology*.

Acknowledgments

The assistance of the U.S. Army Edgewood Chemical Biological Center (ECBC) Library Staff—Patsy d'Eramo, Edward Gier, Corky Smith, and Carolyn Sullivan—is greatly appreciated, as are the contributions of Richard Gilman, Judith Hermann, Mary Frances Tracey, and Susan Briggs of the Chemical and Biological Information Analysis Center (CBIAC). In addition, the assistance of Teresa Rudd is gratefully acknowledged.

About the Editors

Harry Salem is chief scientist at the U.S. Army Edgewood Chemical Biological Center. He has been a visiting professor at Drexel University, Rutgers University, Temple University, and the University of Pennsylvania, and he has held a variety of positions in the pharmaceutical industry and in commercial toxicology laboratories. He is the editor in chief of the *Journal of Applied Toxicology*. He was the 2001 Society of Toxicology's Congressional Fellow and is also a Fellow of the New York Academy of Sciences, the American College of Clinical Pharmacology, the American College of Toxicology, and the Academy of Toxicological Sciences.

Sidney A. Katz is professor of chemistry at Rutgers University. He has been a visiting professor in Canada, England, Germany, Hungary, Slovenia, and South Africa. He has held research positions with E.I. duPont de Nemours and Company, Inc., and with the R.M. Hollingshead Corporation, and he has been a research fellow at the U.S. Army Edgewood Chemical Biological Center. His research activity is in environmental bioanalytical chemistry. From 1997 to 2002, he was a New Jersey commissioner for hazardous waste facilities siting.

Contributors

Michael Adler
U.S. Army Research
Institute for Chemical Defense
Aberdeen Proving Ground, Maryland

Jamie B. Anderson
U.S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland

J. Steven Anthony
U.S. Army Edgewood Chemical and
Biological Center
Aberdeen Proving Ground, Maryland

Bryan Ballantyne
Consultant
Charleston, West Virginia

Bernard J. Benton
U.S. Army Edgewood Chemical and
Biological Center
Aberdeen Proving Ground, Maryland

David M. Bernstein
Toxicology Consultant
Geneva, Switzerland

Malcolm Blackwell
Sequani Limited
Ledburt, Hereford, United Kingdom

Joseph F. Borzelleca
Medical College of Virginia
Richmond, Virginia

William J. Brock
Brock Scientific Consulting
Montgomery Village, Maryland

R.F.R. Brown
Defense Science and Technology Laboratory
Porton Down, Surrey, United Kingdom

Edward J. Calabrese
University of Massachusetts
Amherst, Massachusetts

Finis Cavender
Center of Toxicology and
Environment Health
Hendersonville, North Carolina

Yong Joo Chung
University of North Carolina
Chapel Hill, North Carolina

Christopher R.E. Coggins
Carson Watts Consulting
King, North Carolina

Nancy D. Connell
University of Medicine and Dentistry of
New Jersey
Newark, New Jersey

Ronald B. Crosier
U.S. Army Edgewood Chemical
Biological Center
Aberdeen Proving Ground, Maryland

Charles L. Crouse
Geo-Centers
Abingdon, Maryland

Paul A. Dabisch
U.S. Army Edgewood Chemical
and Biological Center
Aberdeen Proving Ground, Maryland

Pamala Dalton
Monell Chemical Senses Center
Philadelphia, Pennsylvania

Darol E. Dodd
Alion Science and Technology
Fairborn, Ohio

Michael D. Dunkel
OptiMetrics
Bel Air, Maryland

Nabil M. Elsayed
Hurley Consulting Associates
Chatham, New Jersey

Gregory L. Finch
Pfizer Global Research and Development
Groton, Connecticut

Gary L. Foureman
U.S. Environmental Protection Agency
Research Triangle Park, North Carolina

Shayne C. Gad
Gad Consulting Services
Cray, North Carolina

Hans-Juergen Haussmann
Philip Morris Research Laboratories
Cologne, Germany

François Huaux
Université Catholique de Louvain
Brussels, Belgium

Ann F. Hubbs
National Institute for Occupational Safety and Health
Morgantown, West Virginia

Vera W. Hudson
National Library of Medicine
Bethesda, Maryland

Stanley W. Hulet
U.S. Army Edgewood Chemical and Biological Center
Aberdeen Proving Ground, Maryland

Matthew I. Hutton
OptiMetrics
Bel Air, Maryland

Mario A. Inchiosa
New York Medical College
Valhalla, New York

Rudolph J. Jaeger
C H Technologies, Inc.
Westwood, New Jersey

Edward M. Jakubowski
U.S. Army Edgewood Chemical and Biological Center
Aberdeen Proving Ground, Maryland

J. Jenner
Defense Science and Technology Laboratory
Porton Down, Surrey, United Kingdom

B.J.A. Jugg
Defense Science and Technology Laboratory
Porton Down, Surrey, United Kingdom

Sidney A. Katz
Rutgers University
Camden, New Jersey

Michael O. Kierzewski
U.S. Army Edgewood Chemical and Biological Center
Aberdeen Proving Ground, Maryland

Kannan Krishnan
Université de Montréal
Montréal, Quebec, Canada

Erica D. Kuligowski
National Institutes of Standards and Technology
Gaithersburg, Maryland

Samantha R. Lane
University of Cardiff
Cardiff, Wales, United Kingdom

Barbara C. Levin
National Institutes of Standards and Technology
Gaithersburg, Maryland

Jessica M. Mann
University of Medicine and Dentistry of New Jersey
Newark, New Jersey

Kathy L. Matson
Geo-Centers
Abingdon, Maryland

James J. McGrath
McGrath Associates
Chapel Hill, North Carolina

Robert J. Mioduszewski
U.S. Army Edgewood Chemical and Biological Center
Aberdeen Proving Ground, Maryland

John B. Morris
University of Connecticut
Storrs, Connecticut

Paul J. Nicholls
University of Cardiff
Cardiff, Wales, United Kingdom

Andy Nong
Université de Montréal
Montréal, Quebec, Canada

Kevin P. O'Connell
U.S. Army Edgewood Chemical Biological Center
Aberdeen Proving Ground, Maryland

Stanley T. Omaye
University of Nevada
Reno, Nevada

Richard Opiekum
Monell Chemical Senses Center
Philadelphia, Pennsylvania

Brian M. Paddle
Platform Sciences Laboratory
Fisherman's Bend, Victoria, Australia

Kyong H. Park
U.S. Army Edgewood Chemical and Biological Center
Aberdeen Proving Ground, Maryland

Nirmala A. Pinto
OptiMetrics
Bel Air, Maryland

Raymond Poon
Health Canada
Ottawa, Ontario, Canada

Dale W. Porter
National Institute for Occupational Safety and Health
Morgantown, West Virginia

George M. Rusch
Honeywell International, Inc.
Morristown, New Jersey

Harry Salem
U.S. Army Edgewood Chemical and Biological Center
Aberdeen Proving Ground, Maryland

Walter K. Schlage
Philip Morris Research Laboratories
Cologne, Germany

Raymond R. Schleef
Philip Morris Research Laboratories
Cologne, Germany

Alfred M. Sciuto
U.S. Army Medical Research Institute for Chemical Defense
Aberdeen Proving Ground, Maryland

MaryJane K. Selgrade
U.S. Environmental Protection Agency
Research Triangle Park, North Carolina

Janet Weeks Sekowski
U.S. Army Edgewood Chemical and Biological Center
Aberdeen Proving Ground, Maryland

Robert D.E. Sewell
Cardiff, Wales, United Kingdom

Susan G. Shami
Environmental Medicine, Inc.
Davis, California

Robert Snyder
Rutgers University
Piscataway, New Jersey

Douglas R Sommerville
U.S. Army Edgewood Chemical and Biological Center
Aberdeen Proving Ground, Maryland

Sandra A. Thomson
U.S. Army Edgewood Chemical and Biological Center
Aberdeen Proving Ground, Maryland

Sher-Janel T. Todd
University of Nevada
Reno, Nevada

Liana Tsenova
Public Health Research Institute
Newark, New Jersey

John J. Vandenberg
U.S. Environmental Protection Agency
Research Triangle Park, North Carolina

Marcel Van Raaij
Rutgers University
Piscataway, New Jersey

Patrick M. Vanscheevwijck
Philip Morris Research Laboratories
Lueven, Belgium

Robert W. Wannemacher
U.S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland

Marsha D.W. Ward
U.S. Environmental Protection Agency
Research Triangle Park, North Carolina

Elizabeth K. Weisburger
National Library of Medicine
Bethesda, Maryland

Christopher E. Whalley
U.S. Army Edgewood Chemical and
Biological Center
Aberdeen Proving Ground, Maryland

John E. Whalan
U.S. Environmental Protection Agency
Washington, DC

Contents

Part I

Inhalation Toxicology: Methods and Measurements

1 Inhalation Risk Assessment at the Environmental Protection Agency[1]

John E. Whalan, Gary L. Foureman, and John J. Vandenberg

CONTENTS

[1] The views expressed in this chapter are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency.

1.1 RISK ASSESSMENT PRINCIPLES AND PRACTICES

1.1.1 Introduction

The potential adverse health effects of human exposures to environmental pollutants are characterized through a process called health risk assessment. The National Research Council, in *Risk Assessment in the Federal Government: Managing the Process*, defined risk assessment as "the use of the factual base to define the health effects of exposure of individuals or populations to hazardous materials and situations" (NRC, 1983). Risk assessment is based on a conceptual framework wherein research, risk assessment, and risk management are linked (Figure 1.1). The purpose of this chapter is to describe the use of risk assessment in environmental protection programs and the data, methods, models, and guidelines used in health risk assessment for inhalation end points.

"Risk assessment" is a term applied both to the *process* of risk assessment and to the *product* of an assessment. In this chapter the emphasis will be on the process of risk assessment. It is not unusual, however, to encounter reference to a risk assessment of a particular chemical or site or emissions stream.

The risk assessment process is used in several ways by regulatory agencies such as the U.S. Environmental Protection Agency (EPA, the Agency) (U.S. EPA, 1988a, 1990a, 1990b; Sexton, 1992; Loehr et al., 1992; Browner, 1995; NRC, 1996; U.S. EPA, 2004a). First, risk assessment provides a framework to permit data utilization. As will be demonstrated below, risk assessment is by its nature an interdisciplinary effort, thus the coherent and consistent organization of diverse information is an essential feature of the process. Second, risk assessment provides information useful for establishing regulatory and research priorities. In the regulatory arena, risk information can help determine which types of pollutants, emissions, or exposures are of greater or lesser concern, thereby focusing scarce resources on the highest risks. Establishing priorities for research can result from organizing existing information and identifying gaps in methods and data that affect confidence in assessment results. Research can be targeted to address the areas of greatest uncertainty. Finally, risk information is used in establishing standards of human exposures to environmental pollutants. Not surprisingly, this use of risk assessment information is more controversial because of the economic and societal consequences frequently associated with such decisions and the inherent uncertainties in risk assessments. Some examples of approaches to risk evaluation and regulatory authority are shown in Table 1.1 (see also U.S. EPA, 2004a).

The process and products of risk assessment are of interest to many groups. Within EPA, risk information is used by the major regulatory programs, including the air, water, pesticide, toxic substances, hazardous waste, and Superfund programs. External stakeholders in risk assessment include industry, Congress, environmental groups, academia, the legal community, and other governmental as well as private research organizations.

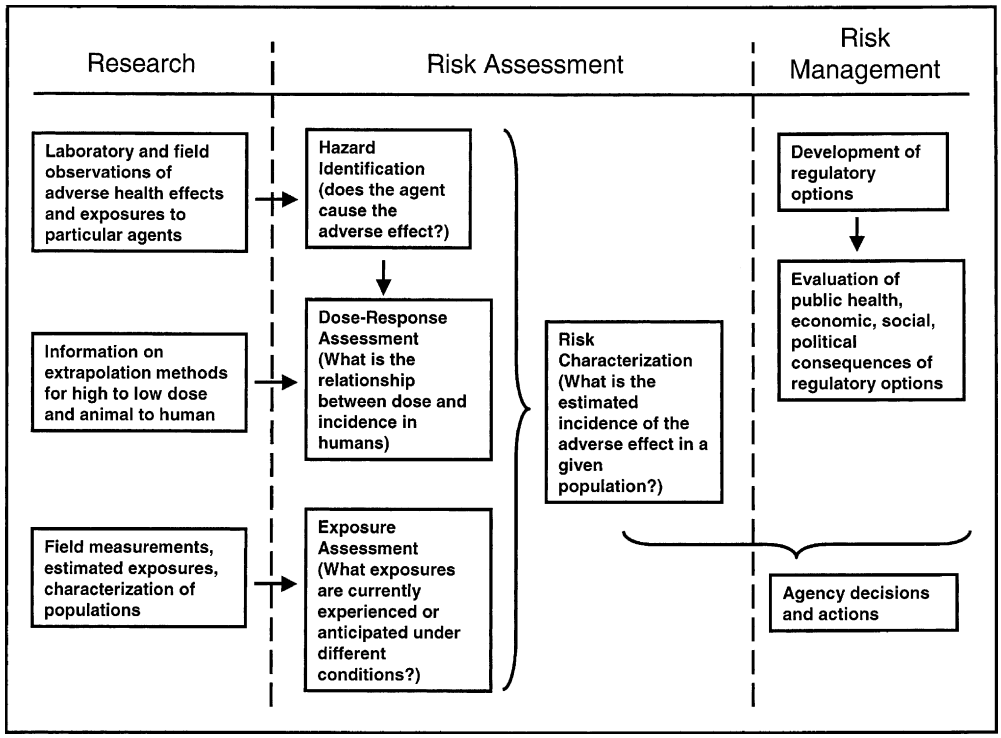


FIGURE 1.1 Elements of risk assessment and risk management (from NRC, 1983).

TABLE 1.1 Selected Examples of Environmental Health Risk-Related Provisions in Federal Laws

Statute	Regulatory Scope	Risk Provisions
Clean Air Act	National ambient air quality standards for criteria pollutants	Set ambient air standards to protect public health with an adequate margin of safety
	Hazardous air pollutants	Protect public health with an ample margin of safety through technology-based maximum achievable control technology followed by residual health risk-based emissions standards
Federal Insecticide, Fungicide, and Rodenticide Act	Pesticides	Protect from unreasonable risks to health and the environment balancing risks against economic benefits to pesticide users and society
Toxic Substances Control Act	Existing chemicals in commerce	Protect from unreasonable risks to health and the environment balancing risks against economic benefits, considering alternative technologies
Safe Drinking Water Act	Drinking water	Protect the public from known or anticipated effects on human health by setting a maximum contaminant level goal with an adequate margin of safety and defining a maximum contaminant level as close as feasible to the goal

The source of information used in risk assessment is similarly diverse, with information coming from universities, government laboratories (e.g., EPA, the National Institute of Environmental Health Sciences, the National Laboratories of the Department of Energy), industry-supported laboratories (e.g., the CIIT Centers for Health Research, Research Triangle Park, NC) regulated industry (e.g., pesticide and chemical manufacturers), consulting companies, environmental and public health organizations (e.g., the American Lung Association), and trade organizations (e.g., Formaldehyde Council).

1.1.2 Environmental Toxicology

The fundamental question addressed by environmental toxicology is whether and how pollutant exposures result in human disease. A series of toxicological events link exposure and disease (Figure 1.2). These events include pollutant uptake, distribution to target tissues, molecular interactions, cellular responses, and altered tissue function that may ultimately progress to disease if repair and adaptation mechanisms are insufficient.

This event series can be separated into pharmacokinetics, which conceptually is the effect of the body on the pollutant, and pharmacodynamics, which is the effect of the pollutant on the body. Pharmacokinetics focuses on the disposition of the pollutant after an exposure. Pharmacokinetic information includes data on the magnitude and time course of absorption and distribution of a pollutant to various body tissues (e.g., to fat, reproductive organs, liver), metabolism to more or less toxic components, and rates of elimination. Such data can be organized in mathematical models to quantitatively estimate the magnitude, duration, and type of exposure to individual target tissues. The pharmacodynamics or toxic effects of exposure of individual target tissues to pollutants and their metabolites depends on many factors, including the capacity of the tissues to be repaired and to maintain normal tissue function through homeostasis. If protective mechanisms are insufficient, toxicity may ensue and lead to disease, manifest as an increase in morbidity or mortality.

To assess risks associated with environmental pollutants, one would ideally like to have information on impacts measured directly in humans exposed at environmental levels. For most environmental pollutants, substantial gaps exist in the available toxicological data. The series of events linking exposure to disease is rarely well characterized. More frequently, the limited evidence shows that high-level exposures can produce an increase in the incidence of morbidity or mortality in laboratory animals. The challenge to regulatory agencies is to determine what actions, if any, are required or warranted to protect public health when toxicological and exposure information for a chemical or source are uncertain or incomplete. To help address this situation, the U.S. EPA and other organizations have developed consistent procedures and guidelines for chemical testing and risk assessment.

1.1.3 Risk Assessment Paradigm

The four key components of risk assessment are hazard identification, dose–response assessment, exposure assessment, and risk characterization (NRC, 1983). **Hazard characterization** is the determination of whether a particular chemical is or is not causally linked to particular health effects by a particular route of exposure. This determination involves evaluation of epidemiological data, animal bioassay data from chronic exposures, short-term studies, and comparisons of molecular structures. The process to determine the validity and significance of toxicity data and the conclusions to be drawn regarding the weight of the evidence that a substance is causally linked to toxic effects are determined by using published risk assessment guidelines (see U.S. EPA, 1986, 1991, 1992). For most chemicals the only toxicity data available are from laboratory studies on animals. The use of these data is based on a long-standing assumption that effects in humans can be inferred from effects in animals.

Dose–response assessment is the determination of the relationship between the magnitude of exposure and the probability of occurrence of the health effects in question (NRC, 1983) (Figure 1.3). Dose–response[2] assessment entails several steps, including identification and selection

[2] The term "dose-response" is commonly used as described subsequently in this chapter. The term "dose" refers to the internal or target-tissue dose whereas "exposure" refers to the external conditions that result in the (internal) dose.

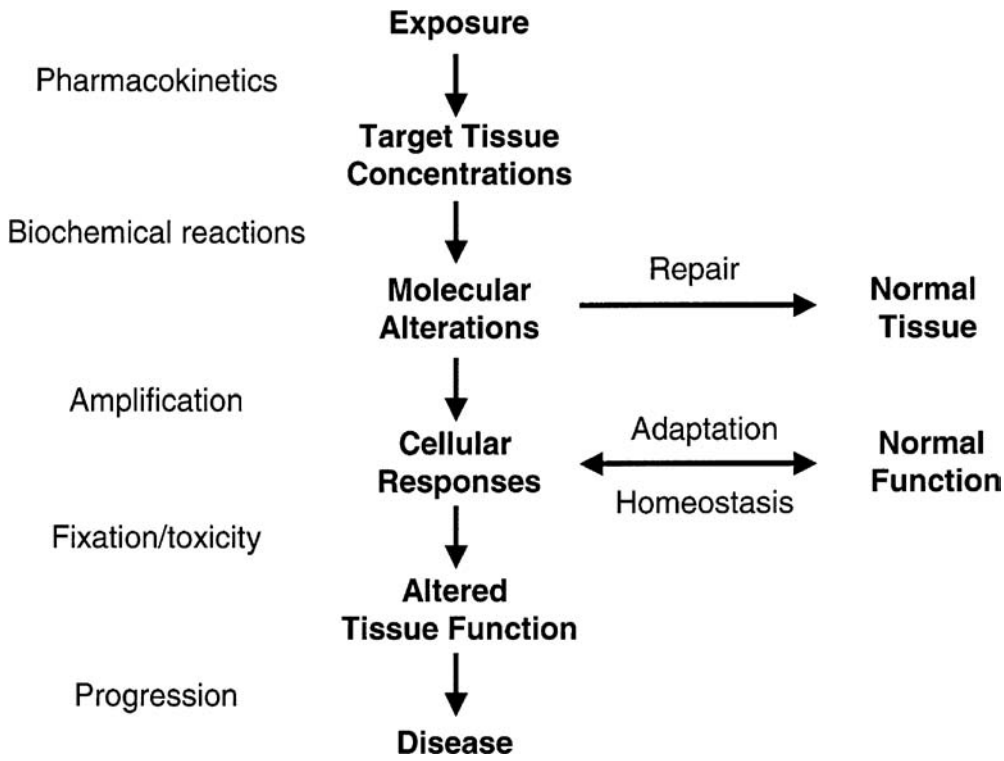


FIGURE 1.2 Toxicological events linking exposure to disease.

of appropriate data, determination of equivalent exposure units between species, and choice of an extrapolation model. The risk assessment guidelines describe these procedures. A key consideration in dose–response assessment, as well as hazard identification, is that extrapolation is needed from the observable range to typically much lower environmental levels. Fundamental differences in the assumptions and dose–response methods used for cancer and noncancer end points are described below.

Exposure assessment is the determination of the extent of human exposure before or after application of controls (NRC, 1983). Quantitative determination of the magnitude, frequency, duration, and timing of human exposure to pollutants involves evaluation of chemical and biological monitoring and modeling.

Questions central to exposure assessment include:

- Where is the substance found?
- Who and how many people are exposed?
- Are there highly exposed subgroups?
- What are the routes (e.g., inhalation, drinking water) of exposure?
- What is the degree of absorption by various routes of exposure?

Possible indicators of exposure potential range from crude estimates of production volume to biomarkers of exposure obtained from human tissues.

Risk characterization is the description of the nature and often the magnitude of human risk, including attendant uncertainty (NRC, 1983, 1996). Risk estimates may include statements regarding the estimated number of cancer cases or noncancer health effects that could result from pollutant exposures, the risks to highly exposed subgroups, and the distribution of risks in the population. Results are accompanied by statements regarding the data and methods employed and

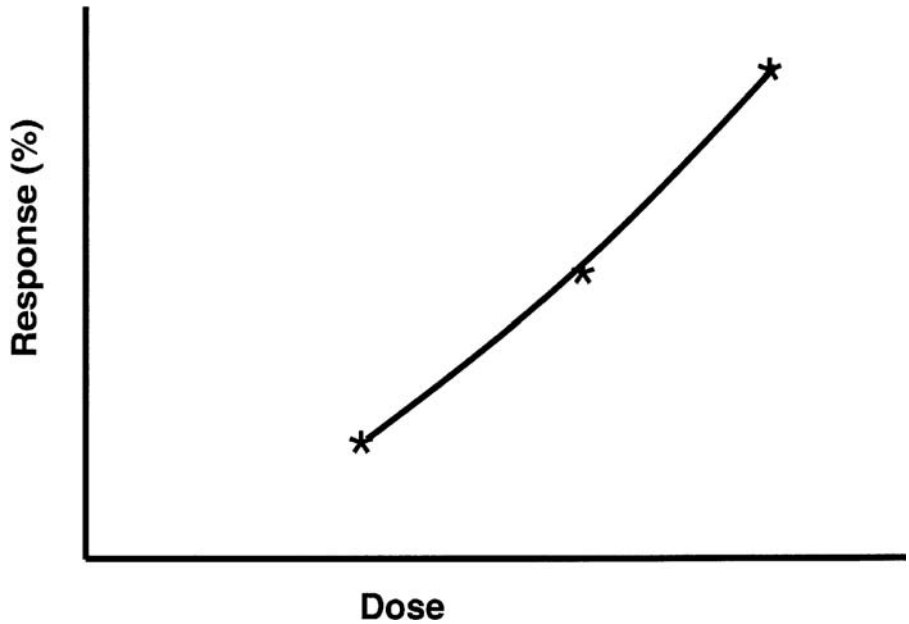


FIGURE 1.3 Dose–response assessment characterizes the quantitative relationship between magnitude of response and the dose inducing the response.

the assumptions and uncertainties affecting the degree of confidence associated with the assessment process and results. The EPA has developed policy and guidance on qualitative and quantitative approaches and considerations for effectively communicating risk information to the public and others (U.S. EPA, 1995a, 1995b).

1.1.4 Extrapolations, Science Policy, and Risk Assessment Guidelines

Because of knowledge gaps, extrapolations and science policy are an integral part of the risk assessment process. This is true for many agents to which humans are exposed via inhalation, such as the hazardous air pollutants. A notable exception, however, is for the so-called "criteria" air pollutants. For these ubiquitous air pollutants, which include airborne particulate matter, tropospheric ozone, carbon monoxide, sulfur dioxide, nitrogen dioxide, and lead, extensive data from human exposures (including, in some cases, controlled human exposures) are available; hence the risk assessment process is different for these pollutants than what is described below (see U.S. EPA, 2004b).

Extrapolation methods and science policy positions developed by regulatory agencies are sources of considerable difference in opinion, and, therefore, controversy, and represent areas of significant research interest.

The extrapolations frequently incorporated into risk assessments include:

- Extrapolating from high-dose experimental levels to much lower environmental levels
- Extrapolating from laboratory species to humans
- Extrapolating from healthy human volunteers to the diverse human population
- Extrapolating from one route of exposure to another (e.g., air to water)
- Extrapolating across widely varying exposure conditions (e.g., duration)

In Agency noncancer risk assessment procedures (following), these extrapolations are viewed and treated as areas of uncertainty and are addressed by the application of uncertainty factors or UFs.

Science policy positions regarding these and other extrapolations are described in the EPA risk assessment guideline series (U.S. EPA, 1986, 1991, 1992). These guidelines present a number of default assumptions that are used, after thorough consideration of available information, in the absence of information to the contrary. Thus, research results can provide information to be used in lieu of the default assumptions, although specific criteria need to be clarified regarding when such information is sufficient to replace the defaults (NRC, 1994).

1.1.4.1 Cancer

Examples of default risk assessment positions for cancer are listed below. These positions address the hazard identification, dose–response assessment, and exposure assessment components of the risk assessment paradigm, and the extrapolations noted previously. Used in the absence of information to the contrary, they include:

- In general, a combination of benign and malignant tumors.
- Use of responses from the most sensitive species and from the most sensitive tissues.
- Scale dose across species based on body weight (raised to the 0.75 power).
- Use of the multistage model to identify a point of departure (POD), then use a linear extrapolation from the POD through the origin (hence, no dose threshold below which the probability of a response is zero).
- Estimation of exposure as an average daily exposure prorated over a nominal 2-year life span for rodents and a 70-year life span for humans.
- Assumption that exposure to multiple carcinogens results in an additive response.

A hypothetical dose–response assessment for cancer is represented in Figure 1.4. The 95% lower confidence interval on dose is used to identify the POD at a nominal 1, 5, or 10% response level, depending on the data being modeled. The cancer slope factor used in most risk assessments is the slope of the line from the POD through the origin.[3] Multiplying the cancer slope factor by estimates of exposure results in a risk estimate for essentially any level of environmental exposure.

When using default cancer risk assessment procedures, note that there is no nonzero dose level below which the risk of cancer is zero. This is based on a no-threshold assumption. Although the mechanism of cancer induction is not known with certainty, it is hypothesized that it may be caused by pollutant-induced alterations in genetic material leading to uncontrolled cell division and tumor formation. This hypothesis provides one basis for the no-threshold default assumption.

Also note that there is no adjustment or consideration of variability in potential response across the human population. The no-threshold assumption and consideration of intrahuman variability are significant points of distinction between cancer and noncancer risk assessment procedures.

The EPA is currently engaged in revising the cancer risk assessment guidelines, thus some of the default assumptions and risk assessment procedures may change soon.

1.1.4.2 Noncancer

For noncancer risk assessment a central assumption is that a range of exposures from zero to some finite level can be tolerated with essentially no effect, that is, a threshold assumption. The assumption of a threshold for noncancer end points is based on evidence that homeostatic, compensating, and adaptive mechanisms exist that must be overcome before toxicity is manifested (Barnes and Dourson, 1988). The concept of threshold is important in that risk assessors are essentially striving to determine an appropriate threshold dose in humans. Because the heterogeneous human population is highly variable in preexisting conditions and in response to exposure to toxic agents, the identification of a threshold at the population level is arguably not possible.

[3] Some risk assessments based on human epidemiological data use the maximum likelihood estimate rather than the lower confidence limit on dose to derive the point of departure.

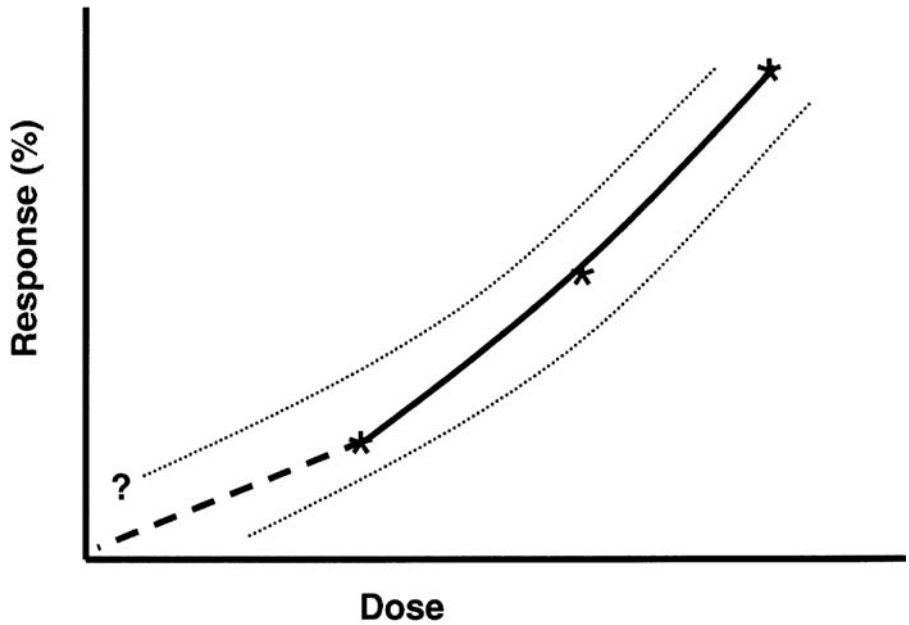


FIGURE 1.4 Hypothetical dose–response curve for a carcinogen. Dotted line is modeled 95% confidence interval.

Nonetheless, a threshold assumption is generally considered to be reasonable for noncancer risk assessment. A typical noncancer dose–response assessment assuming a threshold response is represented in Figure 1.5.

End point-specific guidelines for the risk assessment of noncancer end points have been developed (U.S. EPA, 1986, 1991). These include guidelines for developmental toxicity, reproductive toxicity, and neurotoxicity (U.S. EPA, 1988b, 1988c), which are complemented by methods for estimating reference doses (Barnes and Dourson, 1988) and reference concentrations (Jarabek, 1994).

In the noncancer approach, qualitatively acceptable data are evaluated to determine which species and end point(s) show effects of concern at the lowest doses. For example, the highest dose noted without an observable biologically significant adverse effect is defined as the no observed adverse effect level (NOAEL). This dose level, or some other suitable POD, is then divided by a composite value of UFs applied to reflect the various areas of extrapolation explained above.

The number of UFs applied depends on the data available for an assessment. The individual uncertainty factors are multiplied to provide a combined factor. For example, it is commonplace to encounter a combined factor of 1000 when subchronic animal toxicity data are used (i.e., 10-fold factors each for extrapolations involving human variability, animal-to-human considerations, and duration [subchronic to chronic extrapolation]).

Dividing a POD by the combined UF results in a reference dose (RfD) when exposures are via oral or dermal absorption, and in a reference concentration (RfC) when exposure is via air. The RfD and RfC are further explained and defined in the next sections of this chapter.

Effectively, exposures to levels below the RfD or RfC are of relatively low concern. Concern increases as exposure levels exceed the RfD or RfC and approach the LOAEL, a level at which adverse effects have been observed. A hazard index, defined as the ambient exposure level divided by the RfD or RfC, and/or a margin of exposure (discussed below) also may be calculated to provide insights on how human exposures compare to reference levels to help inform decision making.

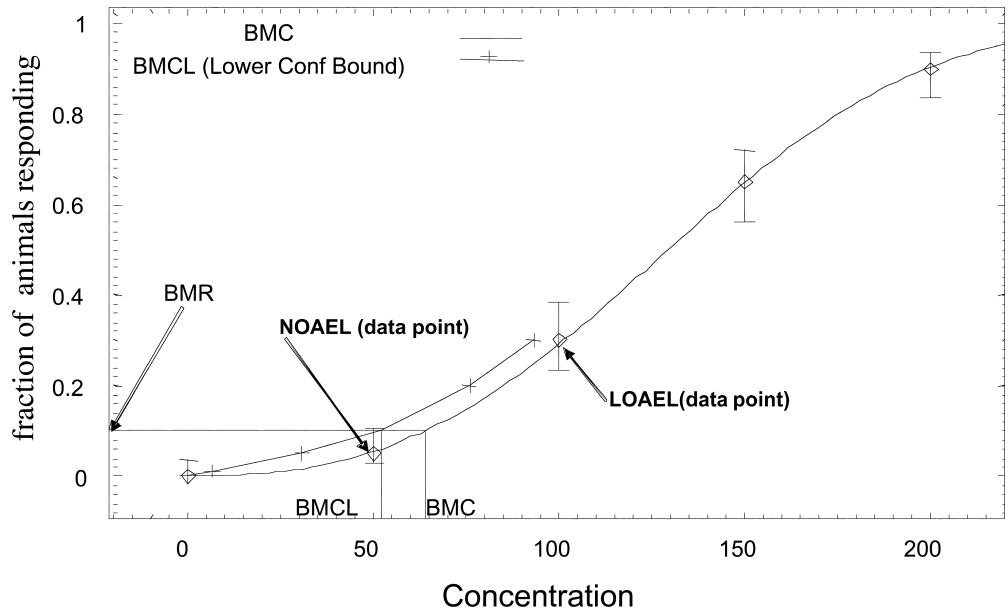


FIGURE 1.5 Calculation of a benchmark concentration (BMC) and the benchmark concentration lower boundlimit (BMCL). This example demonstrates the fitting of a mathematical model to estimate the exposure–response curve (i.e., the BMC) and the 95% confidence bounds (i.e., the BMCL) associated with the fit. This plot is then used to find the exposure corresponding to a designated response level, the benchmark response (BMR). In this case, a BMR of 0.1 (10%) is illustrated along with the concentrations corresponding to the BMC (~64) and the BMCL (~51). The NOAEL (no or low observed adverse effect level) of this data set are shown for comparison. When analyzing information from oral dosing, the terms BMD and BMDL are typically used. (Adapted from a figure provided by EPA colleague Jeffrey S. Gift on oral data.)

1.1.5 Research to Improve Health Risk Assessments

Current risk assessment procedures result from fundamental assumptions about how pollutants cause diseases in humans. These assumptions can be replaced with better scientific information as health effects research is conducted. Given the nature of many current procedures, research advances are anticipated to provide a stronger scientific basis for risk assessment in the future. Society benefits from development of a strong, credible science base for regulatory decisions, and the EPA has been strongly encouraged to advance the scientific basis for risk assessment (Loehr et al., 1992). To this end, EPA for a number of years has been developing new methods, models, and data to improve the scientific basis for risk assessment (U.S. EPA, 1990a). The EPA program has been characterized as a prototype for applied risk assessment research programs (U.S. Congress, Office of Technology Assessment, 1993). The development of physiologically based pharmacokinetic models and biologically based dose–response models represent important advances and opportunities for improving risk assessment procedures.

The preceding description of the concept, derivation, and use of inhalation risk assessment provides a backdrop for the balance of this chapter. What follows is an in-depth, practical description of the Agency processes for estimating health benchmarks from inhaled agents—those used by the Agency-wide Integrated Risk Information System (IRIS) program in derivation of the RfC, and those followed by the Office of Pesticide Programs in derivation of the margin of exposure (MOE) for inhaled pesticides.

1.2 U.S. EPA'S INHALATION REFERENCE CONCENTRATION—THE RfC

1.2.1 Some Background Information on the RfC and IRIS

Current approaches for noncancer dose–response assessment for inhalation exposure currently focus on chronic (lifetime) exposures, although approaches for other exposure durations, in particular, acute, are being actively pursued. In the Agency, this procedure is refined and described in the derivation of the human health inhalation guideline value, the inhalation RfC, as part of the Agency's IRIS database. The current RfC definition (U.S. EPA, IRIS Glossary) for this value is given in the accompanying text box. The specifics in the derivation of an RfC are described in the 1994 Agency publication, "Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry" (*the Methodology*) (U.S. EPA, 1994a), in Jarabek (1994) and Hanna et al. (2001), and in an accompanying publication on the expansion of the IRIS process (Mills and Foureman, 1998). Note that an RfC is expressed as an air concentration (in mg/m^3), which the human population breathes continuously and that no assumptions are made as to the intake of agent (as in milligrams per kilogram per day).

Reference Concentration (RfC)

An estimate of an inhalation exposure for a given duration to the human population (including susceptible subgroups) that is likely to be without an appreciable risk of adverse health effects over a lifetime. It is derived from a BMCL (a statistical lower confidence limit on the benchmark concentration), a NOAEL, a LOAEL, or another suitable point of departure, with uncertainty and variability factors applied to reflect limitations of the data used.

Before the expansion of the IRIS process (Mills and Foureman, 1998), an RfC was prepared individually with the oral RfD or cancer assessment for the given chemical done independently and often at different times. The RfC appeared on IRIS as a summary document with supporting information abstracted within the summary. RfCs currently appear as part of a complete IRIS assessment, including an oral and cancer assessment for the agent, all done simultaneously, and all supported by an accompanying formal technical document, the *Toxicological Review.* The procedures and applications described in this chapter are those that would be incorporated initially into a *Toxicological Review* for purposes of deriving an RfC. Under current IRIS operating procedures, an RfC *Summary* document would then be derived from the *Toxicological Review* document. Both now appear on IRIS.

1.2.2 The Fundamentals of the Process

The fundamental methodology for derivation of an RfC has been built on established practices of risk assessment used both by the risk assessment community, in general, and by the Agency, and is exemplified by the generalized equation:

$$\text{Health guideline value} = \text{point of departure} \div (\text{uncertainty or variability})$$

For the Agency, the health guideline value is the route-specific inhalation RfC, for which the unit is an air concentration in mg/m^3. The POD is the level of inhalation exposure, also in mg/m^3, associated with any of a variety of health effect end point values identified from toxicological studies in either animals or humans. More specifically, the POD is chosen from an ordered array of various levels of exposures[4] to the toxicological agent and the corresponding toxicological responses,

[4] Throughout this section the term "dose" refers to the internal or target-tissue dose. "Exposure" refers to the external conditions that result in the (internal) dose.

(i.e., from analysis of a dose–response relationship). For this reason, an RfC is more correctly termed an "exposure–response" assessment rather than a "risk" assessment, with the latter being more broadly defined (NRC, 1983).

This exposure–response array, and indeed the entire assessment, should always strive to be based on and reflect the events causing the toxicity (i.e., the underlying mode of action of the observed toxicity). Furthermore, the assessment needs to provide to the reader as clear an understanding of those mechanisms as the currently available data and information will reasonably support.

In deriving the RfC, the POD is refined, when possible, to more accurately reflect what humans would be exposed to under the stated definition of the RfC, such that it is termed a "human equivalent concentration" (HEC). Various procedures for this extrapolation to an HEC, discussed generally in this chapter, are given in detail in the *Methodology.* Application of these various procedures are guided based on the physical form of the agent (i.e., gas or particle) and on the site of the action by the agent (e.g., respiratory tract and systemic or "extrarespiratory" sites). Application of these procedures to a POD obtained from an animal exposure–response curve allows the POD to be termed a POD_{HEC}.

In the RfC derivation, uncertainty and variability factors (UFs) are typically used for purposes such as compensating for information that is not known or extrapolating for other unknowns such as animal-to-human (interspecies) considerations or for assumed levels of variability in the human population. The POD_{HEC} is divided by the numerical product of these factors such that the greater the value ascribed to uncertainty, the lower the health guideline value.

The above generalized equation may be written more specifically for the inhalation RfC:

$$RfC = POD_{HEC} \div UF$$

The purpose of the RfC is contained within the definition, as is the application, that is, "... to the human population (including sensitive/susceptible subgroups)". The RfC is also meant to consider critical life events. This aspect is reflected in the overall criteria used to evaluate the confidence in an RfC including toxicity information available on the biological milestones of development and reproduction. More recently, the Agency has indicated that this consideration should possibly be expanded to include more life stages, such as childhood and old age (EPA, 2002a).

This portion of the chapter elaborates on the components of the RfC equation and on the specifics of how the components are chosen, derived, and interpreted. This will be accomplished by following generally the outline of the formal technical support document, the *Toxicological Review,* relating to the inhalation RfC.

1.2.3 Understanding the RfC Equation: The POD—Identification from the Exposure–Response Curve

1.2.3.1 Analysis of Exposure–Response Information

An in-depth inspection of the world's literature for toxicological effects associated with the agent of interest has and should always remain fundamental to any assessment process and to identification of the POD for any given agent. To be of use and scientific merit, such inspections should be thorough; they should be conducted from a viewpoint of what is known about the nature of the toxicity and the reason for its manifestations at the most basic biological level, that is, from the viewpoint of the mode(s) of action of the observed toxicity. Availability of a full-scale chronic animal study for a given agent, conducted under stringent guidelines and showing a clear dose–response relationship, may become available and be seen as a reason to undertake a new assessment or to possibly revise an existing assessment. However, the use and applicability of such a thorough and extensive study may be altered or possibly negated by a less extensive mechanistic-type study demonstrating, for example, that the toxicology reported in the chronic study may be of only marginal relevance to humans.

Once the relevant toxicological information, including mechanistic, has been identified, further analysis for information on exposure–response should proceed. Although it is likely that both human and animal information may be identified in such a process and although human data clearly are from the most relevant species, no clear decision-type analysis can exist to always utilize human data over animal data in deriving the exposure–response relationship and POD. Animal toxicity studies of chronic duration and less are typically conducted under stringent and known conditions of exposure to an isolated and controlled population. On the other hand, occupational studies often report exposure in only the most general terms such as "low" or "high" that apply only to workers in uncontrolled settings. Other difficulties, such as exposure to other agents that cause the same end point (e.g., smoking and lung cancer or heart disease) often occur in these types of studies, making them problematic for establishing a clear unconfounded exposure–response. This above comparison is not intended to advocate excessive reliance on animal information and studies. Such studies are often conducted at extraordinarily high concentrations of a given agent, producing effects in exposed animals that may not even occur at concentrations of concern to the assessment, which are typically much lower. Rather, this narrative demonstrates some of the many pitfalls and difficulties that exist in analyzing scientific data, most of which were not generated for the purpose for which they were attempted to be utilized, that is, establishing a quantitative dose–response relationship for risk assessment. This comparison is also given to encourage the use of all information available to the full extent to which it is informative.

The outcome of this analysis for exposure–response information will vary widely. A complete array of exposure–response information would ideally be available. Such an array may include, for example, an animal inhalation study conducted under conditions of well-controlled chronic exposure showing a reasonably complete spectrum of response from low exposures, where no accompanying toxicity for a given end point is observed, to higher exposures, where more and more quantifiable toxicity occurs. And this study may be accompanied by informative studies in humans showing the same or similar target organ toxicity, studies reinforcing the presumed mode of action, and other animal or human studies that may support this exposure–response relationship. Alternatively, only a minimum of barely useful information may be sufficient, perhaps, to support only a qualitative description rather than a quantitative analysis of the dose–response relationship. The former, more ideal, situation will be assumed in describing the next step in the RfC process.

1.2.3.2 Additional and Supplemental Studies

In addition to studies that may be used to construct an array, other information may be available on the agent of interest that could aid in the interpretation of the array or in the ordering of the array with respect to the elements that should underlie its construction. A primary consideration for considering and deciding on such supplemental information is what they may provide in the way of information about key determinants of the toxicity, that is, mode of action.

IRIS assessments have specific information requirements. For example, all available toxicity information relating to toxicity of the agent in developmental and reproductive settings, and in children as well as gender-specific effects, are reported in designated sections of the *Toxicological Review.* Sections in this portion of the assessment may be expanded to accommodate any specific toxicity of the agent, such as neurotoxicity or immunotoxicity. These data may be used in several aspects of the assessment, including exposure–response, and in the assessment of uncertainty factors. Human information that may have insufficient exposure–response data to make it useful may also be placed here.

The information described in this section (along with basic chemical and physical information of the subject chemical) is summarized and appears in the front portion of the *Toxicological Review.*

1.2.4 Designation of a POD from an Exposure–Response Curve—Qualitative and Quantitative Analyses

1.2.4.1 The Qualitative Analysis

The end points and the exposure–response information on end points identified in the foregoing processes are considered in both a qualitative and quantitative analysis. The qualitative analysis is where these end points are considered from the perspective of what is known about the mode of action of the agent. Some of the end points identified may be of questionable toxicological significance, such as lung cancers in rats from overload exposure conditions at the affected site. The statistical versus biological significance of certain effects may also be discussed here. Evidence for the progression of effects from across several studies may be placed in perspective here. This analysis may also serve to refine or focus the quantitative analysis to follow. The sections in the *Toxicological Review* designated for this purpose are the "*Synthesis and Evaluation of Major Noncancer Effects*" and the "*Weight-of-Evidence Evaluation and Cancer Characterization.*"

1.2.4.2 The Quantitative Analysis

1.2.4.2.1 The NOAEL Approach

An historical view of the ideal dose–response curve is that it is typically sigmoidal. Most toxicity-testing strategies utilize this assumption in the planning of exposures to be tested—such that a series of relatively low exposures may produce little or no toxicological response, a higher exposure may produce a clear response, and yet higher exposures may produce still more of a toxicological response. In this ordered dose–response array, the lowest tested exposure producing a toxicological response that is considered adverse is termed the lowest observed adverse effect level or LOAEL. The next lower exposure from the LOAEL is termed the no observed adverse effect level or NOAEL.

The RfC methodology obligates the examination of all exposure–response arrays in a database and designation of all PODs, including NOAELs, for all toxicological end points that may exist. The NOAELs for these various end points may then be adjusted, both for purposes of the RfC as being continuous (i.e., 24 hr) and for "human equivalency" (described below), and compared to determine selection of the appropriate experimental NOAEL for the "critical" toxic end point, that is, that which occurs first as exposure concentration increases. This NOAEL for the critical toxic end point is designated as the POD.

As evident from this description, the assumption underlying the NOAEL approach and a sigmoidal exposure–response curve is the existence of a threshold. A threshold exposure would be the highest exposure below which an adverse effect is not expected (i.e., a NOAEL). With the assumption of the existence of a threshold, its estimation becomes the object of the exposure–response assessment. This focus on threshold has liabilities as the definition of threshold is complicated because it includes elements of the end use of the assessment (e.g., legislative mandates and implementation policy) as well as elements of data interpretation and science policy, such as questions of adversity, severity, individual versus population thresholds, and biological versus statistical significance. Also, as more and more exposure–response relationships become known and techniques to detect and assign adversity become more and more refined in toxicology, the very concept of a threshold in dose–response at low exposures becomes less and less robust.

1.2.4.2.2 The Benchmark Concentration Approach

The Agency has historically used the NOAEL approach. During this time, however, criticisms of aspects of the approach have accumulated. These include that its designation (i.e., as adverse) is subjective, that it is limited to a single data point of those tested (even though the entire exposure–response curve may be well elucidated), that a NOAEL may not be present in a study (i.e., the lowest dose tested exhibited an "adverse" effect), that it does not reflect the nature of the slope of the exposure–response curve, and that it does not reflect the size or power of the study. In fact, fewer animals in a

given experiment tend to result in higher and higher NOAELs. This is because differences that are statistically significant at a given dose on exposure level testing a large number of animals tend not to be significant with a smaller number of animals, causing this dose level not to be chosen over the next or even higher exposure level (Crump, 1984).

On the other hand, mathematical exposure–response modeling of data can be used to predict a response level that will then serve as the initial basis of the health assessment. Since Crump (1984) proposed the benchmark dose (BMD) method, a great deal of interest and an increasing level of activity have surrounded its use in dose–response analysis that has lead to the development of EPA BMD software (EPA, 2004c). For inhalation scenarios, this method is termed the benchmark concentration (BMC) approach. It is accomplished by fitting a mathematical model to a dose–exposure–response data set. Once this has been accomplished and a predetermined response (the benchmark response or BMR) within the observable range of existing data level chosen, the BMC can be determined by using the fitted curve to find the concentration corresponding to the BMR. Variability and uncertainty may be addressed in this approach by deriving the lower bound on the BMC (the BMCL) predicted by the model that corresponds to the defined BMR response, such that choice of a 10% BMR would result in a $BMCL_{10\%}$. After adjustments as per the NOAEL, BMCLs may be arrayed, analyzed, and designated as PODs. An illustration of the benchmark process and the associated terms is given in Figure 1.5.

Compared with the NOAEL approach, the BMC method utilizes more information from the exposure–response curve, is less influenced by experimental design (e.g., exposure concentration spacing), is sensitive to the influence of sample size (narrower and narrower confidence bounds would result in smaller and smaller BMCLs), and may be utilized even when a NOAEL does not exist. In addition, the BMC method can consider the variability of the response in the experimental population when a continuous variable (e.g., respiratory rate) is modeled (Crump, 1984, 1995; Dourson et al., 1985). This approach is not without controversy, however. Debate continues on the rationale for choice of a response level, how near to the existing data the POD should be, and the criteria for the minimum data set that should be modeled (see Filipsson et al., 2003). Nevertheless, the BMC approach has been applied to the derivation of several RfCs in the IRIS database for a variety of toxic end points.

Other dose–response methods are currently under consideration for use in risk assessment by the Agency. These methods include the categorical regression approach, which is a procedure that involves fitting a mathematical model to response data that have been categorized by severity. The model then determines the probability of obtaining a response of a particular severity, not only at certain exposure concentrations, but also at specific durations (Guth et al., 1997). As the BMC approach has the capacity to elucidate and make the entire exposure–response curve available to the risk assessor, categorical regression has the capability to make extensive portions of the duration–response curve available. This approach is anticipated to contribute greatly to assessments in which duration of exposure is a principal consideration, such as for acute and other less-than-lifetime exposures.

1.2.5 Understanding the RfC Equation: The POD_{HEC}

1.2.5.1 Orientation—Inhalation Dosimetry and the "Target-Tissue Dose"

PODs are typically derived from experimental results in laboratory animal studies where inhalation exposure has been to either gases or particles. The risk assessment process uses these PODs to derive human health guideline values. Historically, this transformation was accomplished by considering this issue as one of uncertainty (i.e., animal-to-human) by simply dividing the animal exposure value by 10 (Dourson and Stara, 1983), implying, among other things, that humans are 10-fold more sensitive than animals.

Thought and analysis applied to this area of uncertainty have reasonably deduced that this transformation comprises two general components, one dealing with what the animal or human body does to the toxic agent (i.e., pharmacokinetics), and the other with what the toxic agent does to the body (i.e., pharmacodynamics). The *Methodology* offers a suite of procedures falling under the

TABLE 1.2 Hierarchy of Model Structures for Dosimetry and Interspecies Extrapolation

Optimal model structure:
Structure describes all significant mechanistic determinants of chemical disposition, toxicant-target interaction, and tissue response
Uses chemical-specific and species-specific parameters
Dose metric described at level of detail commensurate to toxicity data
Default model structure:
Limited or default description of mechanistic determinants of chemical disposition, toxicant-target interaction, and tissue response
Uses categorical or default values for chemical and species parameters
Dose metric at generic level of detail

Source: U.S. EPA, 1994.

descriptive category of inhalation dosimetry intended to address the pharmacokinetic component of this uncertainty for toxic agents in gas or particle form. These inhalation dosimetric procedures, discussed only generally here, have as their basis the concept of the internal dose, more specifically the dose to the target tissue. As recognized by NRC (1994): " target tissue dose is . . . the ultimate determinant of risk . . . ". Dosimetry is the means for estimating the target tissue dose. The result of application of inhalation dosimetry to exposures of laboratory animals is an estimate of an externally applied concentration that would result in the same internal target tissue dose in humans as in animals. The outcome of inhalation dosimetry procedures is aptly termed a HEC. Moreover, because application of dosimetry leads to an estimate of an internal target tissue dose from an externally applied concentration, an assessment based on this estimate could be termed appropriately as an "exposure-dose–response" rather than just an exposure–response assessment.

The suite of options for performing inhalation dosimetry in the *Methodology* provides "optimal" and "default" procedures for the process (Table 1.2), the optimal approach requiring and utilizing extensive chemical-specific information. This hierarchy provides an incentive for research and development of relevant data such that a default option for any given chemical may at some point be reevaluated under an optimal approach.

With their extensive requirement of species- and chemical-specific data, physiologically based pharmacokinetic (PBPK) models may be regarded as an optimal model structure. Because the information available for the majority of environmental agents do not attain this level of information, the discussion following will be concerned with the default model structures that are described in more detail in the *Methodology.*

The respiratory tract is often the target tissue in inhalation risk assessment so that dosimetry in and about this anatomical structure becomes prominent. Consistent with the underlying principal of determining risk (i.e., the internal dose), dosimetry is applied to the target tissue in the area or region where the toxicity occurs. For purposes of dosimetric adjustment in inhalation of both gases and particles (which behave differently in these regions), the *Methodology* divides the respiratory tract into three regions (see Figure 1.6).

In this respiratory tract-based scheme, the remainder of the body is termed the extrarespiratory (ER) or systemic region. Estimates of the surface area of these individual regions of the respiratory tract (i.e., ET, TB, and PU) and other physiological parameters necessary for the dosimetric adjustment process (e.g., minute volumes, default body weights, numerous allometric equations) are included in the *Methodology* for several species and for humans.

Information on the surface areas of these regions along with other critical determinants of dose in an inhalation scenario, that is, ventilation rates, partitioning of the agent into the tissues (for gases),

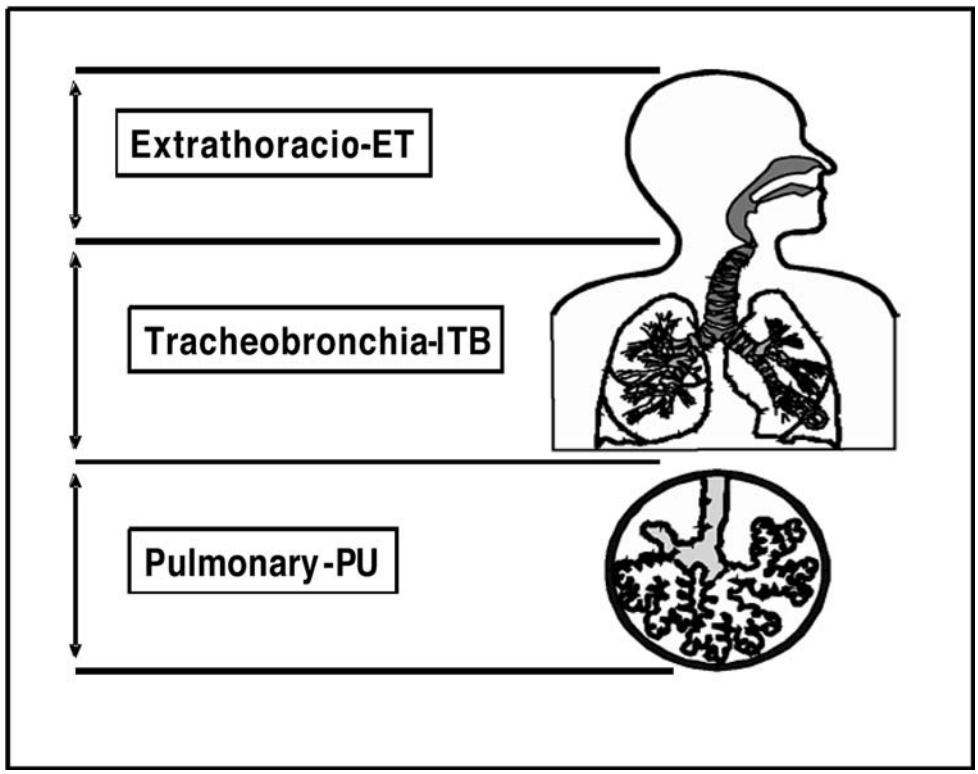


FIGURE 1.6 Diagram of the three regions of the respiratory tract, including the extrathoracic (ET), the tracheobronchial (TB), and pulmonary (PU) regions. (Adapted from U.S. EPA, 1994.)

and characteristics of the particles being inhaled, are combined into a dosimetric adjustment factor, a DAF, a process elaborated on below.

1.2.5.1.1 POD_{HEC}—Mathematical Derivation

Mathematical derivation of the POD_{HEC} consists of the following:

$$POD_{HEC} = POD_{adj} \times DAF$$

where DAF = RGDR or RDDR, **RGDR** is the regional gas dose ratio, and **RDDR** is the regional deposited dose ratio.

The remainder of this section will expand on the explanation of the terms POD_{adj}, DAF, RGDR, and RDDR.

1.2.5.1.2 POD_{adj} (Adjusted Point of Departure)

Nearly all inhalation studies with laboratory animals are conducted under a less-than-continuous daily exposure. To adjust these less-than-continuous daily exposure scenarios to those applicable to the intent for the RfC (i.e., a continuous daily exposure), the procedure of duration adjustment is regularly applied to both animal and human studies.

Duration adjustment, which is actually duration extrapolation, is accomplished by normalizing to a $C \times 24$ hr product and will therefore nearly always result in a lowering of the concentration (C), for example, 100 ppm $\times$ 6 hr = 25 ppm $\times$ 24 hr. Application of this procedure has implications regarding the nature of the exposure associated with the response. The measure of dose associated with a

$C \times t$ product is typically some aspect of the concentration-time curve (e.g., area-under-the-curve, AUC) measure in the tissue and not just the concentration level attained (e.g., a maximum concentration, C_{max}) in the tissue. Although neither of these may be the appropriate measure of dose, application of this procedure will nearly always result in a lowering of the concentration, thereby providing an automatic margin of protectiveness for chemicals for which C alone may be appropriate. Application of this procedure would also reflect the maximum dose for agents for which total or cumulative dose may actually be the appropriate measure. When considered in this way, this procedure may be regarded as being protective of public health (see U.S. EPA, 2002a).

Duration adjustment would not be considered necessary or appropriate where the end point is highly concentration dependent (e.g., anesthesia), or under conditions of exposure that are continuous relative to the end point or desired goal. For example, duration adjustment would not be applied to exposures in a developmental study where exposures are administered continuously (i.e., 24 hr/day, 7 days/week) throughout specific and known "windows" of developmental processes.

Duration adjustment is intended to apply to days per week as well as hours per day to be completely continuous in nature. Experimental inhalation exposures conducted on 5 of 7 days during a week would be factored by 5/7. A variation on this procedure applies also to human occupational exposures as elaborated on in the *Methodology.*

Thus, application of duration adjustment to the POD would result in the POD being designated as:

$$\mathrm{POD}_{\mathrm{adj}} = \mathrm{POD} \times \left(\frac{\text{hours exposure}}{24\ \text{h}} \right) \times \left(\frac{\text{days exposure}}{7\ \text{days}} \right)$$

1.2.5.1.3 DAF, the Dosimetric Adjustment Factors RGDR and RDDR

Application of dosimetry to the conditions of the inhalation scenario, as outlined and justified above, are applied quantitatively through a factor aptly termed a "dosimetric adjustment factor" or DAF. The nature of the DAF and the method employed to derive the DAF is based on the nature of the agent, that is, whether it is a gas or a particle, and the location of the toxicity, either in some region in the respiratory tract (e.g., PU, TB, or ET) or elsewhere in the body, that is, at an "extrarespiratory" site. For effects observed in the respiratory tract or systemically from exposure to a gas, a regional gas dose ratio or RGDR is derived. For effects observed after inhalation exposure to an agent in particulate form, whether in the respiratory tract or systemically, a regional deposited dose ratio or RDDR is derived. The following text elaborates on these terms and procedures.

1.2.5.2 The Regional Gas Dose and Regional Gas Dose Ratio

After identifying the likely or known region of the toxic effect, based either on the characteristics of the gas or on known experimental results, the *Methodology* provides options for computing a DAF—for gases, a regional gas dose ratio (RGDR). The options in the *Methodology* for computation of an RGDR are in a hierarchy, prioritized upon the amount of information available where options such as application of PBPK models or agent-specific mass transport coefficients (K_g) are described. Default procedures, for the more likely situation in which data are limited, are also given and explained. For the purposes here, the default procedure for derivation of an RGD and the RGDR (i.e., the ratio) are illustrated.

1.2.5.2.1 The RGD

The default option for determination of the regional gas dose (RGD) for gases that have effects in the portal-of-entry tissues, that is, the respiratory tract being the target tissue, is based on the assumption that the major interspecies determinants of dose delivered to the respiratory tract are functions of the concentration of the toxicant in the volume of air breathed in and the surface area of the affected region in the respiratory tract. This relationship, which is actually a surrogate for impingement of

the agent on the surface area of the affected region(s), is expressed by constructing minute volume to surface area ratios, for example, $V_E / SA_{ET, TB, PU, TH \text{ or } TOTAL}$ (see Figure 1.6). A listing of the values for these various factors currently used by the Agency for various species is available in the *Methodology*. Note that these within-species ratios are made under the assumption that the gas is distributed uniformly and everywhere to the surface areas of the designated regions. This assumption is known not to be valid (e.g., Kimbell and Subramaniam, 2001) and attempts continue to offer refinements in the quantitative description of this distribution.

The RGD from an experimental animal exposure to a gas that results in an effect in the ET region, for example, would be calculated as follows:

$$\left(RGD_{ET}\right)_A = \left(\frac{V_E}{SA_{ET}}\right)_{animal}$$

For the default option in determining the RGD for gases having systemic or extrarespiratory effects, where absorption and circulation to the target tissue is implied, the partitioning of the gas into the blood (i.e., $H_{b/g}$) is assumed to be the primary determinant of dose delivered to the body.

1.2.5.2.2 The RGDR

For gases associated with effects in the portal-of-entry tissue, that is, the respiratory tract being the target tissue, the basis of the DAF is the gas dose to the region of interest (an RGD) for both animals (RGD_A) and humans (RGD_H). The ratio of these RGDs (RGD_A / RGD_H) yield the RGDR, that is, the DAF.

$$\left[\frac{\left(RGD_{ET}\right)_A}{\left(RGD_{ET}\right)_H}\right] = RGDR_{ET}$$

The exposure concentration of interest is then factored by this RGDR to make the dosimetric adjustment and yield the HEC. For example, the HEC from an experimental animal $BMCL_{10}$ with an effect in the ET region would be calculated as follows:

$$BMCL_{10(adj)} \times RGDR_{ET} = BMCL_{10(HEC)}$$

In a manner analogous to those gases having the respiratory tract as the target tissue, the animal-to-human ratio of the gas–blood partition coefficient, the $H_{b/g}$, provides the basis for constructing the RGDR for gases having systemic or extrarespiratory effects. Under the default option, the *Methodology* provides detailed direction for deriving the RGDR for these type gases based on the availability of the actual partition coefficients. When neither animal nor human values are available, the *Methodology* states that the values may be assumed to be unity such that the RGDR would be 1. When values are available and the animal value is greater than the human (i.e., the value of the RGDR would be >1), the RGDR reverts to unity, that is, the RGDR = 1.

As can be seen, the RGDR is a normalizing factor that brings the concentration applied to the animal to equal that applied to the human.

1.2.5.3 The Regional Deposited Dose and the Regional Deposited Dose Ratio

The current Agency basis to dosimetrically adjust for inhaled particles with effects occurring within regions of the respiratory tract is an empirical model comprising a system of equations fit to actual experimental deposition data from animals and humans. The models developed from the laboratory animal and human data have been mathematically combined and described in the *Methodology* such

that the fractional deposition (F_r) of a particle distribution (characterized by size and distribution) observed in laboratory animals is used to predict the fractional deposition of that same particle distribution in humans. The F_r is combined with the other factors used as surrogates for the regional deposited dose (RDD), that is, minute volumes and surface areas of the affected regions, to estimate the dose of particulate to the particular species, the RDD.

In a manner analogous to the RGDR, RDDRs are then constructed and calculated for a particular region of the respiratory tract. In practice, this is done by using a computer program.

For systemic or extrarespiratory effects occurring in response to inhaled particles, the calculation is adapted quite logically for these circumstances in which body weight (BW) is substituted for surface area (SA) with the fractional deposition (F_r) becoming the dose to the entire respiratory tract or "total", F_{TOTAL}.

1.2.6 Understanding the RfC Equation: Uncertainty/Variability Factors

1.2.6.1 Discussion and Application

The final step in determining the RfC is application of uncertainty and variability factors, that is, UFs, to the POD_{HEC}. As designated, these factors are not intended to be viewed as "safety" factors. Rather they may be viewed as default "place holders" for missing information. Once that information is obtained or the uncertainty is resolved, in whole or in part, the overall uncertainty and variability factors may be adjusted or even eliminated in a revision of that RfC assessment. Thus, a large factor applied to a POD_{HEC} for this purpose in a given RfC assessment should not be interpreted as that agent being especially toxic and requiring a large degree of safety. Rather, it indicates that the assessment considers that much uncertainty exists about the toxicity of this particular agent.

Applications of these factors are matters of scientific judgment on the extent of data available and on how informative these data are in the particular area of uncertainty. The history and extent of their use by the risk assessment community and the lack of instances in which these factors were insufficient are evidence of their general adequacy. Several investigators are considering the intended purpose and overall adequacy of these factors and how they may best address specific populations (Baird et al., 1996; Hattis et al., 2002). Guidance and implementation of "chemical-specific" or "data-derived" adjustment factors (CSAF) to replace the general default uncertainty/variability in the areas of interspecies extrapolation and human variability is now a focus of the U.S. EPA (IPCS, 2001; Dourson et al., 2002; Renwick et al., 2002) as evidenced by the use of a CSAF in the Agency's boron assessment (U.S. EPA, IRIS, 2004d).

The various uncertainty factors used in the derivation of an inhalation RfC (i.e., a health guideline value for lifetime exposures in the human population including sensitive subpopulations) are (U.S. EPA, 1994):

- UF_A, extrapolation of animal data to humans, or interspecies uncertainty
- UF_H, variation in susceptibility among the members of the human population (i.e., interindividual or intraspecies variability)
- UF_S, extrapolation from data obtained in a study with less-than-lifetime exposure (e.g., from subchronic to chronic exposure)
- UF_L, extrapolation from a LOAEL rather than from a NOAEL
- UF_D, incompleteness of the database.

1.2.6.2 UF_A—Interspecies Extrapolation

This UF is applied to account for the extrapolation of laboratory animal data to humans.

The general underlying components in this area of uncertainty are considered to be pharmacokinetics (PK) and pharmacodynamics (PD) with the value typically assigned to this area uncertainty,

10, equally partitioned between these components. This dichotomization, described in the *Methodology* and elsewhere (e.g., Renwick and Lazarus, 1998), allows for the strategy of reducing this uncertainty by addressing either of these components with information. The *Methodology* provides several options for doing this; the ideal option is the application of an agent-specific, physiologically based pharmacokinetic (PBPK) model that is parameterized for such a purpose, that is, with both animal and human information (see "Optimal Approach" in Table 1.2). The other prominent option from the *Methodology* is the application of dosimetry to estimate an HEC (discussed at length previously). These options provide for the replacement of the PK component of this UF. Options provided for the replacement of the PD component with information are yet to be developed but would greatly improve the RfC and other noncancer methodologies.

Model use and dosimetric adjustments provided for in the *Methodology* are considered to address at least certain aspects of pharmacokinetics, thereby decreasing the uncertainty about interspecies extrapolation. As mentioned previously, one-half the default 10-fold interspecies UF (i.e., $10^{0.5}$) is assumed to be due to PK so that, with an HEC calculated in an assessment, the value for this area of uncertainty is reduced to $10^{0.5}$, which is typically rounded to the nearest whole value of 3.

1.2.6.3 UF_H—Intraspecies Variability

The intraspecies UF, actually a variability factor, is applied to account for variations in susceptibility within the human population (hence the subscript "H") and the possibility (given a lack of relevant data) that the database available is not representative of the dose/exposure–response relationship in the most susceptible subpopulations among the human population.

Various authors have evaluated the intraspecies UF by using data from animal or human studies, as summarized by Dourson et al. (1996). They concluded that the 10-fold default factor seemed adequate to address this area of uncertainty when starting from a median response, by inference a NOAEL assumed to be from an average group of humans.

In a manner analogous to the interspecies UF, this 10-fold UF is also considered to have both PK and PD components. Upon evaluating the composite 10-fold factor to account for variability in both PK and PD, Renwick and Lazarus (1998) concluded that a 10-fold factor would cover the vast majority of the population (>99%). These evaluations are intended to consider children or other potential subpopulations (see Table 4–9 in *Methodology*). Reductions from a default value of 10 for this UF are rare and considered only if relevant data are available from what is clearly demonstrable as the most susceptible subpopulation. One such instance is the application of a chemical-specific adjustment factor (CSAF) employed in the Agency health effect assessment for boron (U.S. EPA, 2004d), wherein data on a critical kinetic parameter in the population of concern, the kinetic elimination rate of boron in pregnant women, was used to address the kinetic component of UF_H.

1.2.6.4 UF_S—Extrapolation from a Subchronic to a Chronic Duration

A duration adjustment or extrapolation currently in use is the application of a UF when only a subchronic duration study is available to develop the chronic RfC. A default value of 10 for this UF is applied to the NOAEL/LOAEL or BMD/BMC from the subchronic study. Implicit in this practice is the assumption that effects from a given compound in a subchronic study would occur at a 10-fold higher concentration than in a corresponding (but absent) chronic study. This factor would be applied subsequent to the adjustment of these subchronic exposures from intermittent to continuous (i.e., duration adjustment).

The specific use of a UF applied to a subchronic study in the derivation of a chronic reference value is reasonable. Some work has been published on this aspect of extrapolation (Lewis et al., 1990; Pieters et al., 1998). As with the other areas of uncertainty, this factor could be replaced when applicable information becomes available (e.g., an adequate animal toxicity of chronic duration).

1.2.6.5 UF_L—Extrapolation from a LOAEL to a NOAEL

An UF with a default value of 10 is typically applied to the scenario in which the POD_{HEC} is a LOAEL with no NOAEL available. The size of this UF may be altered, depending on the magnitude and nature of the response at the LOAEL. For example, if the LOAEL available is for a very mild or minor effect, whose adversity is of questionable biological significance, the value assigned to this UF may be assigned a value of 3 instead of 10. Any judgment in applying this UF should also consider the slope of the dose–response curve in the range of the LOAEL and lower, where the NOAEL (or BMC) may occur. Reports examining LOAEL/NOAEL ratios in studies having both have shown this value to vary from approximately 3-fold to as much as 10-fold, depending on the steepness of the dose–response curve in this NOAEL/LOAEL dose range (Lewis et al., 1990; Faustman et al., 1994).

The advent of the benchmark dose approach discussed above, which depends on a designated benchmark response level versus a NOAEL or LOAEL as a POD (see Figure 1.5), may in time result in a more limited use of this UF.

1.2.6.6 UF_D—Completeness of the Database (Including Route-to-Route Extrapolation)

The database UF is intended to account for the potential for deriving an underprotective RfC as a result of an incomplete characterization of the chemical's toxicity. In addition to the identification of toxicity information that is lacking, review of existing data may also suggest a deficiency in the toxicological characterization of a given agent, for example, on particular end points or for certain life stages. Consequently, in deciding to apply this factor to account for deficiencies in the available data set, and in identifying its magnitude, both the data lacking and the data available should be evaluated and considered. With reference to the minimum database for derivation of an RfC, the *Methodology* states that no RfC be derived if neither an adequate subchronic nor chronic inhalation study is available.

If, in a database for a given agent, the data raise suspicions of developmental toxicity, for example, and signal the need for other types of testing (e.g., specialized neurotoxicity studies, developmental immunotoxicity studies, developmental carcinogenesis studies, or developmental endocrine toxicity studies), then the database factor should take into account whether or not these data have been collected and used in the assessment and their potential to become the critical effect or otherwise influence the POD. The size of the factor to be applied will depend on other information in the database and on the judgment as to how much impact the missing data may have on determining the toxicity of a chemical and, consequently, the POD.

An additional 10-fold factor for infants recommended by the National Research Council (1993) and called for in the 1996 FQPA is similar to the database UF but also incorporates consideration of childhood exposure differences. Often a factor of 3 is applied if either a prenatal toxicity study or a two-generation reproduction study is missing, or a factor of 10 may be applied if both are missing (Dourson et al., 1996). Dourson et al. (1992) examined the use of the database UF by analyzing ratios of NOAELs for chronic toxicity in dog, rat, and mouse studies and reproductive and developmental toxicity studies in rats. They concluded that reproductive and developmental toxicity studies provide useful information for establishing the lowest NOAEL or BMC value, and if one or more bioassays are missing, a factor should be used to address this scientific uncertainty in deriving an RfC. Thus, application of the existing uncertainty factor for database deficiency may be adequate in most cases for protecting children's health.

1.2.6.6.1 Route-to-Route Extrapolation from Oral to Inhalation

For many agents of interest, satisfactory oral RfD assessments with adequate databases exist. A strategy whereby a deficient inhalation database could be augmented would be to utilize the oral information via route-to-route extrapolation. The Proposed Test Rule for Hazardous Air Pollutants (U.S. EPA, 1996) states that "EPA would consider route-to-route extrapolation of toxicity data from

routes other than inhalation when it is scientifically defensible to empirically derive the inhalation risk" which recognizes and supports such a strategy.

The text of the Test Rule goes on to state, however, that "Derivation of the inhalation risk is generally only reasonable when portal-of-entry effects (toxic effects on the respiratory tract from inhalation exposure) and/or first-pass effects can be ruled out or adequately characterized." These practical caveats for performing route extrapolation are also stated and expanded upon in the *Methodology,* in particular, the necessity of having adequate information to evaluate the capacity of the agent to produce toxicity in the respiratory tract as a consequence of inhalation. Current efforts are underway within the Agency to provide guidance as to when such a route-to-route extrapolation should be undertaken and how it may be applied to the development of an RfC.

A PBPK model has been used for purposes of route-to-route extrapolation in the Agency's health effect assessment for vinyl chloride, in which an inhalation exposure was estimated for dose-response information that was observed in the oral route (U.S. EPA, 2004d).

1.2.6.6.2 Characterization of the RfC

The multifaceted nature of the deliberative and subjective aspects of the health effect assessment process and the influence they may have on the final version of the assessment have been described in preceding sections. The identification and discussion of those general areas of uncertainty by no means encompass all the deliberative aspects that may be present in an assessment. For example, even though one may be confident in obtaining from the database sufficient exposure–response information on end points adequate to determine the "critical effect," the actual exposure–response information for that effect may be barely adequate for any further quantitative analysis. This uncertainty may be compounded by uncertainty inherent in the method or model chosen to be used in the analysis of these barely adequate exposure–response data. Furthermore, the ever-moving, self-correcting nature of the scientific process and the basic scientific assumptions that underlie an approach to a given assessment may change or be supplanted by other more current science. Layered on this uncertainty is the varying interpretation of the same data. Thus, nearly every decision made to move the assessment process forward requires some deliberative analysis and judgment. The complexity and diversity of the factors involved in the deliberative aspect practically ensures the occurrence of divergent results in the assessment process.

To acknowledge and incorporate this situation, practitioners of risk assessment provide an integrative discussion of this aspect as a "risk characterization" of an assessment. EPA risk characterization guidelines (U.S. EPA, 1995b) provide for and stress the importance of identifying uncertainties and variability and major assumptions in the subject assessment and presenting them as part of risk characterization. A risk characterization should attempt to be the repository and evaluation of the analytic-deliberative aspects of the assessment. It is intended to present conclusions related to risk and convey information regarding the strengths and limitations of those conclusions. These guidelines set forth fundamental principals to what risk characterization should convey. Those principles, and their essence, are transparency with respect to what policy decisions are and what scientific decisions are, clarity, in regard to the strengths and weaknesses of the assessment, and reasonableness, with respect to the conclusions that have been drawn from the available data.

Assessments, such as the RfC, are intended to be scientific syntheses of what is known about the toxicity of a chemical and the relationship of that toxicity to the human population; as such, assessments are to utilize all available information to the maximum extent possible.

1.2.6.6.3 Other Aspects of the RfC Assessment—Review and Appendices

The entire IRIS assessment, the RfC included, undergoes several review processes. These include an internal Agency review conducted by Agency scientists, a review by Agency program offices (e.g., Office of Water), and an external "peer" review conducted by scientific experts outside the Agency. Comments received from these reviewers may result in changes to the assessment. A summary and response to the major peer review comments can be found in an appendix to the *Toxicological Review.*

Other items that are part of the official assessment, such as calculations and descriptions of models used in the assessment, may be added as appendices.

1.3 INHALATION RISK ASSESSMENTS IN THE OFFICE OF PESTICIDE PROGRAMS

Within the Office of Prevention, Pesticides, and Toxic Substances (OPPTS), the Office of Pesticide Programs (OPP) regulates the use of all pesticides in the United States, establishes maximum levels for pesticide residues in food, and performs risk assessments to prevent injury. Risk assessments are routinely performed for exposure to pesticides by the oral, dermal, and inhalation routes for a variety of pesticides and use patterns.

When a registrant submits a petition for the registration of a new pesticide, it is accompanied by considerable supporting data performed in compliance with OPPTS-harmonized test guidelines. The purpose of these guidelines is to minimize variations among the testing procedures that are performed to meet the data requirements of the U.S. EPA under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. 136 *et seq.*). The following guideline series can be found on the EPA's Web site at *www.epa.gov/opptsfrs/home/guidelin.htm:*

810 Product Performance Test Guidelines
830 Product Properties Test Guidelines
835 Fate, Transport and Transformation Test Guidelines
840 Spray Drift Test Guidelines
850 Ecological Effects Test Guidelines
860 Residue Chemistry Test Guidelines
870 Health Effects Test Guidelines
875 Occupational and Residential Exposure Test Guidelines
880 Biochemicals Test Guidelines
885 Microbial Pesticide Test Guidelines

The 870 series of Health Effects Test Guidelines include a wide variety of toxicity studies—49 in all—ranging from acute studies to developmental toxicity, reproductive toxicity, genotoxicity, and carcinogenicity studies. The two guidelines used for inhalation toxicity studies are 870.1300 for acute inhalation toxicity studies and 870.3465 for subchronic inhalation toxicity studies. OPP requires inhalation toxicity studies for all pesticide active ingredients that may pose an inhalation hazard. In addition, acute inhalation toxicity studies are required for labeling purposes for all pesticide formulations that may pose an inhalation hazard. Subchronic studies characterize inhalation toxicity, and the NOAELs in these studies are used to estimate inhalation risk.

1.3.1 Estimating Pesticide Inhalation Risk

Whereas RfCs are based on lifetime exposure, OPP typically deals with inhalation exposures that are less-than-lifetime in duration. Human exposures can range from minutes to years. Air concentrations may be constant, variable, or episodic. Human exposure scenarios can be as diverse as flying a crop duster, spraying an orchard, working in a greenhouse, a child playing in a pesticide-treated lawn, or a family living in a house treated for vermin. An effort is made to consider all applicable variables when estimating human risk.

An interdisciplinary team, consisting of a toxicologist, a chemist, and an exposure specialist, is assigned to each pesticide risk assessment project. This team characterizes the human exposure scenarios encountered with their pesticide, then selects toxic end points and NOAELs that will be used for short-term (1–30 days), intermediate-term (1–6 months), and long-term (longer than 6 months) inhalation risk assessments. The team's selections are considered by a Risk Assessment Review

Committee (RARC) that either endorses or modifies their choices. For pesticides that are controversial, the RARC may review the completed risk assessment before a regulatory decision is made.

The methods used by OPP to characterize human inhalation risk differ from those used by the rest of the Environmental Protection Agency. Rather than expressing risk in terms of a reference concentration (RfC), inhalation risk is expressed as a margin of exposure (MOE). One reason for this difference is that human exposure is less than lifetime in duration. Two risk assessment methods are used in OPP. The first method—the **route-extrapolated MOE**—is used for nearly every inhalation risk assessment and, as the name implies, always involves route extrapolation. The second method—the **route-specific MOE**—does not require route extrapolation and has only been used a few times to estimate inhalation risk for fumigants.

1.3.2 The Route-Extrapolated MOE

The following equation has been used to estimate inhalation risk for nearly all inhaled pesticides since OPP was established in 1970 (U.S. EPA, 1998c). Note that the NOAEL and human exposure values in this equation are both in milligrams per kilogram per day units rather than air concentrations.

$$\mathrm{MOE_I} = \frac{\mathrm{NOAEL}\left(\mathrm{mg/kg/day}\right)}{\mathrm{Human\ exposure}\ \left(\mathrm{mg/kg/day}\right)}$$

Although inhalation toxicity studies are required for all pesticides that may pose an inhalation hazard, in reality, these studies are lacking for most inhaled pesticides. It is usually during the risk assessment process that this data gap is discovered and studies are requested. Rather than waiting for the submission of inhalation data, inhalation MOEs ($\mathrm{MOE_I}$) are calculated by using oral toxicity data on the assumption that a pesticide's absorption and toxicity are similar by the oral and inhalation routes (U.S. EPA, 1998a). Approximately 80% of pesticides have inhalation risk assessments based on oral data. For the other 20% of pesticides that have inhalation data, the inhalation NOAEL is route-extrapolated to a milligrams per kilogram per day dose so it can be used in the preceding equation.

Human inhalation exposure (the denominator of the $\mathrm{MOE_I}$ equation) is measured as an air concentration, then route extrapolated to a milligrams per kilogram per day dose. Most inhalation risk assessments are performed by using surrogate human exposure values from the Pesticide Handlers Exposure Database (PHED). This is a huge database of actual field-monitoring data that provides inhalation and dermal exposure estimates based on type of pesticide product and application method (U.S. EPA, 1998b). Although the inhalation values are based on air sampler concentrations, they are only available as route-extrapolated milligram per kilogram per day doses. When a registrant provides a human exposure concentration specifically for their pesticide, the concentration is route extrapolated to a milligram per kilogram per day dose before calculating an $\mathrm{MOE_I}$ (U.S. EPA, 1998a). The following equation is used to extrapolate from an air concentration to a milligram per kilogram per day dose (U.S. EPA, 1998c):

$$\frac{mg/L/day \times A \times MV \times D}{BW} = mg/kg/day$$

A The inhalation: oral absorption ratio. In the absence of oral and inhalation absorption data, the default ratio is 1.

BW Body weight (kg) (see Table 1.3 for animal weights). Adult human body weight is a default of 70 kg, whereas child body weight is based on a measured value.

D Duration of daily exposure (hours per day).

MV Minute volume (L/min) (see Tables 1.3 [animal] and 1.4 [human]).

TABLE 1.3 Animal Minute Volumes and Body Weights

	Subchronic						Chronic					
	MV (L/min)			BW (kg)			MV (L/min)			BW (kg)		
Species and Strain	♂	♀	♂/♀	♂	♀	♂/♀	♂	♀	♂/♀	♂	♀	♂/♀
Rats			+			+			+			+
Fisher 344	0.14	0.10	0.12	0.180	0.124	0.152	0.25	0.17	0.21	0.380	0.229	0.305
Sprague–Dawley	0.19	0.15	0.17	0.267	0.204	0.236	0.33	0.23	0.28	0.523	0.338	0.431
Long–Evans	0.18	0.14	0.16	0.248	0.179	0.124	0.30	0.23	0.27	0.472	0.344	0.408
Osborne–Mendel	0.19	0.15	0.17	0.263	0.201	0.232	0.32	0.26	0.29	0.514	0.389	0.452
Wistar	0.16	0.12	0.14	0.217	0.156	0.187	0.30	0.21	0.25	0.462	0.297	0.380
Mice												
B6C3F1	0.037	0.028	0.033	0.032	0.025	0.028	0.044	0.041	0.043	0.037	0.035	0.036
BAF1	0.026	0.023	0.024	0.022	0.020	0.021	0.030	0.026	0.028	0.026	0.022	0.024
Hamsters												
Syrian	0.043	0.042	0.042	0.097	0.095	0.096	0.057	0.061	0.059	0.134	0.145	0.140
Chinese	0.015	0.013	0.014	0.030	0.025	0.028	0.020	0.018	0.019	0.041	0.038	0.040
Guinea pigs												
[Not specified]	0.21	0.19	0.20	0.48	0.39	0.44	0.29	0.28	0.28	0.89	0.86	0.88
Rabbits												
New Zealand	1.09	1.17	1.13	2.86	3.10	2.98	1.37	1.43	1.40	3.76	3.93	3.85

MV = minute volume, the total volume of new air moved into the respiratory passages each minute (mean tidal volume × respiratory rate); BW = body weight, mean body weight.
Source: U.S. EPA, 1998c.

Once an MOE_I has been calculated, it is compared with a ***target MOE***. A target MOE is a cumulative uncertainty factor (UF). In most cases, the inhalation risk is considered acceptable provided the MOE is greater than, or equal to, a target MOE of 100. This includes UFs of 10 each for interspecies extrapolation and intraspecies variability. If a LOAEL is used, an additional UF of 3 or 10 is included in the target MOE (i.e., target MOE of 300 or 1000). When an oral NOAEL or a route-extrapolated human exposure value is used to calculate an inhalation MOE, no additional UFs are applied to account for extrapolation error, the likelihood of respiratory portal-of-entry effects, or the possibility that the inhalation route may be more toxic than the oral route. The following example shows how a route-extrapolated inhalation MOE is calculated with biological values in Tables 1.3 and 1.4:

Example 1: Route-Extrapolated MOE

Rat (Sprague–Dawley)

Inhalation NOAEL = 0.2 mg/L/day (6 h/day exposure duration)
Minute volume = 0.17 L/min (from Table 1.3)
Body weight = 0.236 kg (from Table 1.3)

Human

Inhalation exposure = 0.003 mg/L/day (6 h/day exposure duration)
Minute volume (light activity level) = 16.7 L/min (from Table 1.4)
Minute volume (rest) = 6.7 L/min (from Table 1.4)
Duration of daily exposure = 6 h (360 min)
Body weight = 70 kg (default value for an adult human)

TABLE 1.4 Human Minute Volumes

	Short-Term Exposures				
	Rest (L/min)	**Sedentary Activity (L/min)**	**Light Activity (L/min)**	**Moderate Activity (L/min)**	**Heavy Activity (L/min)**
Children	5.0	6.7	16.7	20.0	31.7
Adults	6.7	8.3	16.7	26.7	53.3

	Long-Term Exposures (24-h means)							
Age	**<1 y**	**1–2 y**	**3–5 y**	**6.8 y**	**9–11 y**	**12–14 y**	**15–18 y**	**19–65+**
L/min	3.1	4.7	5.8	6.9	9.7 ♂9.0 ♀	10.4 ♂8.3 ♀	11.8 ♂8.3 ♀	10.6 ♂7.8 ♀

Rest, lying down; **Sedentary**, sitting, pilot, driving a tractor; **Light**, flagger, mixer/loader (containers <50 lb), pneumatic reel sprayer, lawn treatment, most harvesters; **Moderate**, mixer/loader (containers >50 lb), backpack sprayer (greenhouse applicator, hilly conditions, heavy brush), harvesters using ladders; **Heavy**, generally not applicable to occupational exposure to pesticides; **Minute volume**, total volume of new air moved into the respiratory passages each minute (mean tidal volume × respiratory rate).
Source: U.S. EPA 1998c.

Target MOE

100 (UFs of 10 each for interspecies extrapolation and intraspecies variation)

MOE_I Calculation

$$MOE_I = \frac{\left(\frac{0.2\ mg/L/day \times 1 \times 0.17\ L/\min \times 360\min}{0.236\ kg}\right)}{\left(\frac{0.003\ mg/L/day \times 1 \times 16.7\ L/\min \times 360\min}{70\ kg}\right)} = \frac{51.9\ mg/kg/day}{0.26\ mg/kg/day} = 200$$

An MOE of 200 is greater than the target MOE of 100, so this inhalation risk is generally considered acceptable.

The validity of a route-extrapolated MOE depends on the route extrapolation method used. The route extrapolation in Example 1, which considers only one pharmacokinetic factor (minute volume), is clearly not as robust as one based on a PBPK model.

1.3.3 The Route-Specific MOE

Although OPP uses RfDs to estimate oral risk, it does not use RfCs to estimate inhalation risk. Consequently, a route-specific MOE equation for estimating inhalation risk was developed in 1994 (U.S. EPA, 1998d). It is similar to the Agency's RfC method except that it yields an MOE, which OPP prefers. This equation uses air concentrations (milligrams per liter per day) instead of milligram per kilogram per day units, so it is not hindered by the hazard assumptions and

missing pharmacokinetic data that can make route extrapolations unreliable. Whenever possible, a dosimetric adjustment factor or DAF (a $RDDR_r$ for aerosols or a $RGDR_r$ for gases and vapors) should be used to improve accuracy and allow for use of a lower target MOE. A route-specific inhalation MOE is calculated as follows:

$$MOE_I = \frac{NOAEL_I \times DAF_r \times D_A}{Human\ exp.\ conc. \times D_H \times \left(\frac{Human\ MV_{actual}}{Human\ MV_{rest}} \right)}$$

$NOAEL_I$ No observed adverse effect level from an animal inhalation toxicology study in units of air concentration (milligrams per liter per day, milligrams per cubic meter per day and parts per million per day).

Human Exp. Conc. Human air concentration (measured or surrogate) in the same concentration units as the animal NOAEL.

D_A Duration of daily animal exposure (minutes/day).

D_H Duration of daily human exposure (minutes/day).

DAF_r Dosimetric adjustment factor for respiratory tract region (r); either a regional deposited dose ration ($RDDR_r$) for aerosol particles or a regional gas dose ratio ($RGDR_r$) for gases and vapors.

MV_{actual} Human minute volume (L/min) at an actual level of activity.

MV_{rest} Human minute volume (L/min) at rest (see Table 1.4).

When this method was presented to FIFRA the Science Advisory Panel (SAP) in 1997, they recommended that appropriate route-specific data be requested from pesticide registrants to minimize or eliminate the need for route-to-route extrapolations "fraught with many uncertainties." They endorsed the route-specific method for use with all inhaled pesticides and encouraged the inclusion of dosimetric adjustment (FIFRA SAP, 1997). OPP began using the route-specific MOE equation in 2002, but only to estimate inhalation risk for fumigant gases, presuming that they pose a particular inhalation concern.

When dosimetric adjustment is not used, the route-specific inhalation MOE is typically compared with a target MOE of 100, which includes UFs of 10 each for interspecies extrapolation and human variability. An additional UF of 3 or 10 may be added to the target MOE if a LOAEL is used instead of a NOAEL, or if uncertainties exist regarding the quality of the toxicity or exposure database. A target MOE would likely be lower if a DAF_r were used.

Example 2 shows how a route-specific MOE is calculated using the same biological data as in Example 1. At this writing, OPP has yet to use a DAF_r, so one is not included in this example:

Example 2: Route-Specific MOE

Target MOE

100 (UFs of 10 each for interspecies extrapolation and intraspecies variation)

MOE_I Calculation

$$MOE_I = \frac{0.2\ mg/L/day \times 360\,\text{min}_A}{0.003\ mg/L/day \times 360\,\text{min}_H \times \left(\frac{16.7L/\text{min}_{actual}}{6.7L/\text{min}_{rest}} \right)} = 27$$

Because an MOE_I of 27 is less than the target MOE of 100, the inhalation risk is considered unacceptable.

Although Examples 1 and 2 were both calculated by using the same data, the route-extrapolated MOE_I in Example 1 was 7.4-fold greater than the route-specific MOE_I in Example 2. Whereas the route-extrapolated MOE_I of 200 would be considered acceptable because it is greater than the target MOE of 100, the more reliable route-specific MOE_I of 27 reveals that the inhalation risk is, in fact, unacceptable. These examples show that a route-extrapolated MOE may make a pesticide appear "safer" than it really is. Using oral data to estimate inhalation risk further compounds the uncertainty of a route-extrapolated MOE_I.

1.3.4 The Food Quality Protection Act (FQPA) of 1996

By a unanimous vote, Congress passed the Food Quality Protection Act. President Bill Clinton signed it into law (P.L. 104–170) on August 3, 1996. This law amended the two major federal statutes used by EPA to regulate pesticides. These are the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), by which the EPA registers pesticides and prescribes labeling and other regulatory requirements to prevent unreasonable adverse effects on health or the environment, and the Federal Food, Drug, and Cosmetic Act (FFDCA), by which the EPA establishes tolerances (maximum legally permissible levels) for pesticide residues in food.

The FQPA mandates a single, health-based standard for all pesticides in all foods; provides special protections for infants and children; expedites approval of safer pesticides; creates incentives for the development and maintenance of effective crop protection tools for American farmers; and requires periodic reevaluation of pesticide registrations and tolerances to ensure that the scientific data supporting pesticide registrations will remain up to date in the future. The following are highlights of the law:

1. The FQPA establishes a single safety standard under FFDCA for setting tolerances; it is not a risk/benefit standard (with some exceptions).
2. Assessments must include aggregate exposures, including all dietary exposures, drinking water, and nonoccupational exposures (e.g., residential, including oral, dermal, and inhalation).
3. When assessing a tolerance, EPA must also consider cumulative effects and common mode of toxicity among related pesticides, the potential for endocrine disruption effects, and the appropriate safety factor to incorporate.
4. The Act requires a special finding for the protection of infants and children, including a 10-fold safety factor to further protect infants and children unless reliable information in the database indicates that it can be reduced or removed.
5. The Act establishes a tolerance reassessment program and lays out a 10-year schedule whereby EPA must reevaluate all tolerances that were in place as of August 1996.
6. The Act requires a minor use program and provides that special considerations be afforded minor use actions.
7. The Act requires review of antimicrobial actions within prescribed time frames.
8. EPA must review every pesticide registration every 15 years.
9. Tolerances must be set for the use of pesticides under emergency exemptions (FIFRA Section 18).

1.3.5 Risk Aggregation

When two or more routes of exposure induce the same toxic effects, the risk for these routes can be aggregated. OPP's traditional way of aggregating was to add all oral, dermal, and inhalation exposure values (all in milligram per kilogram per day units) and to divide the sum into an oral NOAEL

to yield an aggregate MOE (U.S. EPA, 1998a). This method has two disadvantages: first, an oral NOAEL must suffice for all three routes, and second, extrapolating an inhalation concentration into a milligram per kilogram per day dose introduces error.

To overcome these shortcomings, two new aggregation methods were developed that aggregate route-specific MOEs for the oral, dermal, and inhalation routes. Because MOEs are unitless, there is no need for route extrapolation (U.S. EPA, 1998d). The **aggregate MOE (MOE_A)** is used when all routes of exposure have the same target MOE, and the **aggregate risk index (ARI)** is used when the routes of exposure have dissimilar target MOEs. A more detailed description of OPP's methods for aggregating risk for single chemical, multiroute, and multisource assessments can be found in its Aggregate Guidance (U.S. EPA, 2001).

1.3.5.1 Aggregate MOE (MOE_A) Method

The following equation was developed in 1994 as a means to aggregate "unit-less" MOEs into an **aggregate MOE** (**MOE_A**, formerly called a total MOE or MOE_T). This method was endorsed by FIFRA's SAP in 1997 (FIFRA SAP, 1997).

$$MOE_A = \frac{1}{\frac{1}{MOE_1} + \frac{1}{MOE_2} + \cdots + \frac{1}{MOE_n}}$$

This method is used when the target MOE for each route of exposure is the same as in this example:

Route	MOE	Target MOE
Oral	100	100
Dermal	200	100
Inhalation	70	100

$$MOE_A = \frac{1}{\frac{1}{100_o} + \frac{1}{200_D} + \frac{1}{70_I}} = 34.1$$

The MOE_A is always lower than the lowest individual MOE and decreases with every MOE in the equation because each additional route of exposure increases the hazard. The MOE_A of 34.1 in this example is of concern because it is less than the target MOE of 100.

1.3.5.2 Aggregate Risk Index (ARI) Method

The aggregate risk index (ARI) was devised in 1998 as a way to aggregate MOEs that have dissimilar target MOEs (described here and in the Aggregate Guidance as UFs). It has been used in OPP since February 1998. MOEs for each route of concern are compared with UFs that reflect the nature, source, and quality of the data and the FQPA mandate to protect susceptible infants and children. This can result in differing UFs such as these:

	Oral	Dermal	Inhalation
MOE:	$\frac{300}{1000}$	$\frac{100}{100}$	$\frac{1000}{300}$
UF:			

MOEs can only be combined if they have a common UF. If the MOE/UF ratios for each route are treated as fractions (as shown above), they can be adjusted to a common denominator of 1. This is accomplished by dividing each MOE by its UF to yield a **risk index** (**RI**):

	Oral	**Dermal**	**Inhalation**
RI:	0.30	1.0	3.3

The RIs can then be combined to yield an **aggregate risk index (ARI).**

$$ARI = \frac{1}{\frac{1}{RI_1} + \frac{1}{RI_2} + \cdots + \frac{1}{RI_n}}$$

$$ARI = \frac{1}{\frac{1}{0.30_O} + \frac{1}{1.0_D} + \frac{1}{3.3_I}} = 0.22$$

RIs and ARIs are always compared against 1. This allows for direct comparisons between routes and between chemicals. As a general rule, an RI or ARI $\geqslant 1$ is of little concern, but an RI or ARI <1 suggests a risk of concern. In this example, the ARI (0.22) suggests a risk of concern because it is <1. The oral exposure, with its low RI of 0.30, is the major route of concern.

Just as risk increases as the MOE decreases, so also does risk increase as the RI or ARI decreases. The ARI method automatically considers each route's potency when route-specific NOAELs are used. The following equation is a simplified way of calculating a chemical's ARI in a single step.

$$ARI = \frac{1}{\frac{UF_1}{MOE_1} + \frac{UF_2}{MOE_2} + \cdots + \frac{UF_n}{MOE_n}}$$

Oral hazard is often expressed as the "percent of RfD" instead of as an MOE. Because the UF for the oral route is used to define the oral RfD, the percent of RfD (expressed as a decimal) can be inserted directly in the equation (e.g., oral exposure for a pesticide is 80% of the RfD, i.e., 0.8):

$$ARI = \frac{1}{\%RfD_O + \frac{UF_D}{MOE_D} + \frac{UF_I}{MOE_I}}$$

$$ARI = \frac{1}{0.8_O + \frac{100_D}{100_D} + \frac{300_I}{1000_I}} = 0.48$$

Percentages of reference doses (RfDs) and reference concentrations (RfCs) for all routes may also be aggregated:

$$ARI = \frac{1}{\%RfD_O + \%RfD_D + \%RfC_I}$$

1.3.6 Waiving the Requirements for Inhalation Toxicity Studies

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) gives OPP the authority to request inhalation toxicity studies for any pesticide that may pose an inhalation hazard. Occasionally, the

requirement for an inhalation toxicity study should be waived for ethical or scientific reasons. For example, it is a waste of animals and resources to perform an inhalation toxicity study when a pesticidal product has a very low potential for human inhalation hazard during handling or application.

A document entitled *Waiver Criteria for Multiple-Exposure Inhalation Toxicity Studies* describes scenarios in which waivers may be appropriate (U.S. EPA, 2002b). All waiver requests are considered on a case-by-case basis, and the burden of proof lies entirely with the pesticide registrant. If no significant inhalation hazard is identified during risk characterization and risk assessment, a waiver may be granted. Four criteria may be used to justify a waiver.

1.3.6.1 Criterion 1: Severe Irritation and Corrosivity

An active ingredient that causes severe irritation or corrosion of the skin or eye will also damage the sensitive respiratory mucosa if inhaled. Waivers should be granted for active ingredients that are corrosive (pH <2 or >11.5) or severely irritating. Waivers should not be granted for active ingredients that are slight to moderate irritants. Inhalation toxicity studies of irritants can quantify the sensitivity of this route and characterize portal-of-entry effects. This information is essential in an inhalation risk assessment.

1.3.6.2 Criterion 2: Low Volatility

Waivers will be considered for nonvolatile active ingredients that are not aerosolized (i.e., generated as mist, fog, dust, smoke, fumes), heated, evaporated, or otherwise made inhalable as a gas or vapor. Nonvolatile active ingredients are defined as having vapor pressures $<1 \times 10^{-5}$ kPa (7.5×10^{-5} mmHg) for indoor uses, and $<1 \times 10^{-4}$ kPa (7.5×10^{-4} mmHg) for outdoor use at 20–30°C. Waiver candidates based on volatility may include, but are not limited to, viscous liquids (under conditions of use), waxes, resins, lotions, and caulks. Waivers for formulated products such as animal dips, shampoos, pour-ons, slow-release collars, ear tags, and tree injections will be considered.

1.3.6.3 Criterion 3: Large Aerosol Particle Size

An **inhalable particle** is capable of entering the respiratory tract via the nose or mouth. A **respirable particle** evades capture in the upper respiratory tract and reaches the lungs. The larger the particle, the less likely it is to be inhalable or respirable. Waivers will be considered for active ingredients that do not pose a significant inhalation hazard because the particles are too large to be inhaled.

Large particles have the potential to do considerable local damage if they are absorbed because of the volume of material they contain. Table 1.5 demonstrates that with each 10-fold increase in particle diameter, there is a 1000-fold increase in particle volume. Compared with a 0.1 μm particle, a 100 μm particle has 1000 times the diameter and a billion times the volume.

An aerosol for a product formulation or application method can be considered essentially noninhalable provided $\geqslant$99% of the particles are >100 μm in diameter. Although aerosols that meet this criterion are candidates for waivers, the registrant is responsible for providing data on aerosol size distribution. Waiver candidates based on large particle size include, but are not limited to:

- Microencapsulated formulations that are not readily fractured, dissolved, time released, leaky, or small enough to be respirable during mixing/loading or application. Evidence of capsule durability must be provided.
- Granular products placed in or on the soil and baits applied by hand or during seed planting. Although granular products are inherently noninhalable, they may pose a significant inhalation hazard if attrition occurs. **Attrition** is the breaking down of a material into smaller particles as can occur during shipping, handling, pouring, and application. A product susceptible to attrition is said to be **friable.** A friable product may pose a significant inhalation hazard if it produces a measurable quantity of dust when poured or scattered.

TABLE 1.5 A Comparison of Aerosol Particle Diameters and Volumes

Particle Diameter (μm)	Δ Diameter	Particle Volume (μm^3)[a]	Δ Volume
0.1	—	0.000524	—
1.0	10	0.524	1000
10	100	524	1,000,000
100	1000	523,599	1,000,000,000

[a] Volume of a sphere $= \frac{4}{3}\pi r^3$.

A registrant requesting a waiver on the basis of particle size must demonstrate that their product contains large, noninhalable particles that are resistant to attrition. This can be accomplished by using the latest version of the American Society of Testing Materials (ASTM) *Test Method E728-91—Standard Test Method for Resistance to Attrition of Granular Carriers and Granular Pesticides*. This test method is not available from the EPA but can be purchased from ASTM (100 Barr Harbor Drive, West Conshohocken, Pennsylvania 19428-2959, *www.astm.org*).

1.3.6.4 Criterion 4: Toxicity Category IV and an Extrapolated MOE

Toxicity categories I, II, III, and IV are assigned for labeling purposes based on the 4-h LC_{50} of an active ingredient; toxicity category IV is the least toxic (LC_{50} >2 mg/L). Although pesticides with low oral toxicity also tend to have low toxicity by the inhalation route, waivers are not granted for active ingredients based solely on low *oral* toxicity because:

- Toxicity by the inhalation route tends to be more severe than by other routes.
- Inhaled chemicals bypass the metabolic protection of the liver (portal circulation).
- Oral data cannot be used to predict respiratory portal-of-entry effects (e.g., irritation, edema, cellular transformation, degeneration, and necrosis).
- The use of route extrapolation in a risk assessment minimizes the true inhalation risk.
- The application rate is usually higher for pesticides with low oral toxicity, so a potential exists for high inhalation exposure.

Nevertheless, a waiver may be granted for an active ingredient that is toxicity category IV for inhalation (4-h $LC_{50} \geqslant 2$ mg/L) provided an extrapolated inhalation MOE (based on an oral NOAEL) exceeds a target MOE of 1000 or greater. The target MOE may include the conventional UF of 100; an additional UF of 10–100 to account for unknown pharmacokinetic and pharmacodynamic differences between the oral and inhalation routes in animals and humans, and respiratory portal-of-entry effects; and any other additional assigned UF (e.g., for use of a LOAEL). The waiving of inhalation toxicity studies is the only instance when OPP includes an extra uncertainty factor to account for uncertainties introduced by route extrapolation.

1.3.7 What the Future Holds for Inhalation Risk Assessments in OPP

In early 2005, the Office of Pesticide Programs performed risk assessments for several fumigants using the Agency RfC guidelines. OPP is now willing to use RfCs in its risk assessments of all inhaled pesticides that have adequate inhalation toxicity data.

We thank Ayaad Assaad, Robert Benson, William Burnam, and Robert Hetes of the U.S. Environmental Protection Agency for reviewing this chapter for scientific and regulatory accuracy and providing useful comments.

REFERENCES

Barnes, D.G. and Dourson, M., Reference dose (RfD): description and use in health risk assessments, *Regul. Toxicol. Pharmacol.,* 8, 471–486, 1998.

Browner, C., Transcript of statement of Carol Browner before the U.S. House of Representatives Committee on Science, January 6, 1995.

Crump, K.S., A new method for determining allowable daily intakes, *Fundam. Appl. Toxicol.,* 4, 854–871, 1984.

Crump, K.S., Calculation of benchmark doses from continuous data, *Risk Anal.,* 15, 79–90, 1995.

Dourson, M.L., Felter, S.P., and Robinson, D., Evolution of science-based uncertainty factors in noncancer risk assessment, *Regul. Toxicol. Pharmacol.,* 24, 108–120, 1996.

Dourson, M.L., Hertzberg, R.C., Hartung, R., and Blackburn, K., Novel methods for the estimation of acceptable daily intake, *Toxicol. Ind. Health,* 1, 23–33, 1985.

Dourson, M.L., Knauf, L.A., and Swartout, J.C., On reference dose (RfD) and its underlying toxicity data base, *Toxicol. Ind. Health.,* 8, 171–189, 1992.

Dourson, M., Miller, M., Murray, J., Davis, R., Naumann, B., Meek, B., Stern B., Ohanian, E., Schwartz et al., A national and international debate on default uncertainty factors vs. data-derived uncertainty factors, *Hum. Ecol. Risk Assessment*, 8, 895–911, 2002.

Dourson, M.L. and Stara, J.F., Regulatory history and experimental support of uncertainty (safety) factors, *Regul. Toxicol. Pharmacol*., 3, 224–238, 1983.

Faustman, E.M., Allen, B.C., Kavlock, R.J., and Kimmel, C.A., Dose-response assessment for developmental toxicity. I. Characterization of database and determination of no observed adverse effect levels, *Fundam. Appl. Toxicol*., 23, 478–486, 1994.

FIFRA Science Advisory Panel, A set of scientific issues being considered by the Agency to discuss and evaluate the inhalation risk assessments and the combining of margins of exposure. Transmittal of the final report of the FIFRA scientific advisory panel meeting for March 19 and 20, 1997, April 28, 1997.

Filipsson, A.F., Sand, S., Nilsson, J., and Victorin, K., Benchmark dose method—review of available models, and recommendations for application in health risk assessment, *Crit. Rev. Toxicol.,* 33, 505–542, 2003.

Guth, D.J., Carroll, R.J., Simpson, D.G., and Zhou, H., Categorical regression analysis of acute exposure to tetrachloroethylene, *Risk Anal*., 17,.321–332, 1997.

Hanna, L.M., Lou, D.-R., Su, S., and Jarabek, A.J., Mass transport analysis: inhalation RfC methods framework for interspecies dosimetric adjustment, *Inhalation Toxico1.,* 13, 437–463, 2001.

Hattis, D., Baird, S., and Goble, R., A straw man proposal for a quantitative definition of the RfD, *Drug Chem., Toxicol*., 25, 403–436, 2002.

IPCS, Guidance document for the use of data in development of chemical-specific adjustment factors (CSAF) for inter-species differences and human variability in dose/concentration response assessment, WHO/IPCS/01.4, draft final, 2001. Available from www.who.int/ipcs/en.

Jarabek, A.J., Inhalation RfC methodology: dosimetric adjustments and dose-response estimation of noncancer toxicity in the upper respiratory tract, *Inhalation Toxicol.,* 6 (suppl), 301–325, 1994.

Kimbell, J.S., and Subramaniam, R.P., Use of computational fluid dynamics models for dosimetry of inhaled gases in the nasal passages, *Inhalation Toxicol.,* 13, 325–334, 2001.

Lewis, S.C., Lynch, J.R., and Nikiforov, A.I., A new approach to deriving community exposure guidelines from "no-observed-adverse-effect levels," *Regul. Toxicol. Pharmacol.,* 11, 314–330, 1990.

Loehr, R.C., Goldstein, B.D., Nerode, A., and Risser, P.G., Safeguarding the future: credible science, credible decisions. The report of the expert panel on the role of science at EPA, Environmental Protection Agency, Washington, DC, March 19, 1992.

Mills, A. and Foureman, G.L., U.S. EPA's IRIS pilot program: establishing IRIS as a centralized, peer-reviewed data base with agency consensus, *Toxicology,* 127, 85–95, 1998.

NRC (National Research Council), Risk assessment in the Federal Government: managing the process, Committee on the Institutional Means for Assessments of Risk to the Public Health, Commission on Life Sciences, National Academy Press. Washington, DC, 1983.

NRC (National Research Council), Guidelines for developing community emergency exposure levels for hazardous substances, NRC Committee on Toxicology, National Academy Press, Washington, DC, 1993.

NRC (National Research Council), Science and judgment in risk assessment, National Academy Press, Washington, DC, 1994.

NRC (National Research Council), Understanding risk: informing decisions in a democratic society, Committee on Risk Characterization. Commission on Life Sciences. National Academy Press, Washington, DC, 1996.

Pieters, M.N., Kramer, H.J., and Slob, W., Evaluation of the uncertainty factor for subchronic-to-chronic extrapolation: statistical analysis of toxicity data, *Regul. Toxicol. Pharmacol*., 27, 108–111, 1998.

Renwick, A.G. and Lazarus, N.R., 1998. Human variability and noncancer risk assessment—an analysis of the default uncertainty factor., *Regul. Toxicol. Pharmacol.,* 27, 3–20, 1998.

Renwick, A., Ohanian, E., Dourson, M., Lake, B., Naumann, B.D., and Vu, V., Guidelines for application of chemical-specific adjustment factors in dose/concentration—response assessment, *Toxicology,* 181, 115–120, 2002.

Sexton, K., The role of scientific research in risk assessment and risk management decisions, *Otolaryngol. Head Neck Surg.* 106, 635–641, 1992.

U.S. Congress, Office of Technology Assessment (OTA), Researching health risks, OTA-BBS-570, U.S. Government Printing Office, Washington, DC, 1993.

U.S. EPA (Environmental Protection Agency), Risk assessment guidelines for carcinogenicity, mutagenicity, complex mixtures, suspect developmental toxicants, and estimating exposures, *Federal Register,* 51, 33992–34054, Sept. 24, 1986.

U.S. EPA (Environmental Protection Agency), Future risk: research strategies for the 1990's, SAB-EC88–040, Washington, DC, 1988a.

U.S. EPA (Environmental Protection Agency), Proposed guidelines for assessing reproductive risk, *Federal Register*, 53, 24834–24847, 1988b.

U.S. EPA (Environmental Protection Agency), Proposed guidelines for assessing male reproductive risk and request for comments, *Federal Register* 53, 24850–24869, 1988c.

U.S. EPA (Environmental Protection Agency), Research to improve health risk assessments (RIHRA) program, EPA/600/9-90-038, Washington, DC, 1990a.

U.S. EPA (Environmental Protection Agency), Reducing risk: setting priorities and strategies for environmental protection, SAB-EC-90-021, Washington, DC, 1990b.

U.S. EPA (Environmental Protection Agency), Guidelines for developmental toxicity risk assessment, *Federal Register*, 56, 63798–63826, 1991.

U.S. EPA (Environmental Protection Agency), Guidelines for exposure assessment, *Federal Register*, 57, 22888–22938, 1992.

U.S. EPA (Environmental Protection Agency), Methods for derivation of inhalation reference concentrations and application of inhalation dosimetry, Prepared by the Office of Research and Development, EPA/600/8-90/066F, Washington, DC, 1994a.

U.S. EPA (Environmental Protection Agency), Policy for risk characterization, Memorandum from U.S. Environmental Protection Agency Administrator Carol M. Browner, dated March 21, 1995, Washington, DC, 1995a.

U.S. EPA (Environmental Protection Agency), Guidance for risk characterization, Policy paper dated February 1995, Science Policy Council Washington, DC, 1995b.

U.S. EPA (Environmental Protection Agency), Proposed test rule for hazardous air pollutants; extension of comment period on proposed rule and extension of period for receipt of proposals for enforceable consent agreements for pharmacokinetics studies, *Federal Register*, 61 FR 33178, FRL-4869-1, 1996.

U.S. EPA (Environmental Protection Agency), Jess Rowland, Executive Secretary, Hazard Identification Assessment Review Committee, Hazard identification—toxicology endpoint selection process; a guidance document, August 11, 1998, Health Effects Division, Office of Pesticide Programs, Office of Prevention, Pesticides, and Toxic Substances, Washington, DC, 1998a.

U.S. EPA (Environmental Protection Agency), Office of Pesticide Programs—PHED surrogate exposure guide, August 1998, Office of Pesticide Programs, Office of Prevention, Pesticides, and Toxic Substances, Washington, DC, 1998b.

U.S. EPA (Environmental Protection Agency), Route-to-route extrapolations, Memorandum from J.E. Whalan and H.M. Pettigrew to M. Stasikowski, Health Effects Division, October 10, 1998, Health Effects

Division, Office of Pesticide Programs, Office of Prevention, Pesticides, and Toxic Substances, Washington, DC, 1998c.

U.S. EPA (Environmental Protection Agency), Inhalation risk characterizations and the aggregate risk index (ARI), Memorandum from J.E. Whalan and H.M. Pettigrew to M. Stasikowski, Health Effects Division, November 25, 1998, Health Effects Division, Office of Pesticide Programs, Office of Prevention, Pesticides, and Toxic Substances, Washington, DC, 1998d.

U.S. EPA (Environmental Protection Agency), General principles for performing aggregate exposure and risk assessments, November 28, 2001, Office of Pesticide Programs, Office of Prevention, Pesticides, and Toxic Substances, Washington, DC, 2001.

U.S. EPA (Environmental Protection Agency), A review of the reference dose and reference concentration processes, prepared by the Risk Assessment Forum, EPA/630/P-02/002F, 2002a.

U.S. EPA (Environmental Protection Agency), Guidance: waiver criteria for multiple-exposure inhalation toxicity studies, August 15, 2002, Health Effects Division, Office of Pesticide Programs, Office of Prevention, Pesticides, and Toxic Substances, Washington, DC, 2002b.

U.S. EPA (Environmental Protection Agency), Report of the risk assessment task force, Office of Research and Development, Washington, DC, 2004a.

U.S. EPA (Environmental Protection Agency), Air quality criteria document for particulate matter, EPA/600/P-99/002a-bF, 2004b.

U.S. EPA (Environmental Protection Agency), Benchmark dose software, Office of Research and Development, Washington, DC, *www.epa.gov/nceawww/bmds.htm,* 2004c.

U.S. EPA (Environmental Protection Agency), Integrated Risk Information System (IRIS), www.epa.gov/iris, 2004d.

U.S. EPA (Environmental Protection Agency), Inhalation RfC methodology: an inhalation dosimetry method (in press).

U.S. EPA (Environmental Protection Agency), IRIS glossary, www.epa.gov/iris/gloss8/htm.

2 The Development and Application of Acute Exposure Guideline Levels for Hazardous Substances

George M. Rusch

CONTENTS

2.1 INTRODUCTION

The tragic accidental release of methylisocyanate in Bhopal, India in 1985, resulted in the death of over 2000 people (U.S. EPA, 1988). This incident lead to the realization by both the chemical industry and the regulatory community of the need to pool resources for the development of emergency response plans for areas near chemical production sites (Gephart et al., 1986; Gephart and Moses, 1989). These activities have been occurring at many levels around the world. In Europe, for example, the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), formed a task force that published a guide for reviewing chemicals and estimating the hazard associated with an accidental release (ECETOC, 1991).

In the United States, under Superfund Amendments and Reauthorication Act (SARA) Title III and similar regulations, local communities are required to set up emergency response plans in locations where potential hazards exist, such as nuclear power plants or chemical-manufacturing operations (U.S. Environmental Protection Agency [U.S. EPA], 1987). These plans frequently include utilization of emergency response teams, which may include fire fighters, first aid professionals, and police. Where chemicals are involved, it is important to know the identity of the chemical, the

toxicity of the chemical, its physical properties (vapor pressure, flammability, reactivity), and the amount used or stored at the plant. It is also important to know the area that could be affected if the chemical were accidentally released and the airflow patterns around that area (Fleischer, 1980; Hanna and Drivas, 1987; National Research Council [NRC], 1993). With this information, local emergency response units can estimate potential dispersion patterns and plan for emergency evacuation routes if they are needed. In addition, the teams need to know how to monitor for the chemicals potentially involved in a local release, what constitutes appropriate personnel protective equipment, and what are effective first aid procedures. Finally, the local team needs to know if there is an appropriate remediation to disperse or neutralize the cloud. For example, with many reactive or corrosive chemicals, such as hydrofluoric acid, methyl isocyanate, nitric acid, and phosgene, creating a water mist screen can be an effective means for removing them from the air. However, for substances such as chlorine gas, benzene, carbon tetrachloride, and 1,1,1,2-tetrafluoroethane, creating a water mist screen would not be an effective approach. Most of this information should be well known to the plant safety personnel, especially current information on protective equipment, monitoring, and containment practices. Information on meteorological conditions must be obtained locally. Although the larger chemical companies may have the resources to provide information on toxicity, frequently this information is not available or it may be subject to skepticism by the local community if it comes from the company.

In the past 20 years two groups have been formed to address this issue. The first is the Emergency Response Planning Committee, a group sponsored by the American Industrial Hygiene Association (Rusch, 1993). This group was created in 1988 to review toxicology summary documents, originally prepared by toxicologists within the chemical industry and now written by committee members or other interested parties. The committee developed three advisory levels for a one-hour exposure to these substances. Emergency Response Planning Guideline (ERPG) 1 is a level that could be associated with slight irritation. ERPG-2 is a level that could be associated with developmental or subchronic toxicity. ERGP-3 is a level that is frequently associated with acute lethality.

The other committee, the Acute Exposure Guideline Level for Hazardous Substances, is a National Advisory Committee (NAC) sponsored by the EPA with membership drawn from many federal and state agencies and academia and industry (Table 2.1). Recently member participation has been expanded to include representatives from Germany, France, The Netherlands, and Russia. This committee first met in 1996 (Rusch et al., 2000). The development and review of the information required to conduct a risk assessment for a possible chemical exposure is a time-consuming and costly process. All stakeholders participate in the selection of chemicals for review and in the development of the Acute Exposure Guideline Level (AEGL) values. Thus, when the process has been completed, it has broad support and is applicable to many different situations.

To meet the needs of the many groups represented on this committee, three values are developed for each of several time periods, 10 min, 30 min, 1 h, 4 h, and 8 h. These AEGLs represent threshold exposure limits for the general public and can be applied to emergency exposure periods that would occur infrequently in a person's life (NRC, 2001).

AEGL-1 is the airborne concentration of a substance above which it is predicted that the general population, including sensitive individuals, could experience notable discomfort, irritation, or certain asymptomatic, nonsensory effects. However, the effects are not disabling and are transient and reversible upon cessation of exposure.

AEGL-2 is the airborne concentration of a substance above which it is predicted that the general population, including sensitive individuals, could experience irreversible or other serious, long-lasting adverse health effects, or an impaired ability to escape.

AEGL-3 is the airborne concentration of a substance above which it is predicted that the general population, including sensitive individuals, could experience life-threatening health effects or death.

Airborne levels below AEGL-1 represent exposure levels that could produce mild and progressively increasing but transient and nondisabling odor, taste, and sensory irritation or certain

TABLE 2.1 Composition of the Acute Exposure Guideline Level Committee

U.S. Federal Agencies
Agency for Toxic Substances and Disease Registry
Centers for Disease Control
Department of Defense
Army
Navy
Air Force
Department of Energy
Department of Transportation
Environmental Protection Agency
Office of Pollution Prevention & Toxics (OPPT)
Superfund
Regional Risk Assessors
Office of Research and Development (ORD)
Federal Emergency Management Agency
Food and Drug Administration
National Institute for Occupational Safety and Health
Occupational Safety and Health Administration
Department of Labour

Organizations
American Federation of Labor-Congress of Industrial Organization (AFL-CIO)
American Association of Poison Control Centers
American Association of State and Territorial Health Officials
American Industrial Hygiene Association
American College of Occupational and Environmental Medicine
Institute for Children's Environmental Health (ICEH)
STAPPA/ALAPCO(state local air quality)[a]
Environmental Justice Representative

States	Academia
California	Oregon State
Illinois	Rutgers University
Minnesota	University of Idaho
New York	
Texas	
Vermont	

Industry	International Liaison/Members
Honeywell International	
Arch Chemical	Germany
Exxon/Mobile	The Netherlands

[a] STAPPA/ALAPCO, State and Territorial Air Pollution Program Administrators/Association of Local Air Pollution Control Officials.

asymptomatic, nonsensory effects. With increasing airborne concentrations, above each AEGL, the likelihood of occurrence and the severity of effects described for each corresponding AEGL level progressively increases. Although AEGLs represent threshold levels for the general public, including susceptible subpopulations, such as infants, children, the elderly, persons with asthma, and those with other illnesses, it is recognized that individuals subject to unique or idiosyncratic responses could experience the effects described at concentrations below the corresponding AEGL.

In reviewing toxicology data and performing a risk assessment, one typically evaluates all the available pertinent data, selects key end points for each AEGL level, and applies an uncertainty factor to the threshold levels identified from those data. The result is a guidance exposure level that should protect the "exposed population" from the adverse effects associated with overexposure. In the development of AEGL values, the committee is concerned with the rare, accidental exposure and focuses its attention on the results from short-term studies. The results from studies involving multiple exposures are reviewed to provide a more comprehensive understanding of the toxicity of the substance under review. When multiple studies are available, especially studies involving different animal species, attention is paid to the relative agreement among these studies. If several studies involving multiple species give generally similar results, there is a high degree of confidence that a similar exposure to humans would have a similar result. In this case, a small uncertainty factor may be used. Also, attention is paid to the slope of the dose–response curve. If the slope is steep such that small changes in dose will move from a marked effect to a no-observed-effect level, again one may use a small uncertainty factor. However, a slope that is flat implies that effects may be seen over a wide span of doses. In this case a large uncertainty factor would be used.

Another important consideration is the number of animals used. For a study using small group sizes, a larger uncertainty factor would be used than for a study with either larger group sizes or more groups.

When greater variability exists, the committee tries to use information on mechanisms of action and species sensitivity to determine the most relevant species to use as the basis for understanding the risk to humans. Although frequently this will be the most sensitive species, occasionally, other species are selected as being more biologically relevant to humans (NRC, 2001).

2.2 TOXICOLOGICAL END POINTS USED TO DERIVE AEGL VALUES

Most often different studies will be used to serve as the basis for the three AEGL values defined above. If data are available from human studies, they will most frequently apply to AEGL-1 values. This level can be considered to represent an annoyance level. Above AEGL-1, symptoms of discomfort such as headache, strong objectionable odor, mild sensory irritation, and nausea can be noted. Below AEGL-1, some effects such as odor, taste, or mild sensory irritation may be observed. Studies that look at sensory irritation, altered pulmonary function, central nervous system (CNS) depression in humans, asymptomatic or nonsensory effects such as methemoglobin formation or miosis have been used to define this level.

For AEGL-2, one is concerned about effects that are serious or irreversible or that would impair ones ability to escape. Exposures at levels between AEGL-1 and AEGL-2 may result in clinical signs of toxicity, but these effects should be reversible. An AEGL-2 value could be the highest level that does not cause marked liver or kidney toxicity, pulmonary effects, or other systemic toxicity. It could also be just below a level that would be severely irritating or cause CNS depression to a degree that would impair escape or trigger an asthma episode.

AEGL-3 levels are most typically based on animal lethality studies. An estimate for a threshold for lethality is derived and reduced by the appropriate uncertainty factors. This could be based on an LC_{50} study, an approximate lethal concentration study, circulating levels of methemoglobin or carboxyhemoglobin, or a level that could produce cardiac arrhythmias or other potentially lethal acute effects.

2.3 SELECTION OF UNCERTAINTY AND MODIFYING FACTORS

In general, uncertainty factors are applied to cover extrapolations where the sensitivity of the population being protected may be different from the sensitivity of the population from which the data had been drawn. Additional safety factors frequently can be applied when the data are from a short-term study and the exposure will be for a longer time. This latter situation does not apply to AEGL values because they are derived for rare events.

For extrapolations from animals to humans, where there are few data, for example, a few acute studies in a single species, an uncertainty factor of 10 is most often applied. If, however, the data are from multiple species and they tend to give similar responses, or the most sensitive species is used, the uncertainty factor may be reduced to 3. Also if the species tested is known to respond similarly to humans, a factor of 3 or 1 may be used. If the data are developed from approved clinical studies in humans, an uncertainty factor of 1 will be used. Occasionally when the data are good, human equivalent concentrations may be calculated. Wherever data permit estimation of a more precise toxicology ratio between the test system and humans, that value will be used.

For extrapolations that are designed to protect the more sensitive members of the population, again up to a factor of 10 may be applied to the value considered safe for most people. For example, 10 was used to protect infants from exposure to aniline because they are highly sensitive to this substance. Most often, a factor of from 3 to 6 is used. This would be especially true if the data were developed by using approved clinical studies with well-defined protocols and clear end points. In some cases where data are representative of the sensitive members of the population, an uncertainty factor of 1 has been used. This was the case for an evaluation conducted using HCl, where the subjects included exercising asthmatics.

Where data are poor a modifying factor may be used to account for this additional uncertainty. These modifying factors range from 2 to 10. They are clearly indicated as a separate step in the risk assessment process and are applied after the application of the data-driven uncertainty factors. For a more detailed discussion of this approach the reader should consult the Standing Operating Procedures for the Acute Exposure Guideline Committee (NRC, 2001).

2.4 TIME EXTRAPOLATIONS

As has been noted, the committee develops AEGL values for exposures of 10 min to 8 h in duration. Data exist rarely for all these times. Many different approaches are available to the committee to extrapolate AEGL values from one period to another. In performing these extrapolations, the committee relies heavily on an understanding of the mechanism of toxic action of the chemical and empirical extrapolations using real data. Four approaches can be taken.

For cases where a response is viewed as a concentration threshold and independent of time, the same value may be used at all periods. An example would be the AEGL-1 response to an irritant. For ammonia, the current draft AEGL-1 value is 25 ppm for all intervals. This value is based on the irritation caused by exposure to ammonia at levels above 25 ppm and the fact that continuous exposure to ammonia at 25 ppm will not lead to an increase in severity of the effect. The AEGL-2 levels are based on a combination of time extrapolation and a constant exposure. Time extrapolation based on empirical data is used to develop AEGL-2 levels for 10 min to 1 h. These vary from 380 ppm at 10 min to 110 ppm at 1 h. The committee reasoned that exposure to 110 ppm would not be systemically toxic but would lead to impairment of escape as a consequence of the irritation; thus, that value was applied for exposures from 1 h to 8 h . For the AEGL-3 values, the values were derived by using a time extrapolation for all periods. Here, it was reasoned that the exposure levels could result in a systemic toxicity and therefore, as the exposure time increased, the level of exposure must decrease.

Extrapolations involving data derived under one set of time and concentration conditions to another can be estimated with reference to either Haber's rule (Rinehart and Hatch, 1964; Witschi,

1999), which states that the product of concentration times time is equal to a constant. Thus, this concept states that exposure concentration and exposure duration may be adjusted reciprocally to maintain a cumulative exposure constant (k) and that this cumulative exposure constant will reflect a specific quantitative and qualitative response. This relationship appears to be applicable primarily over short intervals and mostly to direct acting chemicals, such as hydrogen chloride and dibutylhexamethylenediamine:

$$\text{Concentration} \times \text{time} = \text{constant}.$$

ten Berge et al. (1986) evaluated this relationship and concluded that the relationship is more general with the product of concentration to the nth power times time being equal to a constant. (Note: This is the same relationship as concentration times time to the nth power.) Ammonia is an example of this relationship where $n = 2$. This has the effect of flattening the curve so concentration is more important than time.

$$(\text{Concentration})^n \times \text{time} = \text{constant}.$$

In this case, the value for the exponent n is derived from the experimental data.

If the data are not adequate, default values of one (from shorter to longer periods) or three (from longer to shorter periods) are used. Because these two values encompassed more than 90% of the n values calculated by ten Berge et al. (1986), they represent conservative approaches in that application of $n = 3$ for shorter periods of time results in a flatter curve and exposures for short periods will be lower than if $n = 1$. Likewise, using $n = 1$ when extrapolating to longer periods results in a lower recommended exposure level than if $n = 3$.

2.5 DRAFTING OF THE TECHNICAL SUPPORT DOCUMENTS

In the United States technical support documents summarizing key toxicology data on the chemical are written by a team of toxicologists at Oak Ridge Laboratory. Those written in the European Union are prepared by other groups of experts such as TNO in the Netherlands and Fobig in Germany. An AEGL chemical review group initially reviews drafts of these documents. This group is composed of three members of the AEGL Committee, a chemical manager, and two reviewers. When they have completed their review, the revised document is sent to the full AEGL committee for review, discussion, and the development of proposed AEGL values. The public is invited to attend these meetings. Adoption of AEGL values requires support from at least two-thirds of those committee members participating in the meeting. The executive summary with proposed AEGL values from the technical support document is then published in the *Federal Register* for comment. Comments are discussed at a full AEGL Committee meeting. Once the Committee feels that the public comments have been addressed, the values are considered to be interim. The technical support document is revised, as appropriate, based on the public comments, and sent to the NRC's Subcommittee on Acute Exposure Guideline Levels for a final peer review.

The NRC Subcommittee on Acute Exposure Guideline Levels conducts an extensive review of the technical support documents and recommended AEGL values. Their comments are considered by the NAC AEGL committee and may lead to modification of the document or modification of the risk assessment process and the values themselves. Upon acceptance by the NRC subcommittee, the AEGL values are considered final and will be published by the NRC. To date twenty chemicals have been finalized (NRC, 2000, 2002, 2003, 2004).

2.6 COMMITTEE MEMBERSHIP

With the participation by representatives from France, Germany, and The Netherlands, the AEGL process has become international. This is vital to the success of the program for two reasons. First,

a chemical spill does not recognize national borders and it would be wrong to have one level of exposure considered to be safe for the people living in one country and a different level considered as safe for the residents of the adjacent country. Also, many of the third world countries do not have the resources to develop their own chemical exposure guideline levels. Yet many of these same countries have extensive chemical production facilities. Faced with conflicting values from other nations, they would not know which values to use. If, however, they could adopt a set of values that represent a consensus standard from several nations they could have confidence that they were appropriately protecting their citizens.

2.7 SPECIFIC APPLICATIONS TO RISK ASSESSMENT

No general approach exists for the development of the Acute Exposure Guideline Levels. Each chemical must be evaluated independently. The rate of uptake and elimination, metabolism, irritant potency, systemic toxicity, degree of reversibility, half-life, and mode of action must all be considered. Some chemicals like ammonia may be predominately irritants; others, like phosgene, may exert delayed effects and not have adequate warning properties; still others, like aniline and tetrafluoroethane, may show an increased severity of a similar effect as concentration increases. Even similar compounds like hydrogen fluoride and hydrogen chloride can have different patterns of toxicity. In this case, both are irritants at low levels, but at higher levels hydrogen chloride is still an irritation, whereas while hydrogen fluoride can cause cardiac effects through depletion of serum calcium (NRC, 2004). Therefore, instead of trying to present a single approach to the development of the AEGLs, several examples of different approaches are given in the following sections. Each represents a somewhat different approach.

2.7.1 Aniline

Aniline (NRC, 2000, pp. 15–51) is an aromatic amine used chiefly in the chemical industry in the manufacture of dyes, dye intermediates, rubber accelerators, antioxidants, drugs, photographic chemicals, isocyanates, herbicides, and fungicides. Production of aniline oil in 1993 was approximately 1 billion pounds. The primary effect of an acute exposure to aniline is the oxidation of the hemoglobin in red blood cells (RBCs), resulting in the formation of methemoglobin. The effect may occur after inhalation, ingestion, or dermal absorption. In conjunction with methemoglobinemia, chronic exposures or exposures to high concentrations may produce signs and symptoms of headache, paresthesia, tremor, pain, narcosis/coma, cardiac arrhythmia, and possibly death.

No reliable information is available on human exposures via the inhalation route.

All the acute exposure guideline level values for analine are based on a study in which rats were exposed to aniline at concentrations of 0, 10, 30, 50, 100, or 150 ppm for 8 or 12 h (Kim and Carlson, 1986). The only reported effect has been methemoglobin formation. The relationship between aniline concentration and methemoglobin formation appeared to be linear. Furthermore, at a constant concentration (100 ppm), the formation of methemoglobin between 3 and 8 h was basically linear, reaching an asymptote at 8 h. Based on the linear relationship between aniline concentration and methemoglobin formation and between methemoglobin formation and time at constant aniline concentration, a linear relationship between concentration and exposure duration ($C^1 \times t = k$) was chosen for time scaling aniline concentrations to the appropriate AEGL exposure durations.

The AEGL-1 was based on the exposure of rats to 100 ppm of analine for 8 h, which resulted in elevation of methemoglobin from a control value of 1.1% to 22%. A review of the published data indicates that methemoglobin levels of 15–20% in humans results in clinical cyanosis but no hypoxic symptoms. Although inhalation data for comparison purposes are not available, oral ingestion data suggest that humans may be considerably more sensitive to methemoglobin-forming chemicals than rats. Therefore, a default uncertainty factor of 10-fold was used for interspecies extrapolation. Several sources also indicate that newborns may be more sensitive to methemoglobin-forming chemicals than

adults. Because of the absence of specific quantitative data on sensitive human subpopulations and the fact that there are data suggesting greater susceptibility of infants, a default uncertainty factor of 10-fold was used for intraspecies extrapolation. It is believed that an intraspecies uncertainty factor of 10 is protective of the general population including susceptible individuals. The default uncertainty factors of 10 for each of the interspecies and intraspecies variabilities are also supported by the small database of information and the lack of reliable human inhalation studies. The data were scaled across time by using $C^1 \times t = k$ because of data indicating a linear relationship between concentration and exposure duration as related to methemoglobin formation.

The AEGL-2 was based on the same study with rats in which a concentration of 150 ppm for 8 h resulted in elevation of methemoglobin from a control value of 1.1% to 41%. This level of methemoglobin was associated with fatigue, lethargy, exertional dyspnea, and headache in humans and was considered the threshold for disabling effects. Since the same mode of action applies to AEGL-2 effects, the 150-ppm concentration was divided by a combined uncertainty factor of 100 and scaled across time by using the same reasons and relationships as those used for the AEGL-1.

Data on concentrations of aniline inducing methemoglobin levels at the threshold for lethality were not available. Because the relationship between the concentration of aniline and methemoglobin formation is linear, the dose–response curve from the study on which the AEGL-1 and AEGL-2 values were based was extrapolated to a concentration resulting in >70% formation of methemoglobin, the threshold for lethality. The concentration of 250 ppm for 8 h was chosen as the threshold for lethality. Because the same mode of action applies to AEGL-3 effects, the 250-ppm concentration was divided by a combined uncertainty factor of 100 and scaled across time using the same reasons and relationships as those used for the AEGL-1.

Several studies with rats support the AEGL-3 values. A 10-min exposure to aniline at 15,302 ppm resulted in no toxic effects, and a 4-h exposure at 359 ppm resulted in severe toxic effects but no deaths. Dividing these values by a total uncertainty factor of 100 and scaling across time by using $C^1 \times t = k$ results in values similar to those derived from the study above. Studies with repeated exposures of rats resulted in additional effects on the blood and spleen, but concentrations up to 87 ppm, 6 h/d, 5 d/weeks for 2 weeks were not disabling or life threatening.

The derived AEGLs are listed in summary table below. Because aniline is absorbed through the skin in quantities sufficient to induce systemic toxicity, a skin notation was added to the summary table. The reported odor threshold for aniline ranges from 0.012 to 10 ppm. Therefore, the odor of aniline will be noticeable by most individuals at the AEGL-1 concentrations. The odor is somewhat pungent but not necessarily unpleasant.

AEGL Values for Aniline[a, b]

Classification	30 min	1 h	4 h	8 h	End Point (Reference)
AEGL-1[c] (nondisabling)	16 (61)	8.0 (30)	2.0 (7.6)	1.0 (3.8)	22% Methemoglobin, cyanosis
AEGL-2 (disabling)	24 (91)	12 (46)	3.0 (11)	1.5 (5.7)	41% Methemoglobin, lethargy
AEGL-3 (lethal)	40 (152)	20 (76)	5.0 (19)	2.5 (9.5)	>70% Methemoglobin, lethality

[a] Values are stated in ppm (mg/m^3).

[b] Cutaneous absorption of the neat material may occur, adding to the systemic toxicity.

[c] The aromatic, aminelike odor of aniline will be noticeable by most individuals at these concentrations.

2.7.2 Phosgene

Phosgene (NRC, 2002, pp. 15–51) is a colorless gas at ambient temperature and pressure. Its odor has been described as similar to new-mown hay. Phosgene is manufactured from a reaction of carbon monoxide and chlorine gas in the presence of activated charcoal. The production of dyestuffs, isocyanates, carbonic acid esters (polycarbonates), acid chlorides, insecticides, and pharmaceutical chemicals

requires phosgene. Manufacture of phosgene is approximately 1 million tons per year in the United States, and more than 10,000 workers are involved in its manufacture and use. Manufacture of phosgene in the United States is almost entirely captive—it is used in the manufacture of other chemicals within a plant boundary.

Inhalation is the most important route of exposure for phosgene. Because of phosgene's mild upper respiratory tract eye, and skin irritancy and mildly pleasant odor, an exposed victim may not actively seek an avenue of escape before lower respiratory tract damage has occurred (Currie et al., 1987; Lipsett et al., 1994). Pulmonary edema is the cause of death after a clinical latency period of <24 hours.

Odor and irritation cannot be used as a warning for potential exposure. The odor threshold is reported to be between 0.5 and 1.5 ppm, a value above or approaching AEGL-2 and AEGL-3 values, and tolerance to the pleasant odor of phosgene occurs rapidly. Furthermore, following odor detection and minor irritation, serious effects may occur after a clinical latency period of <24 h. Therefore, it was deemed inappropriate to set AEGL-1 levels for phosgene.

AEGL-2 values were based on chemical pneumonia in rats (exposure at 2 ppm for 90 min) (Gross et al., 1965). An uncertainty factor (UF) of 3 was applied for interspecies extrapolation because little species variability is observed for lethal and nonlethal end points after exposure to phosgene. A UF of 3 was applied to account for sensitive human subpopulations due to the steep concentration–response curve and because the mechanism of phosgene toxicity (binding to macromolecules and causing irritation) is not expected to vary greatly among individuals. Therefore, the total UF is 10. The 1.5-h value was then scaled to the 30-min and 1-, 4-, and 8-h AEGL exposure periods by using $C^n \times t = k$, where $n = 1$. Haber's law has been shown to be valid for phosgene within certain limits and was originally derived, in part, from phosgene data. The 30-min value was also adopted as the 10-min value, because extrapolation would have yielded a 10-min AEGL-2 value approaching concentrations that produce alveolar edema in rats. Alveolar pulmonary edema was observed in rats exposed to phosgene at 5 ppm for 10 min (Diller et al., 1985). Applying a total UF of 10 to this data point yields a supporting 10-min AEGL-2 value of 0.5 ppm.

The 30-min and 1-, 4-, and 8-h AEGL-3 values were based on the highest concentration not causing mortality in the rat after a 30-min exposure (15 ppm) (Zwart et al., 1990). A UF of 3 was applied for interspecies extrapolation because little species variability is observed for lethal and nonlethal end points after exposure to phosgene. A UF of 3 was applied to account for sensitive human subpopulations because of the steep concentration–response curve and because the mechanism of phosgene toxicity is not expected to vary greatly between individuals. Therefore, the total UF is 10. The value was then scaled to the 1-, 4-, and 8-h AEGL periods using $C^n \times t = k$, where $n = 1$ for the reason discussed above. The 10-min AEGL-3 value was based on the highest concentration not causing mortality in the rat or mouse (36 ppm) after a 10-min exposure.

AEGL Values for Phosgene[a]

Classification	10 min	30 min	1 h	4 h	4 h	End Point (Reference)
AEGL-1 (nondisabling)	NA	NA	NA	NA	NA	NA
AEGL-2 (disabling)	0.60 (2.5)	0.60 (2.5)	0.30 (1.2)	0.08 (0.33)	0.04 (0.16)	Chemical pneumonia rats
AEGL-3 (lethal)	3.6 (15)	1.5 (6.2)	0.75 (3.1)	0.20 (0.82)	0.09 (0.34)	Highest concentration causing no mortality in the rat after a 30-min or 10-min exposure

[a] Values are stated in ppm (mg/m^3).

2.7.3 Hydrogen Chloride

Hydrogen chloride (NRC, 2004) is a colorless gas with a pungent suffocating odor. It is used in the manufacture of organic and inorganic chemicals, steel pickling, food processing, and processing of

minerals and metals. A large amount of hydrogen chloride is released from solid rocket fuel exhaust. It is an upper respiratory tract irritant at relatively low concentrations and may cause damage to the lower respiratory tract at higher concentrations. Hydrogen chloride is soluble in water, and the aqueous solution is highly corrosive.

The AEGL-1 values are based on a 45-min no adverse effect level in exercising adult asthmatics (Stevens et al., 1992). No uncertainty factors were applied for inter- or intraspecies variability because the study population consisted of sensitive humans (asthmatics). Additionally, the same value was applied across the 10- and 30-min, and 1-, 4-, and 8-h exposure time points because mild irritantancy is a threshold effect and generally does not vary greatly over time. Thus, continuous exposure will not result in an enhanced effect.

The AEGL-2 was based on severe nasal or pulmonary histopathology in rats exposed to 1300 ppm hydrogen chloride for 30 min (Stavert et al., 1991). An uncertainty factor of 3 was applied for interspecies variability because the test species (rodents) is two to three times more sensitive to the effects of hydrogen chloride than primates; therefore, a larger uncertainty factor was not warranted. An uncertainty factor of 3 was applied for intraspecies extrapolation because the mechanism of action is direct irritation and the subsequent effect or response is not expected to vary greatly among individuals. An additional modifying factor of 3 was applied to account for the sparse database of effects defined by AEGL-2 and because the effects observed at the concentration used to derive AEGL-2 values were somewhat severe. Thus, the total uncertainty and modifying factor adjustment is 30-fold. It was then timescaled to the specified 10- and 30-min, and 1-, 4-, and 8-h AEGL exposure periods by using the $C^n \times t = k$ relationship, where $n = 1$ based on regression analysis of combined rat and mouse median lethal concentration (LC_{50}) data (1–100 min).

The AEGL-3 was based on an estimated nonlethal level of one-third of a 1-h LC_{50} reported for rats (Wohlslagel et al., 1976; Vernot et al., 1977). Again, an uncertainty factor of 3 was applied for interspecies variability because the test species (rodents) is two to three times more sensitive to the effects of hydrogen chloride than primates. An uncertainty factor of 3 was applied for intraspecies extrapolation because the mechanism of action is direct irritation and the subsequent effect or response is not expected to vary greatly among individuals. A modifying factor was not needed to derive these values. Thus, the total UF is 10. It was then timescaled to the specified 10- and 30-min, and 1-, 4-, and 8-h AEGL exposure periods using the $C^n \times t = k$ relationship, where $n = 1$ based on regression analysis of combined rat and mouse LC_{50} data (1–100 min).

The calculated values are listed in the table below.

AEGL Values for Hydrogen Chloride[a]

Classification	10 min	30 min	1 h	4 h	8 h	End Point (Reference)
AEGL-1 (nondisabling)	1.8 (2.7)	1.8 (2.7)	1.8 (2.7)	1.8 (2.7)	1.8 (2.7)	No-adverse-effect level in exercising human asthmatics
AEGL-2 (disabling)	130 (197)	43 (65)	22 (33)	56.4 (8.1)	2.7 (4.1)	Histopathology in rats
AEGL-3 (lethal)	620 (937)	210 (313)	100 (155)	26 (39)	13 (19)	Estimated NOEL for death from 1-h rat LC_{50}

[a] Values are stated in ppm (mg/m^3).

2.7.4 Hydrogen Fluoride

Hydrogen fluoride (HF) (NRC, 2004) is a colorless, highly irritating, and corrosive gas. Reaction with water is rapid, producing heat and hydrofluoric acid. Hydrogen fluoride is used in the manufacture of artificial cryolite; in the production of aluminum, fluorocarbons, and uranium hexafluoride; as a catalyst in alkylation processes during petroleum refining; in the manufacture of fluoride salts; and

in stainless steel pickling operations. It is also used to etch glass and as a cleaner in metal-finishing processes.

Hydrogen fluoride is a severe irritant to the eyes, skin, and nasal passages; high concentrations may penetrate to the lungs resulting in edema and hemorrhage. Data on irritant effects in humans and lethal and sublethal effects in six species of mammals (monkey, dog, rat, mouse, guinea pig, and rabbit) were available for development of AEGL values. The data were considered adequate for derivation of the three AEGL classifications for five exposure periods. Regression analyses of the reported concentration–exposure durations for lethality for the animal species determined that the relationship between concentration and time is $C^2 \times t = k$.

AEGL-1 was based on a concentration of 3 ppm for 1 h, which was the threshold for pulmonary inflammation, as evidenced by an increase in the percentage of several inflammatory parameters such as CD3 cells and myeloperoxidase in the bronchoalveolar lavage fluid of 20 healthy exercising adult subjects (Lund et al., 1999). There were no increases in neutrophils, eosinophils, protein, or methyl histamine at this or the next higher average exposure concentration of 4.7 ppm. There were no changes in lung function and only minor increased symptoms of irritation at this concentration (Lund et al., 1997). Although healthy adults were tested, several individuals had increased immune factors, indicating atopy. The 3-ppm concentration was divided by an intraspecies uncertainty of 3 to protect susceptible individuals. Because no effects occurred on respiratory parameters of healthy adults at concentrations that ranged up to 6.34 ppm in this study and up to 8.1 ppm for 6 h/d with repeated exposures in a supporting study (Largent, 1960, 1961), it is felt that the calculated AEGL-1 values would be protective of a population of asthmatics. Although the Lund et al. (1999) study was for a 1-h duration, the longer exposures to higher concentrations in the supporting study (Largent, 1960, 1961), support application of the 1-ppm concentration for up to 8 h.

The 10-min AEGL-2 value was based on an absence of serious pulmonary or other adverse effects in rats during direct delivery of HF to the trachea at a concentration of 950 ppm for an exposure period of 10 min. This reported concentration–exposure value of 950 ppm for 10 min was adjusted by a combined UF of 10: an UF of 3 for interspecies variation, because the rat was not the most sensitive species in other studies (but direct delivery to the trachea is a sensitive model), and an intraspecies UF of 3 to protect susceptible individuals. This 10-min value is clearly below serious injury categories of data from tests in monkeys, rats, dogs, mice, guinea pigs, and rabbits.

The 30-min and the 1-, 4-, and 8-h AEGL-2 values were based on a study in which dogs exposed to 243 ppm for 1 h showed signs of more than mild irritation, including blinking, sneezing, and coughing (Rosenholtz et al., 1963). This value is one-fourth of the rat LC_{50} value in the same study. Rats exposed to a similar concentration (291 ppm) developed moderate eye and nasal irritation. The next higher concentration (489 ppm for 1 h) resulted in respiratory distress and severe eye and nasal irritation in the rat, signs more severe that those ascribed to AEGL-2. The moderate eye and nasal irritation observed in dogs at 243 ppm was considered the threshold for impaired ability to escape. The 1-h value of 243 ppm was adjusted by a total UF of 10: a UF of 3 for intraspecies variation, because the dog is a sensitive species for sensory irritation, and a UF of 3 to protect susceptible individuals. The values were scaled across time by using $C^2 \times t = k$, where the value of $n = 2$ was derived from concentration–exposure duration relationships based on lethality in animals studies. Note that the resulting 30-min AEGL-2 value of 34 ppm is similar to the 32-ppm concentration that was tolerated for only several minutes by human subjects in the study of Machle et al. (1934). Using a larger total uncertainly factor such as 30 would reduce the 1-h value to 8 ppm, a concentration that resulted in only slight irritation in healthy adults during repeated, intermittent exposures. Because a timescaled 8-h value of 8.6 ppm was inconsistent with the study of Largent (1960, 1961) in which humans subjects intermittently inhaling 8.1 ppm suffered no greater effects than slight irritation, the 8-h AEGL-2 value was set equal to the 4-h value.

The 10-min AEGL-3 value was based on the reported 10-min lethal threshold in orally cannulated rats of 1764 ppm (Dalbey, 1996; Dalbey et al., 1998). This value was rounded down to 1700 ppm and adjusted by UFs of 3 for interspecies differences (LC_{50} values differ by a factor of approximately 2–4

between the mouse and rat) and 3 to protect susceptible individuals. The total adjustment for UFs for the 10-min AEGL-3 value was 10. Application of a larger UF such as 30 would reduce the 10-min AEGL-3 to below the 10-min AEGL-2.

The 30-min and the 1-, 4-, and 8-h AEGL-3 values were derived from a reported 1-h exposure resulting in no deaths in mice (Wohlslagel et al., 1976). The data indicated that the value of 263 ppm was the threshold for lethality. A comparison of LC_{50} values among species in several studies indicated that the mouse was the most sensitive species in lethality studies. The 1-h value of 263 ppm was adjusted by an interspecies UF of 1, because the mouse was the most sensitive species, and an intraspecies UF of 3 to protect susceptible individuals. A modifying factor of 2 was applied to account for the fact that the highest nonlethal value was close to the LC_{50} of 342 ppm. The resulting value was scaled to the other AEGL-specified exposure periods using $C^n \times t = k$, where $n = 2$. A total uncertainty plus modifying factor of 6 is reasonable and sufficient, because application of a total uncertainty and modifying factor of 20 (3 each for inter- and intraspecies uncertainties and 2 as a modifying factor) would reduce the predicted 6-h AEGL-3 level to 5.4 ppm, a concentration below the 8.1 ppm concentration which produced only slight irritation in humans when reached intermittently during daily 6-h exposures (Largent, 1960, 1961). Because HF is well scrubbed at low concentrations and because the timescaled 8-h AEGL-3 value of 15 ppm was inconsistent with data from repeated exposures in animal studies, the 8-h value was set equal to the 4-h value. Rhesus monkeys survived a 6–7 h/d, 50-day exposure to 18.5 ppm (Machle and Kitzmiller, 1935).

Values are in the summary table below.

AEGL Values for Hydrogen Fluoride[a]

Classification	10 min	30 min	1 h	4 h	8 h	End Point (Reference)
AEGL-1 (nondisabling)	1.0 (0.8)	1.0 (0.8)	1.0 (0.8)	1.0 (0.8)	1.0 (0.8)	Threshold, pulmonary inflammation, humans
AEGL-2 (disabling)	95 (78)	34 (28)	24 (20)	12 (9.8)	12 (9.8)	NOAEL for lung effects in cannulated rats[b]; sensory irritation in dogs[c]
AEGL-3 (lethal)	170 (139)	62 (51)	44 (36)	22 (18)	22 (18)	Lethality threshold in cannulated rats[d]; lethality threshold in mice[e]

[a] Values are stated in ppm (mg/m^3).

[b] 10-min AEGL-2 value.

[c] 30-min and 1-, 4-, and 8-h AEGL-2 values.

[d] 10-min AEGL-3 value.

[e] 30-min and 1-, 4-, and 8-h AEGL-3 values.

2.7.5 Tetrafluoroethane

Hydrofluorocarbon-134a or 1,1,1,2-tetrafluoroethane (HFC-134a) (NRC, 2003) has been developed as a replacement for fully halogenated chlorofluorocarbons because, compared with chlorofluorocarbons, its residence time in the atmosphere is shorter and its ozone-depleting potential is insignificant. HFC-134a is used in refrigeration and air-conditioning systems as a blowing agent for polyurethane foams and as a propellant for medical aerosols. HFC-134a is a colorless gas with a faint ethereal odor that may go unnoticed by most individuals.

HFC-134a has a very low acute inhalation toxicity. Both uptake and elimination are rapid, but uptake is low, and most of the compound is exhaled unchanged. Consequences of acute HFC-134a inhalation have been studied with human subjects and several animal species, including the monkey, dog, rat, and mouse. Considerable inhalation data from controlled studies with healthy human

subjects and patients with respiratory tract diseases are available. Studies addressing repeated and chronic exposures, genotoxicity, carcinogenicity, neurotoxicity, and cardiac sensitization were also available. At high concentrations, halogenated hydrocarbons may produce cardiac arrhythmias; this end point was considered in development of AEGL values.

Adequate data were available for development of the three AEGL classifications. Based on the observations that (1) blood concentrations in humans, rapidly approach equilibrium with negligible metabolism and tissue uptake and (2) the end point of cardiac sensitization is a blood concentration–related threshold phenomenon, the same concentration was used across all AEGL periods for the respective AEGL classifications.

The AEGL-1 concentration was based on a 1-h no-effect concentration of 8000 ppm in healthy human subjects (Emmen et al., 2000). This concentration was without effect on pulmonary function, respiratory parameters, the eyes (irritation), or the cardiovascular system. Because this concentration is considerably below that causing any adverse effect in animal studies, an intraspecies UF of 1 was applied. The intraspecies UF of 1 is supported by the absence of adverse effects in therapy tests with patients with severe chronic obstructive pulmonary disease and adult and pediatric patients with asthma who were tested with metered-dose inhalers containing HFC-134a as the propellant. Because blood concentrations in this study approached equilibrium after 55 min of exposure and effects are determined by blood concentrations, the value of 8000 ppm was made equivalent across all periods. The AEGL-1 of 8000 ppm is supported by the absence of adverse effects in experimental animals that inhaled considerably higher concentrations. No adverse effects were observed in rats exposed at 81,000 ppm for 4 h or in rats exposed repeatedly at 50,000 or 100,000 ppm for 6 h/d. Adjustment of the 81,000 ppm value by interspecies and intraspecies UFs of 3 each, for a total of 10, results in essentially the same concentration (8100 ppm) as the AEGL-1 based on data from humans.

The AEGL-2 was based on the no-effect concentration of 40,000 ppm for cardiac sensitization in dogs (Hardy et al., 1991). In this test the dog is given an injection of epinephrine just below that which, by itself, would cause a cardiac arrhythmia. This is done both before the exposure and during the exposure to the agent. A positive response is the development of a cardiac arrhythmia during the exposure but not before the exposure. This results in a model that is from 3 to 10 times as sensitive as an untreated dog, and one that appears to be far more sensitive than humans, based on more than 35 years of evaluation (Brock et al., 2003). As a consequence, an interspecies UF of 1 was used in extrapolating from the dog to humans. Although it has been shown that, even with coronary disease, there is no difference in response to account for the possibility that some members of the general population may be highly sensitive to the exposure, an UF of 3 was applied to account for these individuals. Again, using the reasoning that peak circulating concentration is the determining factor in 1,1,1,2-tetrafluoroethane-induced cardiac sensitization, and exposure duration past 5 min was not of importance, the resulting value of 13,000 ppm was applied to all periods.

The AEGL-3 concentration was based on a concentration of 80,000 ppm, which caused marked cardiac toxicity but no deaths in dogs (Hardy et al., 1991). The cardiac sensitization model with the dog is considered an appropriate model for humans; therefore, an interspecies UF of 1 was applied. Because the cardiac sensitization test is highly sensitive as the response to epinephrine is optimized, an intraspecies UF of 3 was applied to account for sensitive individuals. Cardiac sensitization is concentration dependent; duration of exposure does not influence the concentration at which this effect occurs. Using the reasoning that peak circulating concentration is the determining factor in HFC-134a cardiac sensitization, and exposure duration is of lesser importance, the resulting value of 27,000 ppm was applied to all periods.

2.7.6 Sarin (Nerve Agent GB)

The nerve agents for which AEGL analyses have been performed include the G series agents (GA [tabun], GB [sarin], GD [soman], and GF) and nerve agent VX (NRC, 2003). These agents are all toxic ester derivatives of phosphonic acid containing either a cyanide, fluoride, or sulfur substituent group;

AEGL Values for HFC-134a[a]

Classification	10 min	30 min	1 h	4 h	8 h	End Point (Reference)
AEGL-1 (nondisabling)	8,000 (34,000)	8,000 (34,000)	8,000 (34,000)	8,000 (34,000)	8,000 (34,000)	No effects, humans
AEGL-2 (disabling)	13,000 (55,250)	13,000 (55,250)	13,000 (55,250)	13,000 (55,250)	13,000 (55,250)	No effect, cardiac sensitization, dogs[b]
AEGL-3 (lethal)	27,000 (114,750)	27,000 (114,750)	27,000 (114,750)	27,000 (114,750)	27,000 (114,750)	Marked effect, cardiac sensitization, dogs[b]

[a] Values are stated in ppm (mg/m^3).

[b] Response to challenge dose of epinephrine (cardiac sensitization test).

they are commonly termed "nerve" agents because of their anticholinesterase properties. These compounds were developed as chemical warfare agents, and one (agent GB, or sarin) was used by terrorists in the 1995 exposure incident that took place in the Tokyo subway system.

It is a viscous liquid. Toxic effects may occur at vapor concentrations below those of odor detection. However, the vapor pressure and acute toxicity is sufficiently high for the vapor to be rapidly lethal.

Exposure to acutely toxic concentrations of GB can result in excessive bronchial, salivary, ocular, and intestinal secretions and sweating, miosis, bronchospasm, intestinal hypermotiity, bradycardia, muscle fasciculations, twitching, weakness, paralysis, loss of consciousness, convulsions, depression of the central respiratory system, and death. Minimal effects observed at low vapor concentrations include miosis (contraction of the pupils of the eye, with subsequent decrease in pupil area), tightness of the chest, rhinorrhea, and dyspnea (Dunn and Sidell, 1989).

The results of agent GB vapor exposure studies conducted with human volunteers indicate that the threshold for miosis and other minimal toxic effects falls in the range of 0.05–0.5 mg/m^3 for 10- to 30-min exposures. The findings are based on the results of low-concentration nerve agent exposures of informed volunteers who were under clinical supervision during the periods of exposure and for postexposure periods of several months.

A concern associated with symptomatic exposures to anticholinesterase compounds such as the nerve agents is the possibility of chronic neurological effects. At present, no evidence indicates that asymptomatic exposures to any of the nerve agents result in chronic neurological disorders. In general, the available epidemiological data indicate that most clinical signs of toxicity resolve within hours to days; severe miosis can require several months after exposure for resolution. However, several studies have shown that subclinical signs may persist for longer periods. After the chemical terrorist attacks with nerve agent GB (sarin) that occurred in Japan in 1994 and 1995, clinical signs of agent toxicity were no longer apparent in the surviving victims 3 months after the exposures had occurred; however, several studies conducted on a small number of asymptomatic individuals 6–8 months after the attack revealed subclinical signs of neurophysiological deficits as measured by event-related and visual evoked potentials, psychomotor performance, and increases in postural sway.

In a study of workers who had been occupationally exposed to agent GB, altered electroencephalograms (EEGs) were recorded 1 year or more after the last exposure had occurred. Spectral analysis of the EEGs indicated significant increases in brain beta activity (12–30 Hz) in the exposed group when compared with nonexposed control subjects, and sleep EEGs revealed significantly increased rapid eye movement in the exposed workers; however, those observations were not clinically significant.

Animal data from vapor and oral exposure studies suggest that agent GB does not induce reproductive or developmental effects in mammals. Neither agent GB nor agent VX was found to be genotoxic in a series of microbial and mammalian assays. No evidence indicates that agent GB is carcinogenic.

AEGL-1 values for agent GB were derived from a well-conducted study on adult female Sprague–Dawley rats exposed whole-body in a dynamic airflow chamber to a range of GB vapor concentrations (0.01–0.48 mg/m^3) over three time durations (10 min, 60 min, or 240 min) (Mioduszewski et al.,

2002b). Analysis of rat pupil diameters assessed pre- and postexposure allowed determination of EC_{50} values for miosis (defined as a postexposure pupil diameter of 50% or less of the preexposure diameter in 50% of the exposed population). Blood samples collected from tail vein and heart at 60 min and 7 d postexposure indicated no significant change from preexposure baseline in monitored blood RBC-cholinesterase (ChE), butyrylcholinesterase (BuChE), or carboxylesterase. No other clinical signs were evident throughout the duration of the study. Gender differences (females more susceptible) were statistically significant at 10 min ($p = 0.014$) and 240 min ($p = 0.023$), but not at 60 min ($p = 0.054$). This is a well-defined animal end point in a susceptible gender, and it is transient, reversible, and nondisabling.

In terms of potential effects on humans, an EC_{50} for miosis is not considered an adverse effect. This degree of miosis is the first measurable change, by modern and reproducible techniques, in the continuum of response to anticholinesterase compounds. In bright daylight or under bright lighting, a 50% reduction in pupil diameter would result in greater visual acuity among some members of the affected exposed population and no marked reduction in visual acuity for most of the affected population. In twilight or dim-light conditions, 50% reduction in pupil diameter in some persons would result in reduced visual acuity and less-than-optimal performance of tasks requiring operation of vehicular controls, monitoring or tracking on computer screens, reading of fine text, or shifts in focus between near and far fields. For individuals with central cataracts, the effects would be more pronounced at all illumination levels. During the Tokyo Subway Incident (terrorist release of GB), persons experiencing $\geqslant$50% reduction in pupil diameter were able to self-rescue and to render aid to others.

The human data of Harvey (1952) and Johns (1952) indicate that some adult humans exposed to concentrations within the exposure range tested by Mioduszewski et al. (2002b) would experience some discomfort (headache, eye pain, nausea, etc.) in addition to miosis, corresponding to $\leqslant$50% pupil area decrement but no disability. Compared with the available human data, the miosis data derived from the study on rats are considered a more reliable data set because they are based on current and multiple analytical techniques for quantifying exposures and measuring miosis and because they apply an experimental protocol incorporating sufficiently large test and control populations.

The weight-of-evidence analysis indicates reasonable concordance among AEGL-1 estimates derived from the female Sprague–Dawley rat, the marmoset, and the human data sets identified above.

The AEGL-2 values for agent GB were derived from a study in which miosis, dyspnea, photophobia, inhibition of RBC-ChE, and changes in single-fiber electromyography (SFEMG) were observed in human volunteers after a 30-min exposure at 0.5 mg/m^3.

Although not considered debilitating or permanent effects in themselves, SFEMG changes are considered an early indicator of exposures that potentially could result in more significant effects. Selection of this effect as a protective definition of an AEGL-2 level is considered appropriate given the steep dose–response toxicity curve of nerve agents (Mioduszewski et al., 2000, 2001, 2002a). The concept of added precaution for steep dose–response is consistent with the emergency-planning guidance for nerve agents that was developed by the National Center for Environmental Health for the Centers for Disease Control and Prevention.

Animals exposed to low concentrations of the G agents exhibit the same signs of toxicity as humans, including miosis, salivation, rhinorrhea, dyspnea, and muscle fasciculations. Studies on dogs and rats indicate that exposures to GB at 0.001 mg/m^3 for up to 6 h/d are unlikely to produce any signs of toxicity.

Because exposure–response data were not available for all the AEGL-specific exposure durations, temporal extrapolation was used in the development of AEGL values for some of the SEGL-specific periods. The concentration–exposure time relationship for many systemically acting vapors and gases may be described by $C^n \times t = k$, where the exponent n ranges from 0.8 to 3.5. The temporal extrapolation used here is based on a log-log linear regression of the LC_{01} lethality of GB in female Sprague–Dawley rats and a log-log linear regression of female SD rat miosis data following GB vapor exposure for durations of 10–240 min (Mioduszewski et al., 2000, 2001, 2002a, 2002b). Regression analysis of the LC_{01} values yields an n value of 1.93 with an r^2 of 0.9948, and regression analysis of

the miosis data yields an n value of 2.00 with an r^2 of 0.4335 (24 data points; see Appendix B). Given that all mammalian toxicity end points observed in the data set for all nerve agents represent different points on the response continuum for anticholinesterase exposure, and that the mechanism of acute mammalian toxicity (cholinesterase inhibition) is the same for all nerve agents, the experimentally derived $n = 2$ from the rat lethality and the miosis data sets was used as the scaling function for all AEGL derivations rather than a default value. However, because of uncertainties associated with some of the exposure measurements in the earlier studies, the rat data of Mioduszewki et al. were determined to be the best source of an estimate for n. The n value of 2 was used to extrapolate for exposure periods for which there were no experimental data. Those included (1) the 8-h AEGL-3 value (extrapolated from experimental data for 6 h); (2) the 30-min and 8-h AEGL-1 values (extrapolated from 10-min and 4-h experimental data); and (3) all the AEGL-1 values (extrapolated from experimental data for 30 min).

In consultation with experimental investigators at Porton Down (United Kingdom) and the TNO Prins Maurits Laboratory (The Netherlands), the analysis has determined that the miotogenic response of mammalian eyes to agent GB vapor exposure is similar across species. The species evaluated include standard laboratory animals (rabbits, rats, guinea pigs), nonhuman primates (marmosets), and humans. As a consequence, the interspecies UF for critical AEGL-1 end point of miosis is considered equal to 1. To accommodate known variation in human cholinesterase and carboxylesterase activity that may make some individuals susceptible to the effects of cholinesterase inhibitors such as nerve agents, a factor of 10 was applied for intraspecies variability (protection of susceptible populations). Thus, the total UF for estimating AEGL-1 values for agent GB is 10.

The fact that AEGL-2 analyses for agent GB are based on data from human volunteers (Baker and Sedgwick, 1996) precludes the use of an interspecies UF. As in the AEGL-1 estimations, a factor of 10 was applied for intraspecies variability (protection of susceptible populations). A modifying factor is not applicable. Thus, the total UF for estimating AEGL-2 values for agent GB is 10.

AEGL-3 values for agent GB were derived from recent inhalation studies in which the lethality of GB vapor in female Sprague–Dawley rats was evaluated for 10-, 30-, 60-, 90-, 240-, and 360-min periods (Mioduszewski et al., 2000, 2001, 2002a). Both experimental LC_{01} and LC_{50} values were evaluated. The use of the rat data set resulted in selection of an interspecies UF of 3; the full default value of 10 was not considered appropriate because the mechanism of toxicity in rats and humans is the same, and lethality represents one point on the response continuum for these anticholinesterase

AEGL Values for Nerve Agent GB[a]

Classification	10 min	30 min	1 h	4 h	8 h	End Point (Reference)
AEGL-1 (nondisabling)	0.0012 (0.0069)	0.00068 (0.0040)	0.00048 (0.0028)	0.00024 (0.0014)	0.00017 (0.0010)	EC_{50} for miosis observed in adult female SD rats[b] exposed to a range of GB vapor concentrations (0.01–0.48 mg/m^3) for 10, 60, and 240 min
AEGL-2 (disabling)	0.015 (0.087)	0.0085 (0.050)	0.0060 (0.035)	0.0029 (0.017)	0.0022 (0.013)	Miosis, dyspnea, RBC-ChE inhibition, SFEMG changes in human volunteers exposed at 0.5 mg/m^3 for 30 min
AEGL-3 (lethal)	0.064 (0.38)	0.032 (0.19)	0.022 (0.13)	0.012 (0.070)	0.0087 (0.051)	Based on experimental SD rat lethality data (LC_{01} and LC_{50}); whole-body dynamic exposure

[a] Values are stated in ppm (mg/m^3).

[b] SD rats, Sprague–Dawley rats.

compounds. The full default value of 10 for intraspecies uncertainty was considered necessary to protect susceptible populations. Because a modifying factor is not applicable, the composite UF for AEGL-3 determination for agent GB is equal to 30.

2.7.7 Nerve Agent VX

Insufficient data were available from which to directly derive AEGL values for VX (NRC, 2003) from human or animal inhalation toxicity studies. The few studies available are historical and are considered nonverifiable because of flawed study design, poor sampling techniques, or suspect contamination of sampling and detection apparatus. Nevertheless, available literature clearly indicates that inhibition of cholinesterase activity is a common mechanism of toxicity shared by the G-series nerve agents and nerve agent VX. Thus, it was possible to develop AEGL estimates for agent VX by a comparative method of relative potency analysis from the more complete data set for nerve agent GB. The concept has been applied before in the estimation of agent VX exposure limits. All estimates in the literature regarding the potency of VX relative to agent GB indicate that vapor toxicity for agent VX is greater than for agent GB. Comparable RBC-ChE_{50} data from clinically supervised human volunteers who were exposed to agents GB and VX during well-conducted studies are available for estimation of relative potency. The human data indicate that agent VX is approximately four times more potent than agent GB or induces the RBC-ChE_{50} end point, which is considered an early and quantitative measure of the response continuum known for those compounds. Thus, the GB:VX relative potency ratio of 4 is considered an appropriate estimate of GB:VX relative potency for all VX AEGL determinations (Grob and Harvey, 1958; Sidell and Groff, 1974).

All mammalian toxicity end points observed in the data set for nerve agent VX and the G-series agents represent different points on the response continuum for anticholinesterase effects. Further, the mechanism of mammalian toxicity (cholinesterase inhibition) is the same for all newer agents. In consequence, the experimentally derived $n = 2$ from the Mioduszewski et al. (2000, 2001, 2002a, 2002b) rat miosis and lethality data sets for agent GB are used as the scaling function for the agent-VX AEGL-1, AEGL-2, and AEGL-3 derivations rather than a default value.

By applying the GB:VX relative potency concept outlined above (the relative potency of GB:VX equal to 4), the AEGL-1 analyses for agent VX are derived from miosis data for adult female Sprague–Dawley rats exposed to GB vapor for three time durations of significance for AEGLs (10, 60, and 240 min). Data from a GB vapor study of nonhuman primates (marmosets, 5-h exposures to GB vapor concentrations of 0.05–150 μg/m^3) and human volunteers (minimal and reversible effects of miosis, rhinorrhea, headache, etc., after 20-min exposure to a GB vapor concentration of 0.05 mg/m^3) are considered secondary and supportive. The same UFs and logic applied in the derivation of AEGL-1 and AEGL-2 values for agent GB (e.g., interspecies UF of 1 intraspecies UF of 10) are used here for estimating AEGL-1 and AEGL-2 values for agent VX. With application of a modifying factor of 3 for the sparse VX data set, the total UF for estimating AEGL-1 values for agent VX is 30.

By further application of the GB:VX relative potency concept outlined above, the AEGL-2 values for agent VX were derived from a GB vapor exposure study of human subjects in which miosis, dyspnea, photophobia, inhibition of RBC-ChE to approximately 60% of individual baseline, and small but measurable changes in SFEMG of the forearm occurred after a 30-min exposure at 0.5 mg GB/m^3 (Baker and Sedgwick, 1996).

The fact that AEGL-2 analyses for agent VX are based on data from clinically supervised human volunteers exposed to GB vapor (Baker and Sedgwick, 1996) precludes the use of an interspecies UF. With application of a factor of 10 for intraspecies variability and a modifying factor of 3 for the sparse VX data set, the total UF for estimating AEGL-2 values for agent VX is 30.

AEGL Values for Agent VX[a, b]

Classification	10 min	30 min	1 h	4 h	8 h	End Point (Reference)
AEGL-1 (nondisabling)	0.000052 (0.00057)	0.000030 (0.00033)	0.000016 (0.00017)	0.0000091 (0.00010)	0.0000065 (0.000071)	Derived by relative potency from EC_{50} for miosis observed in adult female SD rats[c] exposed to a range of GB vapor concentrations.
AEGL-2 (disabling)	0.00065 (0.0072)	0.00038 (0.0042)	0.00027 (0.0029)	0.00014 (0.0015)	0.000095 (0.0010)	Derived by relative potency from study of GB vapor exposure to exercising human volunteers exposed at 0.5 mg/m^3 for 30 min; miosis, dyspnea, inhibition of RBC-ChE, changes in SFEMG.
AEGL-3 (lethal)	0.0027 (0.029)	0.0014 (0.015)	0.00091 (0.010)	0.00048 (0.0052)	0.00035 (0.0038)	Derived by relative potency from experimental SD rat lethality data (LC_{01} and LC_{50}); whole-body dynamic exposure to GB vapor concentrations.

[a] Values are stated in ppm (mg/m^3).

[b] The derived AEGL values are for vapor exposures only. Percutaneous absorption of nerve agent vapors is known to be an effective route of exposure; nevertheless, percutaneous vapor concentrations needed to produce similar adverse effects are greater than inhalation vapor concentrations by several orders of magnitude. (For agent VX, the percutaneous vapor concentration needed to produce similar adverse effects are greater than inhalation vapor concentration by an approximate factor of 10.) Thus, the AEGL values presented are considered protective for both inhalation and percutaneous routes of exposure.

[c] SD rats, Sprague–Dawley rats.

By further application of the GB:VX relative potency concept outlined above, the AEGL-3 values for agent VX were derived from recent inhalation studies in which the lethality of GB to female Sprague–Dawley rats was evaluated for the 10-, 30-, 60-, 90-, 240-, and 360-min periods. Both experimental LC_{01} and LC_{50} values were evaluated. The same UFs and logic applied in the derivation of AEGL-3 values for agent GB (interspecies UF of 3 and an intraspecies UF of 10) are used here for agent VX. With the additional application of a modifying factor of 3 for the sparse VX data set, the total UF for AEGL-3 determination for agent VX is equal to 100.

2.8 CONCLUSIONS

With the increased production and shipment of chemicals, an urgent need exists for international guidance levels applicable to exposures to the general population. These guidance levels are being developed by the National Advisory Committee on Acute Guideline Levels for Hazardous Substances. The international membership of this committee ensures that there will be international recognition for these values. In addition to the benefits of having one standard approach to acute risk assessment for chemical spills or accidental releases in the United States and the European Union, this approach is of great value to the less-developed countries, as it provides them with a set of standards to use both for planning and in the event of a chemical release. To date, the AEGL committee has reviewed more than 130 chemicals. The National Academies have published many standards. A current list of these chemicals is provided in Table 2.2.

TABLE 2.2 Acute Exposure Guideline Levels: Chemicals Reviewed

Chemical Name	CAS No.[a]	Chemical Name	CAS No.
Final AEGLs			
Agent GA (Tabun)	77-81-6	HCFC 141b	1717-00-6
Agent GB (Sarin)	107-44-8	Hydrogen chloride	7647-01-0
Agent GD (Soman)	96-64-0	Hydrogen cyanide	74-90-8
Agent VX	50782-69-9	Hydrogen fluoride	7664-39-3
Aniline	62-53-3	Methyl hydrazine	60-34-4
Arsine	7784-42-1	Methyl isocyanate	624-83-9
Diborane	19287-45-7	Phosgene	75-44-5
1,1-Dimethyl hydrazine	57-14-7	Propylene glycol dinitrate 6423-43-4	106602-80-6
1,2-Dimethyl hydrazine	540-73-8	Sulfur mustard	505-60-2
GF agent	329-99-7		
Interim AEGLs			
Acetone cyanohydrin	75-86-5	Methacrylonitrile	126-98-7
Acrolein	107-02-8	Methanol	67-56-1
Acrylic acid	79-10-7	Methyl ethyl ketone	78-93-3
Allyl alcohol	107-18-6	Methyl mercaptan	74-93-1
Allyl amine	107-11-9	(HFE-7100) Methyl monofluorobutyl ether	163702-07-6
Ammonia	7664-41-7	Monochloroacetic acid	79-11-8
Boron trichloride	10294-34-5	*N*,*N*-Dimethylformamide	68-12-2
Boron trifluoride	353-42-4	Nickel carbonyl	13463-39-3
Bromine	7726-95-6	Nitric acid	7697-37-2
Carbon disulfide	75-15-0	Nitric oxide	10102-43-9
Carbon monoxide	630-08-0	Nitrogen dioxide	10102-44-0
Carbon tetrachloride	56-23-5	Peracetic acid	79-21-0
Chlorine	7782-50-5	Perchloromethyl mercaptan	594-42-3
Chlorine dioxide	10049-04-4	Phenol	108-95-2
Chlorine trifluoride	7790-91-2	Phosphine	7803-51-2
Chloroform	67-66-3	Phosphorus oxychloride	10025-87-3
Chloromethyl methyl ether	107-30-2	Phosphorus trichloride	7719-12-2
cis- and *trans*-1,2-Dichloroethylene	156-60-5	Piperidine	110-89-4
cis-1,2-Dichloroethylene	156-59-2	Proprionitrile	107-12-0
cis-Crotonaldehyde	4170-30-3	Propylene oxide	75-56-9
Cyclohexylamine	108-91-8	Propyleneimine	75-55-8
Dichlorodimethylsilane	75-78-5	Tetrachloroethylene	127-18-4
Epichlorohydrin	106-89-8	Tetranitromethane	509-14-8
Ethylene diamine	107-15-3	Toluene	108-88-3
Ethylene oxide	75-21-8	2,4-Toluene diisocyanate	584-84-9
Ethyleneimine	151-56-4	2,6-Toluenediisocyanate	91-08-7
Fluorine	7782-41-4	*trans*-Crotonaldehyde	123-73-9

(*Continued*)

TABLE 2.2 Acute Exposure Guideline Levels: Chemicals Reviewed (Continued)

Chemical Name	CAS No.[a]	Chemical Name	CAS No.
	Interim AEGLs		
Furan	110-00-9	1,1,1-Trichloroethane	71-55-6
HFC 134A	811-97-2	Trichloroethylene	79-01-6
Hydrazine	302-01-2	Trichloromethyl silane	75-79-6
Hydrogen sulfide	7783-06-4	Uranium hexafluoride	7783-81-5
Iron pentacarbonyl	13463-40-6	Vinyl chloride	75-01-4
Isobutyronitrile	78-82-0	Xylenes	1330-20-7
Jet fuels	70892-10-3		

[a] CAS No., Chemical Abstracts Service Registry Number.

REFERENCES

Baker, D.J. and Sedgwick, E.M., Single fiber electromyographic changes in man after organophosphate exposure, *Hum. Exp. Toxicol.,* 15, 369–375, 1996.

Brock, W.J., Rusch, G.M., and Trochimowicz, H.J., Cardiac sensitization: methodology and interpetation in risk assessment, *Regul. Toxicol. Pharmacol.* 38, 78–90, 2003.

Currie, W.D., Hatch, G.E., and Frosolono, M.F., Pulmonary Alterations in Rats Due to Acute Phosgene Inhalation, *Fundam. Appl. Toxicol.* 8*,* 107–114, 1987.

Dalbey, W., Evaluation of the Toxicity of Hydrogen Fluoride at Short Exposure Times, Petroleum Environmental Research Forum Project 92-09, performed at Stoneybrook Lab. Inc., Pennington, NJ.

Dalbey, W., Dunn, B., Bannister, R., Daughtrey, W., Kerwin, C., Reitman, F., Steiner, A., and Bruce, J., Acute effects of 10-minute exposure to hydrogen fluoride in rats and derivation of a short-term exposure limit for humans, *Regul. Toxicol. Pharmacol. 27,* 207–216, 1998.

Diller, W.F., Bruch, J., and Dehnen, W., Pulmonary changes in the rats following low phosgene exposure, *Arch. Toxicol.,* 57*,* 184–190, 1985.

Dunn, M.A. and Sidell, F.R., Progress in the medical defense against nerve agents, *J. Am. Med. Assoc.,* 262, 649–652, 1989.

Emmen, H.H., Hoogendijk, E.M.G., Klopping-Ketelaars, W.A.A., Muijser, H., Duistermaat, E., Ravensberg, J.C., Alexander, D.J., Borkhataria, D., Rusch, G.M., and Schmit, B., Human safety and pharmacokinetics of the CFC alternative propellants HFC 134a (1,1,1,2-tetrafluoroethane) and HFC-227 (1,1,1,2,3,3,3-heptfluoropropane) following whole body exposure, *Regul. Toxicol. Pharmacol.* 32, 22–35, 2000.

European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) Emergency exposure indices for industrial chemicals, Technical Report 3, Brussels, Belgium, 1991.

Fleischer, M.T., Mitigation of Chemical Spills: An Evaporation/Air Dispersion Model for Chemical Spills on Land, Shell Development Company, Houston, TX, 1980.

Gephart, L.A., Gorton, B., Schurger, M.G., and Ely, T.S., Three-tier emergency action levels for 20 chemicals, *Am. Ind. Hygiene Conf.,* Abstr. 176, 1986.

Gephart, L.A. and Moses, S., An approach to evaluate the acute impacts from simulated accidental releases of chlorine and ammonia, *Plant/Operations Prog.,* 8, 8–11, 1989.

Grob, D. and Harvey, J.C., Effects in man of the anticholinesterase compound sarin (isopropyl methyl phosphonofluoridate), *J. Clin. Invest.,* 37, 350–368, 1958.

Gross, P., Rinehart, W.E., and Hatch, T., Chronic pneumonitis caused by phosgene, *Arch. Environ. Health,* 10, 768–775, 1965.

Hanna, S. and Drivas, P., Guidelines for Use of Vapor Cloud Dispersion Model, Center for Chemical Process Safety (CCPS), American Institute, Chemical Engineering, New York, 1987.

Hardy, C.J., Sharman, I.J., and Clark, G.C., Assessment of the Cardiac Sensitization Potential in Dogs: Comparison of HFA 134a and A-12, Report CTL/C/2521, Huntingdon Research Centre, Huntingdon, Cambridgeshire, England, 1991.

Harvey, J.C., Clinical Observations on Volunteers Exposed to Concentrations of GB, Medical Laboratories Research Report 114, Publication Control 5030–114 (CMLRE-ML-52), Army Chemical Center, MD, 1952.

Johns, R.J., The Effect of Low Concentrations of GB on the Human Eye, Chemical Corps Medical Laboratories Research Report 100, Publication Control 5030–100 (CMLRE-ML-52). Army Chemical Center, MD, 1952.

Kim, Y.C. and Carlson, G.P., The effect of an unusual workshift on chemical toxicity. II. Studies on the exposure of rats to analine*, Fundam. Appl. Toxicol.,* 7, 144–152, 1986.

Largent, E.J., The metabolism of fluorides in man, *AMA Arch. Ind. Health,* 21, 318–323, 1960.

Largent, E.J., *Fibrosis: the health aspects of Fluorine Compounds*, Ohio State University Press, Columbus, OH, 1961, pp. 34–39, 43–48.

Lipsett, M.J., Shusterman, D.J., and Beard, R.R., Phosgene, in *Industrial Hygiene and Toxicology,* 4th ed., Vol. II, Part F, Clayton, G.C., and Clayton, F.E., Ed., John Wiley & Sons, New York, 1994, pp. 4557–4563.

Lund, K., Ekstrand, J., Boe, J., Sostrand, P., and Kongerud, J., Exposure to hydrogen fluoride: an experimental study in humans of concentrations of fluoride in plasma, symptoms, and lung function., *Occup. Environ. Med.,* 54, 32–37, 1997.

Lund, K., Refsnes, M., Sandstrom, T., Sostrand, P., Swarze, P., Boe, J., and Kongerud, J., Increased CD3 positive cells in bronchoaveolar lavage fluid after hydrogen fluoride inhalation, *Scand. J. Work Environ. Health,* 25, 326–334, 1999.

Machle, W. and Kitzmiller, K.V., The effects of the inhalation of hydrogen fluoride. II. The response following exposure to low concentrations, *J. Ind. Hyg.,* 17, 223–229, 1935.

Machle, W., Thamann, F., Kitzmiller, K., and Cholak, J., The effects of the inhalation of hydrogen fluoride. I. The response following the response to high concentrations, *J. Ind. Hyg.,* 16, 129–145, 1934.

Mioduszewski, R.J., Manthei, J., Way, R., Burnett, D., Gaviola, B., Muse, W., Thomson, S., Sommerville, D., and Crosier, R., Estimating the probability of sarin vapor toxicity in rats as a function of exposure concentration and duration, in *Proceedings of the International Chemical Wepons Demilitarization Conference* (*CWD-2000*)*,* The Hague, May 21–24, 2000.

Mioduszwski, R.J., Manthei, J., Way, R., Burnett, D., Gaviola, B., Muse, W., Anthony, J., Durst, D., Sommerville, D., Crosier, R., Thomson, S., and Crouse, C., ECBC Low Level Operational Toxicology Program: Phase I, Inhalation Toxicity of Sarin Vapor in Rats as a Function of Exposure Concentration and Duration, ECBC-TR-183, Edgewood Research, Development and Engineering Center, Aberdeen Proving Ground, MD, Aug. 2001.

Mioduszwski, R.J., Manthei, J., Way, R., Burnett, D., Gaviola, B., Muse, W., Thomson, S., Sommerville, D., and Crosier, R., Interaction of exposure concentration and duration in determining acute toxic effects of sarin vapor in rats, *Toxicol. Sci.* 66, 176–184, 2002a.

Mioduszwski, R.J., Manthei, J., Way, R., Burnett, D., Gaviola, B., Muse, W., Thomson, S., Sommerville, D., Crosier, R., Scotto, J., McCaskey, D., Crouse, C., and Matson, K., Low Level Sarin Exposure in Rats: Effect of Exposure Concentration and Duration on Pupil Size, ECBC-TR-235, Edgewood Chemical Biological Center, U.S. Army Soldier and Biological Chemical Command, Aberdeen Proving Ground, MD, May 2002b.

National Research Council, Guidelines for Developing Community Emergency Exposure Levels for Hazardous Substances, National Academy Press, Washington DC, 1993, pp. 19–20.

National Research Council, Acute Exposure Guideline Levels for Selected Airborne Chemicals, Vol. 1, National Academy Press, Washington, DC, 2000.

National Research Council, Standing Operating Procedures for Developing Acute Exposure Guideline Levels for Hazardous Chemicals, National Academy Press, Washington, DC, 2001.

National Research Council, Acute Exposure Guideline Levels for Selected Airborne Chemicals, Vol. 2, National Academy Press, Washington, DC, 2002, pp. 15–70.

National Research Council, Acute Exposure Guideline Levels for Selected Airborne Chemicals, Vol. 3, National Academy Press, Washington, DC, 2003, pp. 15–300.

National Research Council, Acute Exposure Guideline Levels for Selected Airborne Chemicals, Vol. 4, National Academy Press, Washington, DC, 2004.

Rinehart, W.E. and Hatch, T., Concentration-time product (CT) as an expression of dose in sublethal exposures to phosgene, *Ind. Hyg. J.,* 25, 545–553, 1964.

Rosenholtz, M.J., Carson, T.R., Weeks, M.H., Wilinski, F., Ford, D.R., and Oberst, F., A toxicopathologic in animals after brief single exposures to hydrogen fluoride, *Am. Ind. Hyg. Assoc. J.,* 24, 253–261, 1963.

Rusch, G.M. The history and development of emergency response planning guidelines, *J. Hazard. Mater.,* 33, 193–202, 1993.

Rusch, G.M., Garrett, R., Tobin, P., Falke, E., and Lu, P.-Y., The development of acute exposure guideline levels for hazardous substances, *Process Saf. Prog.,* 19, 98–102, 2000.

Sidell, F.R. and Groff, W.A., The reactivatibility of cholinesterase inhibited by VX and sarin in man, *Toxicol. Appl. Pharmacol.,* 27, 241–252, 1974.

Stavert, D.M., Archuleta, D.C., Behr, M.J., and Lehnert, B.E., Relative acute toxicities of hydrogen fluoride, hydrogen chloride and hydrogen bromide in nose- and pseudo-mouth breathing rats, *Fundam. Appl. Toxicol.,* 16, 636–655, 1991.

Stevens, B., Koenig, J.Q., Rebolledo, V., Hanley, Q.S., and Covert, D.S., Respriatory effects from the inhalation of hydrogen chloride in young adult asthmatics, *J. Occup. Med.,* 34*,* 923–929, 1992.

ten Berge, W.F., Zwart, A., and Appelman, L.M., Concentration-time mortality response relationship of irritant and systematically acting vapors and gases, *J. Hazard. Mater.,* 13, 301–309, 1986.

U.S. Environmental Protection Agency, List of Extremely Hazardous Substances and Their Threshold Planning Quantities, 40 CFR Section 355, Appendix A, U.S. Government Printing Office, Washington, DC, 1987.

U.S. Environmental Protection Agency, Health Assessment Document for Methyl Isocyanate. Environmental Criteria and Assessment Office, Research Triangle Park, NC (Final Draft), Dec. 27, 1988.

Vernot, H.E., MacEwen, J.D., Haun, C.C., and Kinkead, E.R., Acute toxicity and skin corrosion data for some organic and inorganic compounds and aqueous solutions, *Toxicol. Appl. Pharmacol.,* 42, 417–423, 1977.

Witschi, H.P., Some notes on the history of Haber's Law, *Toxicol. Sci.,* 50, 164–168, 1999.

Wohlslagel, J., DiPasquale, L.C., and Vernot, E.H., Toxicity of solid rocket motor exhaust: effects of HCl, HF, and alumina on rodents, *J. Combust. Toxicol.* 3, 61–70, 1976.

Zwart, A., Arts, J.H.E., Klokman-Houweling, J.M., and Schoen, E.D., Determination of concentration-time-mortality relationships to replace LC50 values, *Inhalation Toxicol.,* 2*,* 105–117, 1990.

3 Emergency Response Planning Guidelines (ERPGs)

Finis Cavender

CONTENTS

3.1 INTRODUCTION

3.1.1 Background and Need for Community Protection

In December 1984, the release of 40 tons of methyl isocyanate (MIC) in Bhopal, India, emphatically underscored the need for the development of chemical emergency response plans. The great surprise was that so little was known about the toxicity of MIC. It was primarily used as a captive chemical intermediate in the production of carbaryl, and a toxicological profile had never been developed. The lack of a toxicological base was, in part, because its acute toxicity was so great that long-term studies had never been considered. In this tragedy, more than 3800 residents died and an additional 200,000 suffered adverse health effects (www.Bhopal.com/chrono.htm). The public in India and even worldwide was enraged that an event of this magnitude could happen, because the local community had no idea that such a dangerous chemical was housed in such large quantities, just across the road from their homes (Rusch, 1993; Cavender and Gephart, 1994; Cavender, 2002).

Globally, residents living near chemical plants demanded that they be given information on chemicals being produced or used in their communities. They also insisted that solutions be

found that would prevent similar catastrophes from happening again. As a result, many chemical companies began developing emergency response plans, most of which included health-based exposure values. However, if each company had their own exposure values, the toxicological basis and rationale for each set of values might vary from one company to the next based on the data available to them. Such variance would be confusing and could lead the public to ask, "Which values should we use?" A better approach was for stakeholder chemical companies to work together in developing a single set of emergency response exposure values for chemicals of interest, eliminating the possibility of a multiplicity of numbers which would also be confusing to emergency response planners and managers (Rusch, 1993; Cavender and Gephart, 1994; Kelly and Cavender, 1998; Cavender, 2002).

The outcry of the public in the United States resulted in the promulgation of the Superfund Amendments and Reauthorization Act (SARA) of 1986, which contained provisions entitled Emergency Planning and Community Right-to-Know. This legislation along with provisions under Title III of the Clean Air Act of 1990, required local communities to set up emergency response plans. How was the public to develop emergency response plans without a single set of exposure values? Without a unified set of exposure values based on acute toxicity data, each planning activity would result in differing numbers for every community across the nation. Thus, it was all the more important that a single set of exposure values be developed for each chemical for emergency response planning purposes.

3.1.2 Were Health-Based Numbers Suitable for Emergency Planning Available in 1986?

In 1986, occupational exposure guidelines and standards such as the American Conference of Governmental Industrial Hygienist's threshold limit values (TLVs) (2005), the American Industrial Hygiene Association (AIHA) Workplace Environmental Exposure Levels (WEELs) (AIHA, 2005b), and the Occupational Safety and Health Administration's permissible exposure limits (PELs) (Occupational Safety and Health Administration, 1995) were available for a good number of chemicals. However, these values were inappropriate for evaluating brief emergency exposures to the general public. These values were developed for the protection of healthy workers. They were based primarily on repeated dose studies conducted over months and years to simulate the daily exposure of workers over their working lifetime. The levels were set as a time-weighted average over the usual 8-h workday and serve to protect against both acute and chronic health effects. They were not designed to protect children, the elderly, or otherwise compromised individuals, such as individuals with asthma or alcohol dependency.

In addition to these occupational standards, several types of guidelines have been developed for use in emergency situations involving a single exposure to a specific chemical or mixture that may cause adverse health effects. In the 1950s, the National Research Council (NRC) began setting operational emergency numbers as advisory responses to specific exposure scenarios. These emergency numbers were developed principally for the Department of Defense (DOD) and were intended for young robust individuals. For instance the Navy needed levels for confined spaces such as being submerged in a submarine. In 1964, such exposure levels were termed emergency exposure levels (EELs) (NRC, 1986).

Independently, in 1964, the AIHA Toxicology Committee also introduced the concept of EELs and initially proposed EELs for three chemicals: nitrogen dioxide, 1,1-dimethylhydrazine, and 1,1,1-trichloroethane (AIHA Toxicology Committee, 1964). The EELs were established for a single exposure for periods of 5, 15, 30, or 60 min. They were defined as levels that would not result in irreversible toxicity, impair the ability to perform emergency operations, or impair the ability to escape from the exposure. However, these levels were for healthy workers and were not designed to protect many within the general public. Considered to duplicate the NRC guidelines, no additional AIHA EELs were developed (Jacobson, 1966).

In 1986, the NRC changed the from EELs to Emergency Exposure Guideline Levels (EEGLs), which have been developed for a number of chemicals (NRC, 1986). EEGLs were developed

primarily for military personnel and were not applicable to the general public. Realizing the need for community numbers, NRC introduced the concept of short-term public emergency guidance levels (SPEGLs), but few SPEGLs were developed (NRC, 1986). The NRC also developed continuous exposure guidance levels (CEGLs) for continuous exposure scenarios, for example, in a submarine (NRC, 1986). In the 1990s, similar levels were needed for the confined quarters of spacecraft. Such levels called spacecraft maximum allowable concentrations (SMACs) have been released periodically in a series of publications (SMACs, 1992).

National Institute for Occupational Safety and Health (NIOSH), in the late 1970s, developed a series of values representing exposure levels that were "immediately dangerous to life and health" (IDLHs) (NIOSH, 1994). These values were used for identifying respiratory protection requirements in the NIOSH/OSHA Standards Completion Program. Since 1994, some IDLHs have been reviewed and some documentation is available. One of the problems inherent in using IDLHs is that they were intended for the workplace where protective clothing and equipment are at hand, and they do not take into account exposure of the more sensitive individuals, such as the elderly, children, or people with asthma, living in the surrounding community. In addition, the rationales and documentations were not peer-reviewed and are not generally available.

Other groups have considered the development of short-term exposure guidelines including, the American National Standards Institute (ANSI), the Pennsylvania Department of Health, and the National Fire Protection Association (NFPA). In general, the guidelines from these organizations are directed only to occupational exposure, are no longer being generated, or are merely relative hazard ratings. As such, they do not meet the need for airborne concentrations to be used in planning for emergency situations (AIHA, 2005c).

Although various "emergency numbers" have been recommended by several organizations, none of them have been specifically developed for potential accidental releases to the community with the exception of the few NRC SPEGLs. The process of developing NRC SPEGLs is a thorough process, but it is time consuming. Thus, it became evident that a new set of numbers for emergency planners and managers was needed and that they should be developed rapidly for a wide variety of chemicals.

3.2 THE DEVELOPMENT OF EMERGENCY RESPONSE PLANNING GUIDELINES

3.2.1 The Birth of ERPGs

Without suitable numbers in place in 1986, several companies, responding to internal needs, independently undertook development of these emergency planning guideline numbers. Each had separately reached similar conclusions:

1. The numbers are useful primarily for emergency planning and response.
2. The numbers are suitable for protection from health effects due to short-term exposures.
3. They are not suitable for effects due to repeated exposures nor as ambient air quality guidelines.
4. The numbers are guidelines. They are not absolute levels demarcating safe from hazardous conditions.
5. The numbers do not necessarily indicate levels at which specific actions must be taken.
6. The numbers are only one element of the planning activities needed to develop a program to protect the neighboring community.
7. The selection of chemicals needing emergency planning guidelines should, in general, be based on volatility, toxicity, and releasable quantities.

In discussions among personnel in companies developing these guidelines, it became evident that a consistent approach was needed. Uniform procedures and definitions would provide more

consistent guidelines. In addition, sharing guidelines for different chemicals would avoid redundant efforts and increase the number of available guidelines (Rusch, 1993; Cavender and Gephart, 1994; Kelly and Cavender, 1998; Cavender, 2002; AIHA, 2005c).

3.2.2 The Role of the Organization Resources Counselors, Incorporated

Recognizing this need, Organization Resources Counselors, Inc. (ORC) established a task force to address the need for reliable, consistent, and well-documented emergency planning guidelines. Through the ORC Emergency Response Planning Guidelines (ERPGs) Task Force, companies willing to play an active role in the development of emergency guidance concentrations, by using a consistent procedure, were able to coordinate their efforts. Members of the ORC ERPG Task Force collectively developed methods for establishing emergency exposure guidance levels and a list of chemicals for which ERPGs were believed to be needed. Participating companies made commitments to develop ERPGs for selected chemicals according to the methods developed by the Task Force. Participation in the ORC Task Force was voluntary. (Rusch, 1993; Cavender and Gephart, 1994; Kelly and Cavender, 1998; Cavender, 2002; AIHA, 2005c).

When the Emergency Response Planning Committee was formed, many chemical companies had representatives on the Organizational Resources Counselors (ORC). Chemical companies who needed ERPGs for given chemicals would write a draft document and submit it to the Emergency Response Planning Committee through ORC. This worked well for the first 40 or so documents (Cavender, 2002; AIHA, 2005c).

3.2.3 The Role of the American Industrial Hygiene Association

In looking for an acceptable venue to develop and publish the documents of the Emergency Response Planning Committee, the AIHA seemed to be a logical organization because its membership included both industrial hygienists and toxicologists from stakeholder chemical companies, government agencies, academic institutions, and the public sector. As a result, the Emergency Response Planning Committee (ERP Committee) was formed in 1987, initially as an ad hoc group under the Workplace Environmental Exposure Level Committee, with the specific charge to develop suitable documents for emergency response planning. In 1988, the ERP Committee began producing Emergency Response Planning Guidelines (ERPGs) (Rusch, 1993; Cavender and Gephart, 1994; Cavender, 2002; AIHA, 2005a, 2005b).

It should be obvious that groups other than chemical companies need ERPGs. For instance, the Department of Energy (DOE) includes many nuclear plants, laboratories, and research facilities. Because DOE and numerous other organizations are not represented on ORC, a method was needed whereby other stakeholders could supply a draft document for a chemical for which they needed an ERPG. As a result, ORC has dropped out of the process and all documents are now submitted directly to the ERP Committee. Submissions have been made by the DOE, the DOD, consortia of producers, and committee members. EPRGs have been integrated in risk management scenarios to protect soldiers in noncombat situations. Documents are considered from any source, and the selection of chemicals is based on interest in the chemical, production volume, and physical/chemical properties such as volatility, odor, reactivity, and solubility (Cavender, 2002; AIHA, 2005c).

Almost immediately, ERPGs gained worldwide recognition and acceptance and are currently used throughout the world. In 1991, the European Centre for Ecoltoxicology and Toxicology of Chemicals (ECETOC) published the concept of Emergency Exposure Indices (EEIs) (ECETOC, 1991). However, only three documents were ever published, mainly because of the wide acceptance of ERPGs by emergency planners (ECETOC, 1991; Woudenberg and von der Torn, 1992; AIHA, 2005c). Instead of developing additional EEIs, Dutch scientists joined the ERP Committee and have aided in the development of sound documents.

At the request of the U.S. Environmental Protection Agency and the Agency for Toxic Substances and Disease Registry, NRC convened a Subcommittee on Guidelines for Development of Community Emergency Exposure Levels (CEELs) in 1993. The report of the comprehensive CEEL concept served as a blueprint for risk assessment of short-term exposures to high concentrations of chemical toxicants. This report identified the NRC SPEGLs and the AIHA ERPGs as being standards that might be useful in developing CEELs (NRC, 1993). The NRC's criteria for establishing CEELs were similar to the established methodology used in developing ERPGs. However, no CEEL documents were ever produced (Rucsh, 1993; Cavender and Gephart, 1994; Cavender, 2002; AIHA, 2005c). In an effort to move forward, representatives from the EPA approached the ERP Committee in 1995 and expressed their interest in developing similar numbers for emergency response planning. After several meetings, EPA spearheaded the development of Acute Exposure Guideline Levels (AEGLs) through the NRC/AEGL committee, which was established in 1996 (NRC, 2001). The ERP Committee welcomed EPA's efforts to develop similar exposure values and helped them establish the AEGL committee. From the outset, the plan was to work independently of each other. The AEGLs have a basis very similar to ERPGs as the ERP Committee has worked with EPA in setting up this program. (Kelly and Cavender, 1998, Cavender, 2002; AIHA, 2005c).

3.2.4 What Are the Levels of Concern?

The AIHA ERP Committee has utilized three guidance concentration levels. Each of these levels is defined and briefly discussed below.

3.2.4.1 ERPG-3

ERPG-3 is "the maximum airborne concentration below which it is believed that nearly all individuals could be exposed for up to one hour without experiencing or developing life-threatening health effects."

The ERPG-3 level is a worst-case planning level above which there is the possibility that exposure to levels above ERPG-3 will be lethal to some members of the community. This guidance level could be used to determine the maximum releasable quantity of a chemical should an accident occur. This quantity is used in the planning stages to project possible exposure levels in the community should a release occur. Once the releasable quantity is known (size of the storage tanks, etc.), the steps to mitigate the potential for such a release can be established.

3.2.4.2 ERPG-2

ERPG-2 is "the maximum airborne concentration below which it is believed that nearly all individuals could be exposed for up to one hour without experiencing or developing irreversible or other serious health effects or symptoms which could impair an individual's ability to take protective action."

Above ERPG-2, for some members of the community, there may be significant adverse health effects or symptoms, including lung or liver disease, miscarriage, or cancer. On the other hand, this level could impair an individual's ability to take protective action. These effects might include dizziness, severe eye or respiratory tract irritation, central nervous system (CNS) depression, or muscular weakness.

3.2.4.3 ERPG-1

ERPG-1 is "the maximum airborne concentration below which it is believed that nearly all individuals could be exposed for up to one hour without experiencing other than mild, transient adverse health effects or without perceiving a clearly defined objectionable odor."

ERPG-1 identifies a level that does not pose a health risk to the community but which may be noticeable because of odor, coughing, discomfort, or irritation. In the event that a small, nonthreatening release has occurred, the community could be notified that they may notice an odor or slight irritation but that concentrations are below those which could cause serious health effects. For some materials, because of their properties, an ERPG-1 may not exist. Such cases would include substances for which sensory perception levels are higher than the ERPG-2 level. In such cases, the ERPG-1 level would be given as "not appropriate." It is also possible that no valid sensory perception data are available for the chemical. In this case, the ERPG-1 level would be given as "insufficient data."

All planning activity is to ensure that the daily operations of the plant or process will not result in lethality even if a catastrophic release occurs. The ERPG-2 is the most significant and useful of the ERPGs in emergency planning. This is the level on which decisions to evacuate a region or to shelter in place are based. After collecting and reviewing all the data that have an impact on the ERPG-2 level, setting ERPG-1 and ERPG-3 levels are straightforward.

3.2.5 What Time Period Is Relevant?

One might consider a range of time periods for these guidelines; however, the decision was made to focus on a single period, namely, 1 h. This decision was based on the availability of toxicity information and on a reasonable estimate for an exposure scenario. Most acute inhalation toxicity studies are for either 1 h or 4 h because EPA, the Consumer Product Safety Commission, the Department of Transportation, the Organization of Economic and Community Development, and other government agencies require studies for one or both of these periods. Acute lethality data are rarely available for 10 min, 30 min, or 8 h. Some releases may be shorter or longer than 1 h, depending on volatility and weather conditions, but 1 h should serve the community well in being notified of a release, allow time for suitable rescue operations within the plant, and allow first responders to reach and isolate the site of the release. The Committee has resisted the development of values for periods other than 1 h because, for most exposure scenarios, the 1-h period is sufficient; and when another time period is expected to be important, the emergency planners or managers can develop values specifically for their situation rather than randomly using safety factors or concentration times time ($C \times t$) extrapolations for every chemical. However, when warranted, the ERP Committee has set ERPGs for different time periods (see the 1999 addendum to the hydrogen fluoride ERPG document). The logistics are, in general, against very short periods. It is impossible to report a spill or release, run the air dispersion models, and have the news agencies report the information to the public within 10 min. If the message would be to shelter in place with windows closed and air conditioners turned off to prevent outside air entering the house, the exposure would be over before the public could be alerted to the danger (Kelly and Cavender, 1998; Cavender, 2002; AIHA, 2005c). For emergency managers who need to extrapolate to different time periods, there are two generally recognized approaches. For nonirritants, Haber's rule (concentration $\times$ time = constant, or $C \times t = k$) is usually valid over a three- or fourfold range of time (Habor, 1924). A seemingly better method for all chemicals was developed by ten Berge and is given as ($C^n \times t = k$), where in practice, $n = 3$ in extrapolating to a shorter period or $n = 1$ in extrapolating to a longer period (ten Berge et al., 1986). For the best possible results, n should be derived from the slope of the dose–response curve.

3.2.6 How Are the Numbers Derived?

In developing an ERPG level for a chemical, it is important to emphasize the use of acute or short-term inhalation toxicity data and workplace experience. ERPGs are for a once-in-a-lifetime exposure for 1 h. When evaluating the adverse health effects, both immediate and delayed health effects should be considered. When it is believed that adverse reproductive, developmental, or carcinogenic effects might be caused by a single exposure, these data should be carefully considered in the

derivation of the ERPGs (Rucsh, 1993; Cavender and Gephart, 1994; Kelly and Cavender, 1998; Cavender, 2002; AIHA, 2005c). If carcinogenicity data are used, the mathematical approaches adopted by the NRC are utilized in assessing the risk of developing cancer from a single exposure. (NRC, 1986).

The data for developing an ERPG for any chemical are evaluated on a case-by-case basis because each chemical is likely to have a different dose–response curve and produce significantly different effects. Also, the quantity and quality of data available vary widely. There is no formula for selecting ERPG values and no fixed relationship between the three ERPG values (Rucsh, 1993; Cavender and Gephart, 1994; Kelly and Cavender, 1998; Cavender, 2002; AIHA, 2005c).

It is very important that the rationales for the ERPGs be documented and published. AIHA publishes a pocket-sized handbook (AIHA, 2005c) that contains the numbers for all ERPG documents. In addition, anyone using these numbers for chemicals of concern, should obtain the complete ERPG documents for those chemicals (AIHA, 2005a). These documents provide all the data and rationales used in deriving the ERPGs.

3.2.7 Chemical Selection and Data Requirements

ERPGs are developed for a once-in-a-lifetime exposure for up to 1 h. Thus, acute toxicity data and workplace experience in handling the chemical are important in developing ERPGs. Because most community exposures are anticipated to be via inhalation, inhalation toxicity data are most useful in setting the numbers. A 1-h or 4-h inhalation lethality study (1-h median lethal concentration [LC_{50}] or 4-h LC_{50}) in one or more species, a respiratory depression study in Swiss–Webster mice (RD_{50}), an odor threshold, and workplace exposure or human testing to known concentrations are useful in setting ERPGs. Repeated exposure toxicity data and developmental toxicity data, sensitization data, and carcinogenicity data are also important in setting the final numbers. Toxicity by routes other than inhalation are supportive, and if the only carcinogenicity or developmental studies available are via oral dosing, such studies are carefully considered in setting ERPG-2 levels. Finally, if mechanistic or dose–response data are available, these are applied as appropriate (Rucsh, 1993; Cavender and Gephart, 1994; Kelly and Cavender, 1998; Cavender, 2002; AIHA, 2005c).

Obviously, the more complete the data set, the easier it is to set ERPG numbers and the greater credibility they will have because there will be greater confidence that the effects noted are due to exposure to the chemical. However, it is important that the data be specific and relevant to the derivation of ERPGs. Consider mercury vapor or toluene diisocyanate (TDI). Numerous reports are available on the toxicity of these compounds, but most are not useful in deriving ERPGs. It is common knowledge that mercury vapor is toxic, but no one has conducted a 1-h LC_{50} for mercury vapor. Similarly, there are many reports on the sensitizing properties of TDI, yet these data contribute little to the overall acute toxicity profile of TDI. Finally, note that some very common chemicals are extremely reactive, e.g., hydrogen fluoride (HF). Studies conducted in standard inhalation chambers destroy the chamber because of the extreme reactivity of HF. Such chemicals are studied in special chambers coated or lined to reduce the damage to the chamber.

The documents are arranged in the following sections in the AIHA 2005 Handbook for ERPGs: and WEELs (AIHA, 2005c):

I. Identification
II. Chemical and Physical Properties
III. Animal Toxicity Data
IV. Human Experience
V. Current Occupational Exposure Guidelines
VI. Recommended ERPGs and Rationales
VII. References

3.2.8 The Review Process

For documents that are submitted to the ERP Committee, the author should adhere to the following guidelines:

1. The authoring organization should use a multidisciplinary team, including expertise in industrial hygiene, toxicology, medicine, and other health professions to collect and review data and write a draft ERPG document.
2. The author should identify producers, major users, and industry associations that have a significant interest in the chemical and should request unpublished data and other relevant information from them. Studies of effects in humans or animals at known airborne concentrations are especially useful.
3. A robust literature search should be conducted and should include appropriate on-line databases, including MEDLINE and TOXLINE.
4. The author should make every effort to obtain the original reference for all data because transcriptional errors or significant omissions frequently occur in secondary references.
5. The ERPG document should be drafted by using the format prescribed in the latest edition of the AIHA Handbook on ERPGs (AIHA, 2005c).
6. The authoring company or organization should submit the draft ERPG document, marked "Preliminary Draft," to the ERP Committee.
7. Copies of all the referenced literature must accompany the draft document. For some lengthy publications, such as NTP chronic studies, the full document may not be needed. Unpublished data such as internal industry reports are often an enormous help, representing the majority of the toxicity data on a given chemical. Confidential company reports should not be used unless a summary containing some details of the methods, results, and conclusions is provided.
8. Upon receipt of a draft document, the AIHA ERP Committee will assign a primary and a secondary reviewer who will review the draft in depth, rewriting any sections that do not conform to current practice in developing ERPG documents. The submitting author may be contacted concerning any necessary clarifications or corrections.
9. After this initial review and revision, the document is presented to the full AIHA ERP Committee for a detailed discussion of the data summary, ERPG values, and rationales. A document may be discussed at several meetings before it is approved to ballot.
10. A majority vote of members in attendance is needed before sending the final draft to all members for ballot. Members may vote "yes," "no," or "abstain." "No" votes must be accompanied by a specific explanation. Every attempt to garner unanimous approval is sought for every ERPG document.
11. Before being balloted by the committee, the ERPG numbers, rationales, and references are posted on the AIHA Web site for 45 days to garner any private or public comments concerning the chemical or the document.
12. Following approval via the balloting process, all necessary changes are incorporated into the document and it is then sent along with the entire reference package to AIHA headquarters for publication.
13. The ERPG document is filed at AIHA along with copies of all the referenced literature. These will be made available to the public as needed.
14. ERPGs are updated after seven years, but may be reviewed and revised at any time as relevant new data become available.

3.2.9 Policy for Commenting on ERPGs under Review

1. On a quarterly basis a list of all chemicals for which ERPGs are being reviewed is posted on the AIHA Web site. The following statement will also be posted: "The following

materials are currently being studied for future ERPGs. Information and comments are welcome. If one has any input on candidate or completed ERPGs, he/she should contact the AIHA Department of Scientific and Technical Affairs who will forward all comments to the current Chair of the committee."

2. For chemicals where ERPG levels have been approved to ballot, the ERPG numbers, rationales, and a list of references will be posted on the Web site for a period of 45 days before balloting. This allows time for public review and comment before publishing the document. All suitable comments will be incorporated into the document before balloting. Comments that do not arrive in the 45 days will be incorporated in the next update of the document or if they provide significant new data, the document will be re-reviewed as soon as feasible.
3. The Chair will direct the comments to the primary reviewer with a copy to the secondary reviewer and the Secretary. The primary and secondary reviewers will develop a response and, with the concurrence of the Chair, they will send the response directly to the individual submitting the initial comment. Copies of both comments and responses will be listed in the minutes of the next meeting and will be maintained with the reference packages for the specific ERPG documents. When possible, responses will be made within 30 days after the next Committee meeting.
4. If a responsible individual requests the opportunity to attend a Committee meeting to discuss a specific document, the Chair may, at his discretion, grant permission. In general, the guest is only present for the discussion of the document of interest. The individual should be encouraged but is not required to first submit comments in writing. The Chair has the right to limit discussion, as would be necessary to ensure an orderly, productive meeting.
5. All requests to attend meetings and all comments not presented at meetings must be in writing. Written responses, possibly brief, will be given to all written comments.
6. Although the Committee may elect to incorporate new information into an ERPG document based on these comments, they are under no obligation to do so.
7. Drafts of documents are rarely provided, even if formally requested. For certain working committees or interested government agencies, a single copy of a draft that is so stamped on every page may be given to a responsible individual. In such documents, the tentative numbers are deleted because they do not reflect the Committee's or AIHA's position. The numbers may be communicated verbally, with the caveat that they are only tentative. Draft documents are never published because the draft may contain incomplete or erroneous data that are usually completed or corrected during the review process.
8. ERPG values that have not been approved by ballot by the Committee must not be published. These numbers usually change as data are evaluated during the review.

3.3 APPLICATION OF ERPGs FOR EMERGENCY PLANNING

3.3.1 How Are the Numbers Used?

ERPGs are intended for emergency planning. ERPGs can be applied in a variety of mandated or voluntary emergency response planning programs. These programs generally include accident scenarios in which air dispersion models determine concentration isopleths. ERPGs are also used in programs designed to protect the public from transportation incidents. ERPGs are extremely important for compliance with Emergency Planning and Community Right-to-Know legislation. Individuals using ERPGs include:

Air dispersion modelers
Community Action Emergency Response (CAER) participants
Fire protection specialists

Government agencies
Industrial process safety engineers
Industrial hygienists and toxicologists
Local Emergency Planning Coordinators (LEPCs)
RCRA managers
Risk assessors and risk managers
State Emergency Response Commissions (SERCs)
Transportation safety engineers.

ERPGs may be used with air dispersion models together with information such as vapor pressure and storage volumes, to provide computerized estimates of the direction and speed at which the released plume or cloud will spread over the neighboring terrain. These models will also provide the concentration within the cloud during the time of its dispersion. These models incorporate the quantity and rate of release, volatility, wind speed and direction, temperature, and other environmental conditions. Such models help emergency planners know whom to alert and where first responders should report should a release occur. From these models, action plans can be developed. The plans may vary for any given emergency depending on such things as population density, type of population (e.g., schools), terrain, weather conditions, and other hazards of the released entity (e.g., flammability).

Many air dispersion models, as related to accidental releases of toxic chemicals, stem from assumptions established in *Technical Guidance for Hazard Analysis—Emergency Planning for Extremely Hazardous Substances,* also known as the Green Book, published in 1987 (U.S. Environmental Protection Agency/U.S. Federal Emergency Management/U.S. Department of Transportation, 1987). This reference provides a basis for technical applications for community exposure limits. This and similar references often specify that ERPGs be used to determine where in the community protective actions are needed (sheltering in place, evacuation, or isolation zones).

Although emergency planners need to know the conditions of the release, the magnitude of a potential release can also be predicted by using these models. This allows plant managers to reevaluate possible "worst case" situations that might occur as a result of process or human failures. Since 9/11, industrial sabotage and terrorist activities are also considered. To aid in plant design and community planning, the engineers select the size of tank that will ensure that a potential release will never reach an airborne concentration above the ERPG-2 level or whatever level the planning group selects as its action level. This type of information is also useful in transporting chemicals across the country. The quantity within the tankers or tank car is limited based on the predicted concentrations that could result from an accidental release.

3.3.2 Limitations in Using ERPG Numbers

ERPGs are general reference levels, the best judgment of specialists using the best available data, and are intended to be used as part of an overall emergency planning program. The levels are not to be used as safe limits for repeated exposures, as definitive delineators between safe and unsafe exposure conditions, or as a basis for quantitative risk assessment.

Human responses do not occur at precisely the same exposure level for all individuals but can vary over a wide range of concentrations. The values derived for ERPGs should be applicable to nearly all individuals in the general population; however, in any population there may be hypersensitive individuals who have adverse responses at exposure concentrations far below levels where most individuals would respond. Furthermore, because these ERPG values have been derived as planning and emergency response guidelines, *not* as exposure guidelines, they do not contain the safety factors normally incorporated into exposure guidelines. Instead, they are estimates of the concentrations above which there would be an unacceptable likelihood of observing the defined effects. The estimates are based on

the available data that are summarized in the documentation (AIHA, 2005a). In some cases where the data are limited, the uncertainty of these estimates may be large. Users of the ERPG values are strongly encouraged to carefully review the documentation before applying these values.

Using ERPG values to determine the actions to be taken when planning for or responding to a given emergency requires careful evaluation of site-specific or situation-specific factors. These may include how the 1-h ERPG values might relate to exposures of different duration; whether there are populations at special risks (such as the elderly, the very young, or those with existing illnesses); and other factors such as the volatility and vapor density of the chemical, storage quantities, weather conditions, and terrain.

3.3.3 What Does the Future Hold?

As of December 2005, the ERP Committee published ERPG documents for 114 chemicals and updates for 49 of these chemicals. Although other groups are producing emergency response planning documents, there will always be a need for the ERP Committee because the chemical selection process for government agencies or foreign entities will never include many of the chemicals that are of interest to industry in the United States. So, the ERP Committee, as volunteers working steadily in behalf of communities worldwide, will continue to evaluate data and produce these documents to reduce the risk of such exposures. After all, chemicals are an integral part of our lives and the potential of exposure to chemicals manufactured, transported, or stored within our communities should not pose an undue risk. It would be great to say, "A catastrophe such as Bhopal will never happen again!" However, "never" does not have a statistical basis, and so we plan shrewdly, we consider all of the data available, and we strive to reduce or minimize the risk of significant exposure (Kelly and Cavender, 1998; Cavender, 2002; AIHA, 2005c).

REFERENCES

American Conference of Governmental Industrial Hygienists (ACGIH), *Threshold Limit Values and Biological Exposure Indices,* ACGIH Worldwide, Cincinnati, OH, 2005.

American Industrial Hygiene Association (AIHA), *Documentation of Emergency Response Planning Guidelines,* AIHA Press, Fairfax, VA, 2005a.

American Industrial Hygiene Association (AIHA), *Documentation of Workplace Environmental Exposure Levels,* AIHA Press, Fairfax, VA, 2005b.

American Industrial Hygiene Association (AIHA), *The AIHA 2003 Handbook for ERPGs and WEELs,* AIHA Press, Fairfax, VA, 2000, 2005c.

American Industrial Hygiene Association Toxicology Committee, Emergency exposure limits, *Am. Ind. Hyg. Assoc. J.,* 25, 578–586, 1964.

Cavender, F.L., Protecting the community, one guideline at a time, *The Synergist (AIHA)*, 13, 29–31, 2002.

Cavender, F.L. and Gephart, L.A., Emergency response planning, *J. Air Waste Manage.*, 11, 111–122, 1994.

European Centre for Ecoltoxicology and Toxicology of Chemicals, Emergency Exposure Indices for Industrial Chemicals, Technical Report 43, ECETOC, Mar. 1991.

Haber, F., Funf Vortage aus den Jahren 1920–1923: Geschichte des Gaskrieges, 76–92. Verlag von Julius Springer, Berlin, Germany, 1924, pp. 76–92.

Jacobson, K.H., AIHA short-term values, *Arch. Environ. Health*, 12, 486–487, 1966.

Kelly, D.P. and Cavender, F.L., Emergency response, in *Encyclopedia of Toxicology*, Vol. 1, Wexler, P., Ed., Academic Press, San Diego, CA, 1998, pp. 527–531.

National Institute of Occupational Safety and Health, Documentation for Immediately Dangerous to Life or Health Concentrations (IDLHs), NTIS PB94-195047, NIOSH, Cincinnati, OH, 1994.

National Research Council (NRC), Commission on Life Sciences, Board on Environmental Studies and Toxicology, Committee on Toxicology: Criteria and Methods for Preparing Emergency Exposure Guidance Level (EEGL), Short-term Public Emergency Guidance Level (SPEGL), and Continuous Exposure Guidance Level (CEGL) Documents, National Academy Press, Washington, DC, 1986.

National Research Council (NRC), Commission on Life Sciences, Board on Environmental Studies and Toxicology, Committee on Toxicology: Guidelines for Developing Community Emergency Exposure Levels (CEEL's) for Hazardous Substances, National Academy Press, Washington, DC, 1993.

National Research Council (NRC), Committee on Toxicology: Guidelines for Developing Spacecraft Maximum Allowable Concentrations (SMACs) for Space Station Contaminents, National Academy Press, Washington, DC, 1992.

National Research Council (NRC), Standard Operating Procedures for Developing Acute Exposure Guidline Levels (AEGL's) for Hazardous Chemicals, National Academy Press, Washington, DC, 2001.

Occupational Safety and Health Administration, Permissible Exposure Limits (PELs), Federal Register, 1910–1000, June 29, 1995.

Rusch, G.M., The history and development of Emergency Response Planning Guidelines, *J. Haz. Mater.,* 33, 192–202, 1993.

Spacecraft Maximum Allowable Concentrations (SMACs) for selected airborne contaminants 1994–2000, Vols. I–IV, National Academy Press, Washington, DC.

Superfund Amendments and Reauthorization Act, 1986.

ten Berge, W.F., Zwart, A., and Appleman, L.M., Concentration-time mortality response relationships of irritant and systematically acting vapors and gases, *J. Haz. Mater,* 13, 301–309, 1986.

Title III, Clean Air Act Amendments, 1990.

U.S. Environmental Protection Agency/U.S. Federal Emergency Management Agency/U.S. Department of Transportation, *Technical Guidance for Hazard Analysis—Emergency Planning for Extremely Hazardous Substances,* U.S. Government Printing Office, Washington, DC, Dec. 1987.

Woudenberg, F. and von der Torn, P., Emergency exposure limits: a guide to quality assurance, *Good Pract. Regul. Law,* 1, 249–293, 1992.

4 Directed-Flow Aerosol Inhalation Exposure Systems: Applications to Pathogens and Highly Toxic Agents

Rudolph J. Jaeger, Susan G. Shami, and Liana Tsenova

CONTENTS

4.1 INTRODUCTION

There is a need for technology that allows controlled inhalation exposure of animals to highly pathogenic and highly toxic chemical agents. Thus, this chapter seeks to describe how directed-flow, nose-only inhalation exposure systems can be operated. The reader is also directed to works that may provide additional guidance (Salem, 1994).

Nose-only, directed-flow inhalation exposure systems require less material and occupy relatively little space in the laboratory. They are also easier to operate and decontaminate than traditional whole-body exposure systems. We differentiate directed-flow "nose and snout only exposure" from "flow past exposure." The latter concept consists of a system or chamber having a central space where airflow (whether from above or from the side) is tangential to the axis of the animal's body. Such systems include flowing manifold designs that cause the aerosol to be delivered sequentially to each animal's nose. Examples of these include the Henderson Apparatus (Henderson, 1952) and the ADG type system (Alexander et al., 1997; Fox et al., 2001). We observe that simultaneous exposure is preferred over sequential because the contribution that each animal makes to the inhaled air of the subsequent animal can seriously reduce the exposure dose (Moss and Asgharian, 1994; Moss, 1997).

The history and some aspects of operation of selected inhalation exposure systems for the generation, regulation, and delivery of toxic particles and pathogenic aerosols to animals via the respiratory tract are also described in this chapter. The principles, like the equipment described, can apply as well to gases and vapors. The generation and control of the inhalant and the equipment used to generate them have been described by others (Drew and Laskin, 1973; Leong, 1981; MacFarland, 1983, 1987).

4.2 BACKGROUND

4.2.1 History of Inhalation Exposure Systems

Early work in this field was summarized by Fraser et al. (1959). As noted in the Introduction, a good portion of the standard inhalation exposure literature has since been presented elsewhere and need not be repeated here. Several important works have appeared in the past several decades. These include the pioneering work of Bob Drew and Sidney Laskin (Drew and Laskin, 1973); compendium works such as Leong's 1981 symposium on inhalation toxicology (Leong, 1981), Salem's first edition of *Inhalation Toxicology* (Salem, 1987), and Phalen's text of the same name (Phalen, 1997) deserve special mention. More recently, McClellan and Henderson's ***Inhalation Toxicology,*** second edition, is a comprehensive text on the subject (McClellan and Henderson, 1995). Drs. Pauluhn and Mohr have covered their investigations of whole-body and nose-only exposures along with those of others in a well-documented review article (Pauluhn and Mohr, 2000). Additionally, the reader may wish to review the following works (Willeke, 1980; Gardner and Kennedy 1993; Wong, 1999).

These preceding works are all suggested as basic texts to inform those wishing to practice this art. However, it should be noted that this field, like its parent, namely toxicology as a whole, can be learned in at least two lessons, each one taking 10 or more years (Arnold Lehman, as ascribed to him in Casarett and Doull's toxicology text).

Drew and Laskin (1973) cite a number of early versions of head and nose exposure units in their review. The efforts of Stokinger, D. W. Henderson, Hodge, Laskin, and the Rochester group, among others, to develop and advance the science of inhalation toxicology are cited. Among the early investigators, several stand out as having developed methods for working with pathogens, radioactive aerosols, and tobacco smoke. The design for one such a system, an early New York University (NYU) implementation of a 12-animal, multi-tier nose-only exposure unit with Lucite restraint tubes is illustrated in Drew's review. This design, described by a graphic illustration in the text, shows how an early style of nose-only exposure system might appear. Note that the design pictured is one that is based on a flow-past mode of operation rather than a directed-flow design.

One situation in which Drew and Laskin state a need for nose-only exposure concerns cigarette smoke. Such researchers need to study exposure methods that prevent oral exposure to tobacco tar. Oral exposure by licking and preening occurs routinely in fur-bearing experimental animals. The animal licks its fur as a matter of course and thus, an oral dose will occur due surficial tar deposition during whole-body exposure. These observations, that fur contamination by cigarette smoke and whole-body exposure to radionuclides resulted in a sizeable oral dose, form the basis for the designs of Homburger and colleagues (1967) and later, Dontenwill (1970), among others, to restrict the exposure of the body to toxicants. Thus, early tobacco smoke investigators employed the nose-and-snout-only technique as one means to minimize and reduce oral exposure due to contamination of body parts. Similarly, in infectious diseases research, after exposure to the virulent organisms the exposed animal must be irradiated with ultraviolet light for 30 min to disinfect the animal's skin and hair (Lurie et al., 1950; Middlebrook, 1952; North, 1995).

Based on the above, Coggins and his colleagues at the Battelle Memorial Institute in Geneva, Switzerland developed a multi-tier, directed-flow nose-only exposure system for use in tobacco smoke research (Baumgartner and Coggins, 1980). This Battelle-Coggins-Geneva (BCG) exposure design was fabricated from aluminum and poly (vinyl chloride) (PVC). Two radial openings within each exposure port, one for smoke delivery and one for smoke removal, were employed. The unit was closely coupled to a cigarette-smoking machine (CSM). It allowed fresh, suitably diluted mainstream smoke to be radially distributed to the nose (breathing zone) of each of 72 animals. The animals were individually restrained, each within an open polycarbonate tube. Each tube was located on one of three separable 24-port exposure tiers. A total of 72 animals, mouse, rat, hamster, or guinea pig, could be thus accommodated.

The animal restraint tubes were originally designed by Baumgartner. An example of the available style is shown in Figure 4.1. The tubes are fabricated from polycarbonate and have become

FIGURE 4.1

known in the inhalation toxicology field as "Battelle Restraint Tubes" because of their early use in tobacco smoke research conducted at the Geneva, Switzerland, toxicology laboratory of Battelle. Each open tube has an asymmetric design with vent openings and an easy-to-operate sliding rear pusher (tail piece); each movable pusher is sized for the animal in question and is suitable for use with mice, adult and weanling rats, hamsters, or guinea pigs. Note that the rat, hamster, and guinea pig open-tube designs have three longitudinal slots above the neck of the animal. While useful for cooling, early investigators at Battelle found that the slots permitted the release of static electricity that had built up between the fur and the plastic.

Finally, the design in a closed format is available in several sizes and shapes that can accommodate mice, various sizes of rat and hamster, guinea pigs, and more recently rabbits. Examples of additional restraint tubes can be seen at www.inhalation.org.

Tubes like those pictured in Figure 4.1 have been used in hundreds of studies for at least 20 years. Tubes like these as well as cigarette-smoking machines are available from CH Technologies (USA) Inc. (Westwood, NJ).

Cannon and his colleagues at the Battelle Memorial Institute's Pacific Northwest Laboratory (Cannon et al., 1983) designed another early and widely accepted directed-flow, nose-only inhalation system. This design is illustrated in Figure 4.2.

This top-to-bottom flow design calls for four animals per level with 13 tiers**.** The total capacity is 52 animals or less with sample ports. Multispecies exposures can be performed with such a system. Various Battelle-style open and closed tubes are accommodated by this system. The tubes can also be held in place by wire supports as well as be freestanding by means of a double O-ring tube seal (snout) at each exposure port. The tube snout seal grasps the front protrusion of tube, not the snout of the animal. Sufficient pressure must be applied to rear pusher to assure that the animal places its nose and nares within the narrow end of the tube.

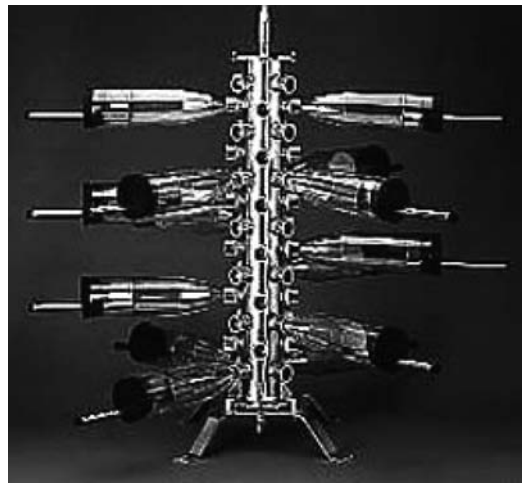


FIGURE 4.2

Early implementations of this patented design have been rendered in a variety of materials. One example is the use of PVC pipe. Some investigators are reported to have "built their own Battelle style system." This may have led to variability in results. For example, the use of different materials among investigators and a range of system sizes could lead to the existence of systematic differences that may change the operating characteristics of the device. To reduce this possibility, commercial standardized design exposure units in brass and stainless steel have been fabricated and sold by the Lab Products Company under license from Battelle. Such systems stand almost 4 feet tall and are supplied with a rotating base. The usual flow of aerosol in this implementation is from the top to the bottom.

This design, a tall device with a potentially stratified central delivery column, may lead to differences in concentration at various points along the inlet column. Dose differences between layers are possible. To reduce this possibility, a modular design was developed (Bernstein et al., 1994, 1995, 1996) for asbestos fibers, and Bernstein's modular system was employed by RCC and others to assess the hazards associated with various fibers. The system is fabricated with either 8 or 16 ports per layer. A large ring and circular tray, similar to that used by Coggins but now more open, was employed as a back tube support and as a feces and urine trap. Such sanitary collection systems were missing in some of the early exposure system implementations. Such collectors do not work with closed tubes and the latter may result in soiling of the animal's fur. Such soiling has been reported to offend some Institutional Animal Care and Use Committees. The nose-only exposure system was used for the first time for tuberculosis infection of mice by Tsenova et al. (1997).

The Jaeger-NYU Inhalation System was designed to provide an inhalation exposure system with reduced height, a smaller, centralized common breathing zone, and less internal dead space as compared with most other existing systems. In an effort to harmonize a variety of needs and wishes, namely ease of cleaning, alternative materials, and an altered airflow pattern, the design selected was one that encompasses and extends the radial form of the Coggins, Bernstein, and Cannon designs so as to construct a reduced-height, nose-only, directed-flow exposure system that allowed 12 animals per level. The U.S. patent for this design titles it as a flow-past system. However, the more recent definition of Moss applies, namely, that this system, along with the systems of Cannon, Coggins, and Bernstein, should be considered a directed-flow system.

The number of animals per layer, 12, was chosen for pragmatic reasons because many studies require 10 animals per sex per group and thus, 12 ports per level allows for the desired group size with two ports for sampling. When sampling at each level is not required, more animals can be accommodated in a small space. The design issues of numbers of animals per level have an impact on the diameter of such exposure systems. The restraint tubes are fixed in length and diameter.

Mouse tubes, being smaller, can be accommodated in a tighter circle (20 inches) than a comparable number of guinea pig tubes (36 inches). Sealed tubes often require an extended pusher to coax the animal forward and thus, 48-inch diameters can be required. Inaccurate placement of the nose cone ports and their associated delivery tubes can lead to potential misalignments of the aerosol delivery tube within the common breathing space (Moss and Asgharian, 1994).

Because there is often no connection between the inner and outer plenum of some designs, problems of assembly and alignment have been reported. These problems will occur unless the aerosol delivery tube location is nested or somehow controlled within the nose port. This is precisely the case with the BCG unit because the delivery tube is integral with the connector port. To prevent misalignment, the Jaeger-NYU design makes the round center hole of the common breathing zone within each animal port serve as a nesting point for a square cross section in the delivery nozzle, also called a trumpet because of the bell-shaped flare at the end (outlet). The four points of the 3/8-inch cross section make contact with the edge of the connector cone that holds the restraint tube (characterized by Owen Moss as a square peg in a round hole). The aerosol is thus directed to the nares of each animal without the possibility for misalignment. Further, choosing different tube lengths can alter the location of the end of the delivery trumpet. Flow rate (velocity actually) can be controlled by choosing delivery diameter sizes ranging from 7.5 mm down to 1.0 mm. Thus, larger openings will have a delivery velocity that is low whereas small holes will have a high velocity for a given pressure difference. Thus, at a constant flow rate, the delivered velocity can be adjusted. At a given location or level, the rate of aerosol delivery can be controlled in this manner.

Based on the preceding considerations, the Jaeger-NYU design (Jaeger, 1994) was created; it is illustrated schematically by S. Shami in Figures 4.3 and 4.4.

This radial, modular stacking design allows for a reduction of the diameter compared with that used in the BCG system to a more manageable size within a standard laboratory hood. To allow for reduced height restriction due to the hood used, the repeat distance between tiers (layers) is decreased to 3.5 inches. This is achieved by staggering the location of tubes in adjoining tiers. A 24-port stackable design with tube offset is illustrated below in a patent drawing but similar results are seen with two by 12-port slices where the tube connector cones are offset by 30 degrees (see Figure 4.5).

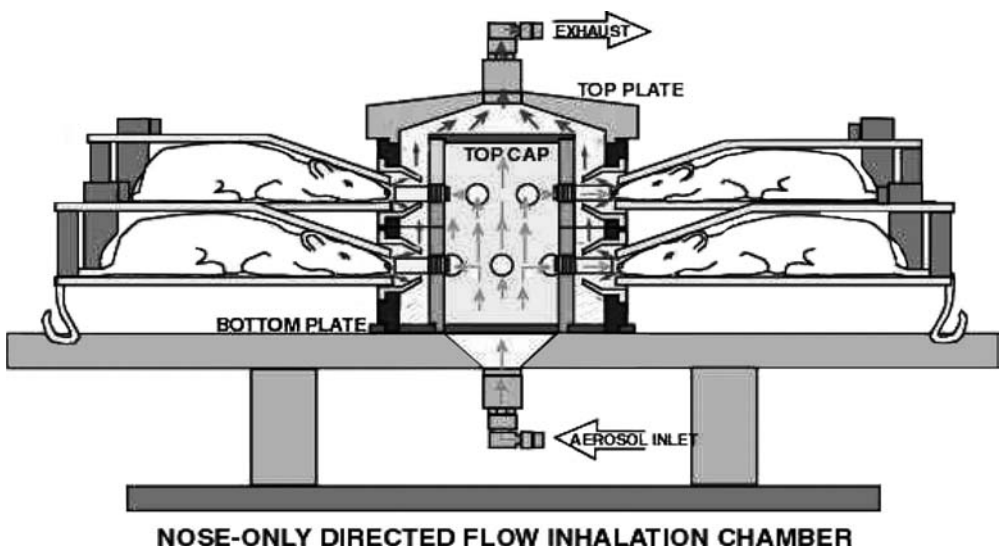


FIGURE 4.3 Representation of the Jaeger-NYU nose-only inhalation chamber. Two levels of 12 exposure ports each are depicted. Green arrows indicate clean air and aerosol. Red arrows indicate exhaled air and aerosol. Any type of aerosol production device may be used at the aerosol inlet including nebulizers or dust generators. An enlarged view of a portion of this chamber shows more detail. (Illustration by Susan Shami.)

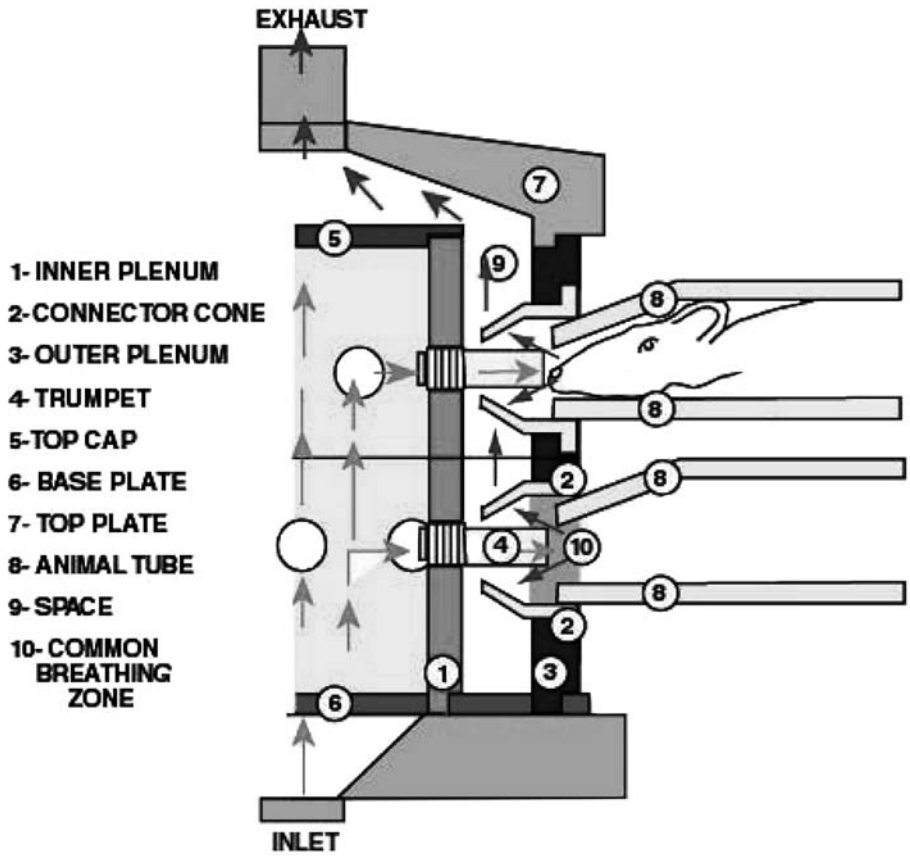


FIGURE 4.4 Aerosol enters inner plenum (1) at inlet on bottom and is delivered to animals via the trumpets (4) which screw into the inner plenum. There are usually 12 trumpets per level. The trumpets then deliver the aerosol to the animal's nose. The animal is held steady in the restraint tube (8). Each restraint tube is held into a port of the outer plenum (3) by means of a connector cone (2). Exhaust and exhaled air enter the space between the inner and outer plenum (9) and are released at the exhaust at the top of the chamber. The inner plenum is sealed with top cap (5) and base plate (6) and O-rings. The outer plenum is sealed with the top plate (7) and base plate and O-rings. The common breathing zone (10) is shown in pink around the nose of each animal. It is the region in which there may be a small amount of mixing of exhaled air and incoming aerosol. (Illustration by Susan Shami.)

Thus, a large number such as six layers or 72 animals can be mounted on a 6-inch-high table base (rotating). This will result in a system height that is less than 30 inches overall. As noted above, pragmatic considerations resulted in a modular directed-flow system based on 12 animals per tier. The airflow pattern that would apply is illustrated in Figure 4.6.

The system of radial aerosol presentation uses a generation system that is physically located below the exposure unit. Other systems such as those fabricated in accord with the designs of Cannon and Bernstein make use of exposure sources or generators that are often located above or at a distance removed from the exposure unit. As a result, significant line losses may occur. Additionally, a larger delivery dead space (tubing and fittings) can make such systems slow to achieve the desired concentration except where greater flows are used. A representation of the

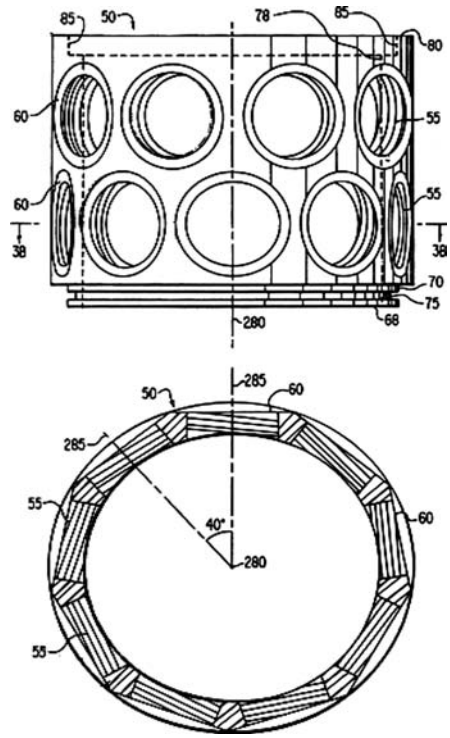


FIGURE 4.5

Jaeger-NYU design with an attached BGI Collison Nebulizer mounted directly beneath the exposure tower is shown in Figure 4.7.

4.2.2 Generation Systems

The next most important consideration after the choice of the animal exposure system used relates to the choice of a generation system for the exposure atmosphere. The system in Figure 4.7 shows a Collison nebulizer for direct delivery or aerosol to the base of the chamber. Desirable and efficient aerosol generation devices need to identified; many of these technologies are outlined by Phalen (1997). Particular attention should be paid to the article by Moss and Cheng (1995). Aerosol exposures are by their nature dynamic; thus, the generation and characterization of the atmosphere must be done in a careful and systematic manner.

Figure 4.8 shows a reduced-height inhalation exposure system that accommodates guinea pigs. The unit allows for simultaneous exposure of 64 animals in sealed tubes. The system height is less than 20 inches, whereas the diameter with internal plugs and sealed tubes is less than 31 inches. Thus, within many standard hoods, even those rated for highly toxic agents and pathogens, large numbers of animals can be exposed simultaneously.

The system pictured in Figure 4.9 is equipped for rats. A 64-port version of this system may be used with mice. Thus, it uses less space, namely a 20-inch diameter. The design shown above uses a sloping inner plenum with straight inner and outer surfaces. Thus, the internal delivery volume at each tier is reduced. Airflow that enters the bottom of the chamber is sent directly to each of the four layers so that the contained volume or delivery space at each level decreases as the input gas or aerosol is delivered to each layer of integrated nose cones. This design and detailed photographs can be found at www.inhalation.org. The pictured unit is undergoing validation at CIIT Centers for Health Research (Research Triangle Park, NC) for use as both an exposure system for mice and as a breath collection unit.

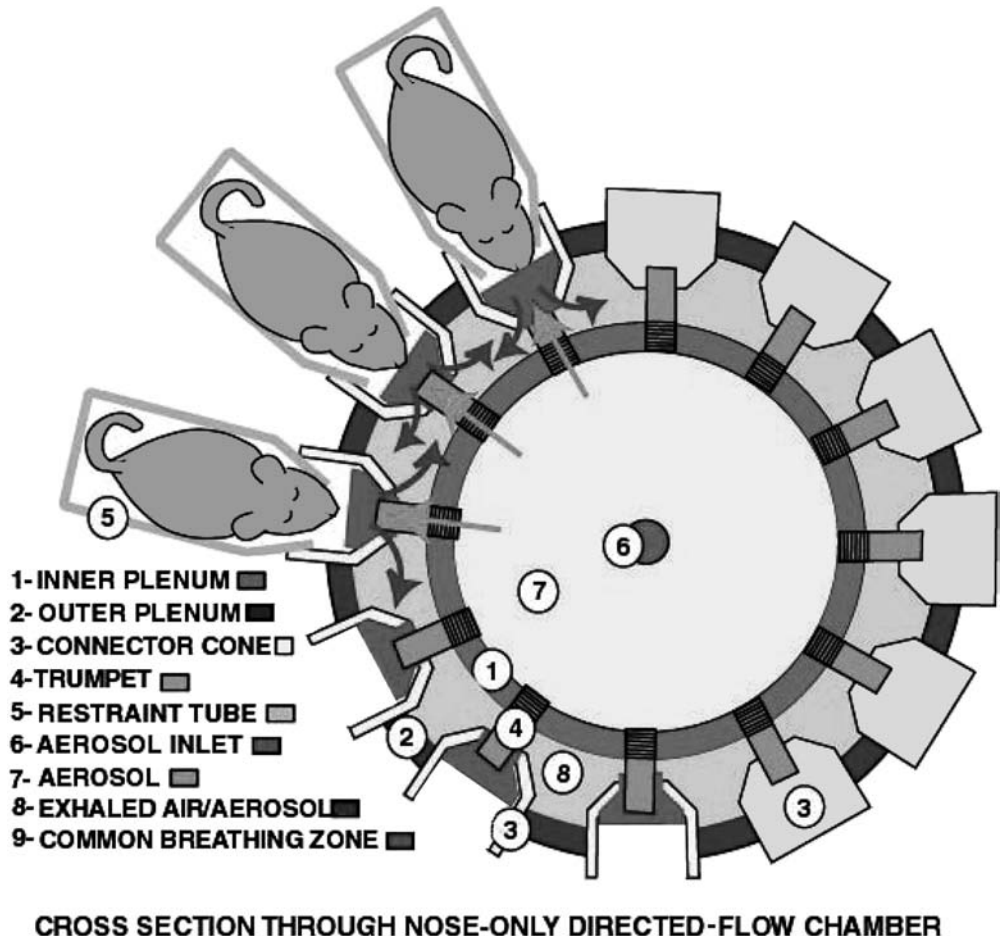


FIGURE 4.6 This cross-sectional diagram through a nose-only directed-flow inhalation chamber demonstrates the relationship between the entering aerosol, exhaled air and the exhaust aerosol, and the common breathing zone. The common breathing zone is the region of aerosol flow where some air exhaled from the animal may mix with the fresh aerosol allowing the animal to rebreath a small amount of mixed exhaust plus air. The Jaeger-NYU chamber has been designed to minimize the volume of this common breathing zone. (Note that the light green region represents the incoming air/aerosol and the light pink region represents the exhaled air and exhaust aerosol. The regions are contained by the inner and outer plenum.) (Illustration by Susan Shami.)

A restraint tube for rabbits is shown in Figure 4.10. The holder for this tube can be seen in Figure 4.11. Both of the systems pictured are experimental and are based on the need for larger animal models for infectious disease research.

The term dynamic inhalation exposure implies regulated flow and the maintenance of a controlled exposure concentration. In the dynamic case, the operative terms, concentration, exposure, and control can be done in several ways. Principally, clean dry air at a suitable pressure is regulated by a device such as a calibrated rotameter or a mass flow controller; a calibrated orifice is applied to a suitable generation system. Such devices include the Collison Nebulizer (Ip and Niven, 1994), the Wright Dust Feeder (www.bgiusa.com/agc/wright.htm), or the newly developed BioAerosol Nebulizing Generator (BANG) (www.inhalation.net/compare.htm).

In some cases, dry powders and fibers have been generated by using a rotating brush, and thus, controlled delivery of powder can be done in the Pallas Rotating Brush Generator (www.pallasdust.de).

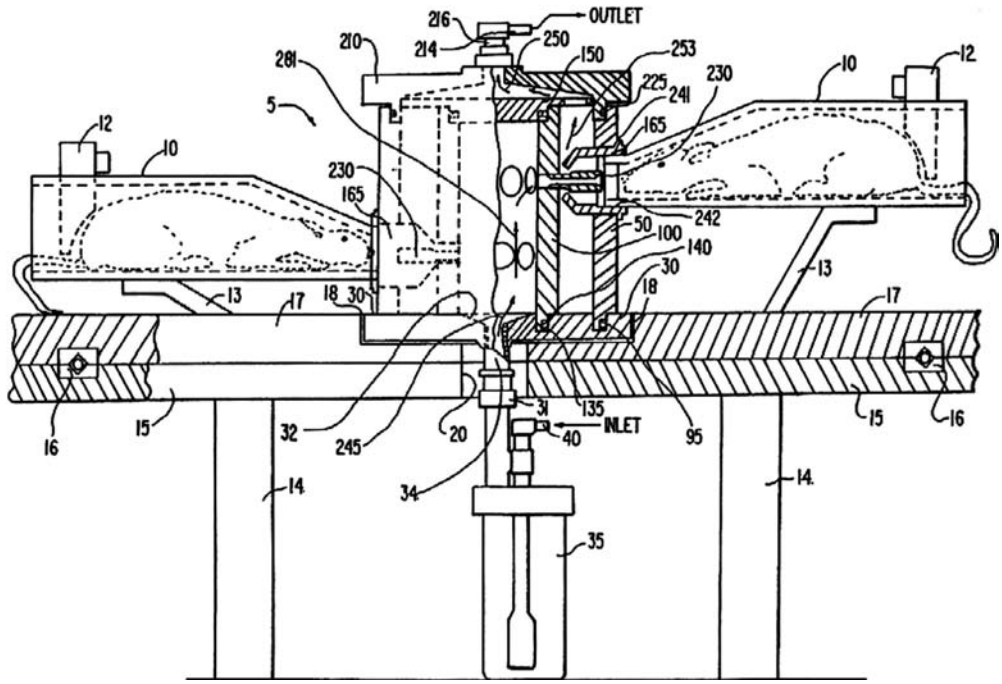


FIGURE 4.7

An extensive discussion of devices and principles used for aerosol generation can be found in the Lung Biology in Health and Disease volume (Hickey, 1996).

Whether the choice is for whole-body or snout/nose-only exposure, whether the choice is between directed-flow or flow-past systems, the inhalation exposure chamber must always be supplied with a source of clean, uncontaminated air before the animals are admitted to it. To start the process, animals are gently and carefully placed within their restraints. For practical reasons of uniformity of delivered dose, they should be allowed a period to accustom themselves to their novel surroundings. In the nose- and/or snout-only exposures, where restraint is required and is thought to cause stress, researchers at NYU have shown that an acclimatization period, 7 days at least, is required for animals to "settle" down, even when using optimally designed animal restraint tubes (Narciso et al., 2003).

4.2.3 Operational Issues

The exposure plan and equipment must fit within the facilities available. One such design is illustrated in Figure 4.12. The design presumes a Jaeger-NYU exposure system but others can be accommodated. The system also uses digital mass flow controllers, a digital control unit or general-purpose computer, along with suitable pressure regulators and air-cleaning devices to maintain aerosol generation while keeping the system negative. Air-cleaning devices would be required for safety even if the system were placed within a BSL-2 or BSL-3 enclosure.

Regardless of the inhalation system chosen by an investigator, a procedure or plan should be followed. Such a plan or procedure is sometimes referred to as a standard operating procedure, or SOP. All investigators and staff should agree about what and how work is to be done. The plan should be tailored for use in a specific institution or physical location. Of course, it must comply with applicable good laboratory practices (GLP) regulations. Anyone not familiar with using SOPs should seek the advice of an expert.

By using a Jaeger-NYU inhalation exposure system as the example and applying a modified version of the technology illustrated above for aerosol infection of 24 mice, the following procedure

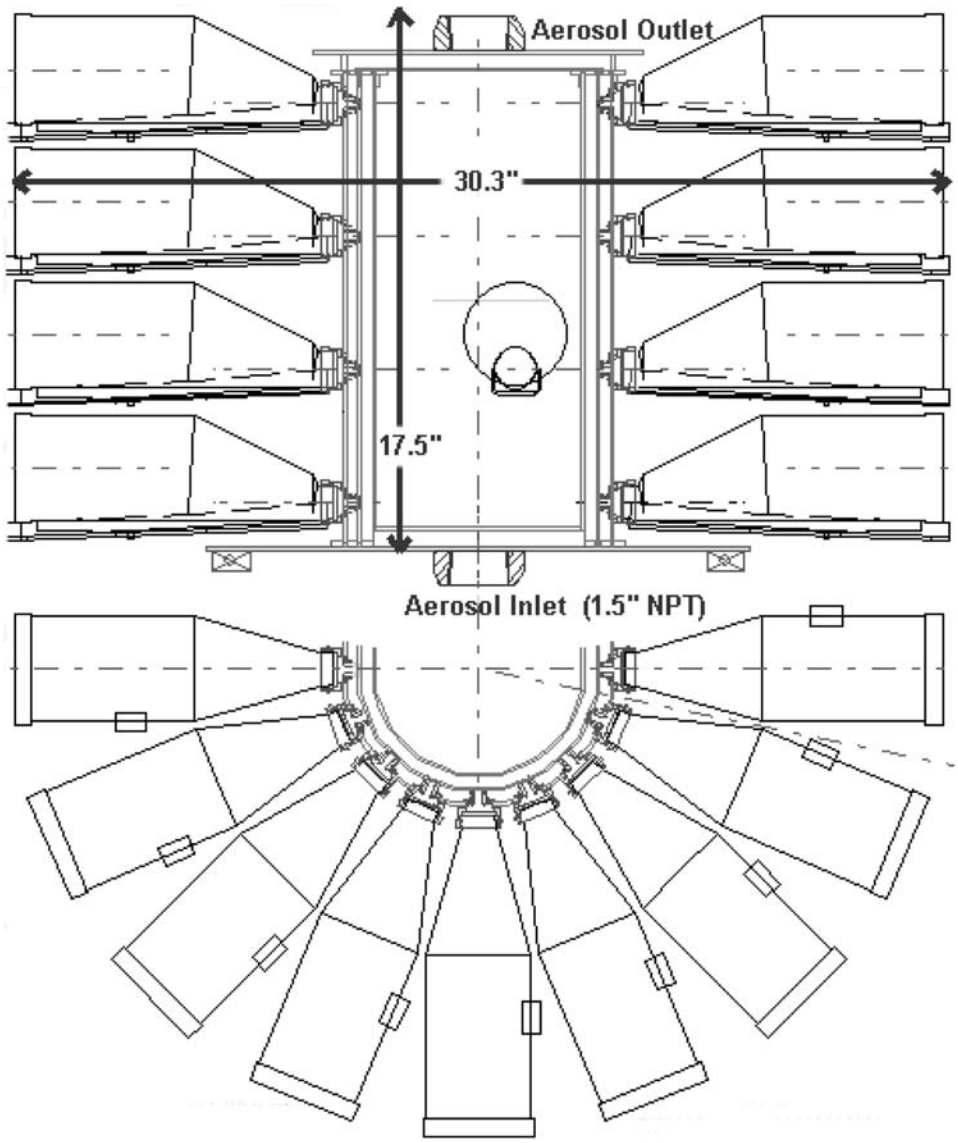


FIGURE 4.8 (Illustration by Henri Baumgartner.)

might be followed. Portions are derived from the work of Mark Buller and the St. Louis University group. This plan is subject to change and modification, depending on the infectious agent and specific institutional conditions. The reader is encouraged to contact the authors, Mark Buller, or others knowledgeable in the field for the latest offerings. See also www.inhalation.net for further discussion.

4.3 PROCEDURE: AEROSOL INFECTION OF MICE

This procedure utilizes the Jaeger-NYU aerosol exposure unit (CH Technologies) for pathogen infection of mice. The unit is a directed-flow, nose-only exposure system with multianimal capacity. This procedure is based on a 12- or 24-port unit. Four illustrations are shown.

The first view (Figure 4.13) shows a single-tier, 12-port design that is similar in concept to that given in Figure 4.12, now with manual controls replacing the digital flow controllers.

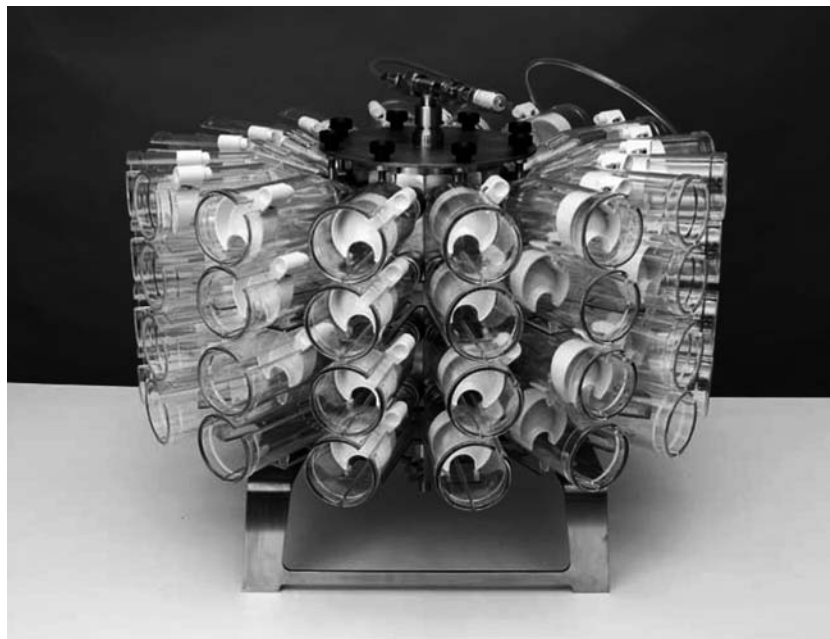


FIGURE 4.9

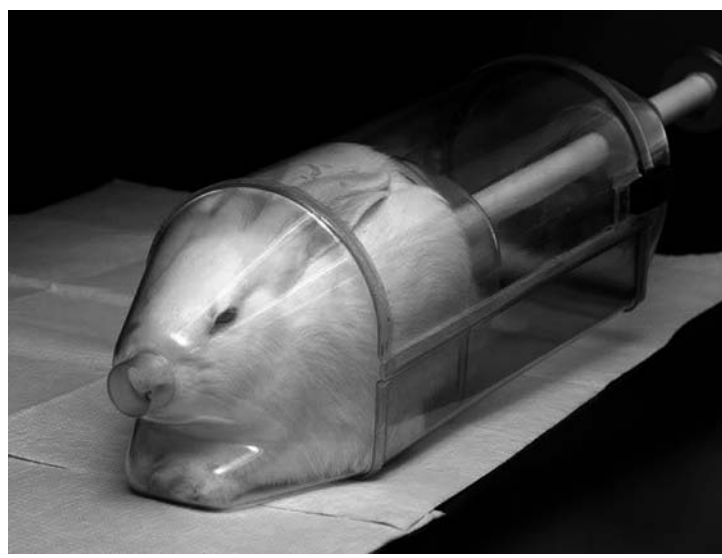


FIGURE 4.10

- The entire exposure apparatus must be placed inside a suitable biosafety cabinet, one specifically constructed or sized to contain the unit. For maximum safety, the entire device must be located within a properly designed and operated biosafety facility.
- An actual system during installation within a BSL-3 hood is shown in Figures 4.14 and 4.15. The front panel of the glove box/hood (Purified Micro-Environments, Ormond Beach, FL) has been removed for installation and illustration purposes.

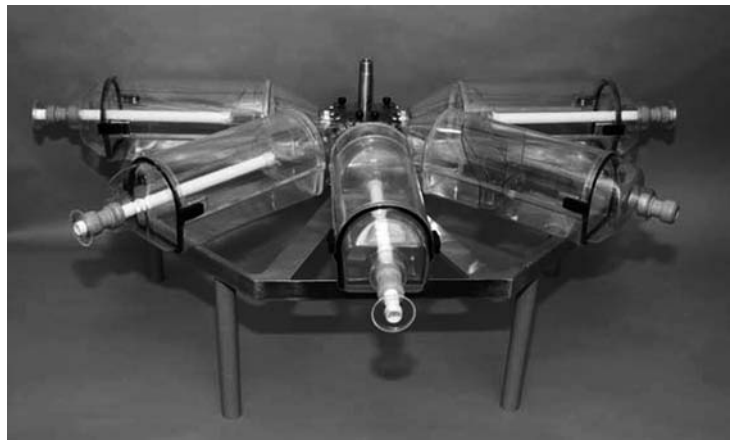


FIGURE 4.11

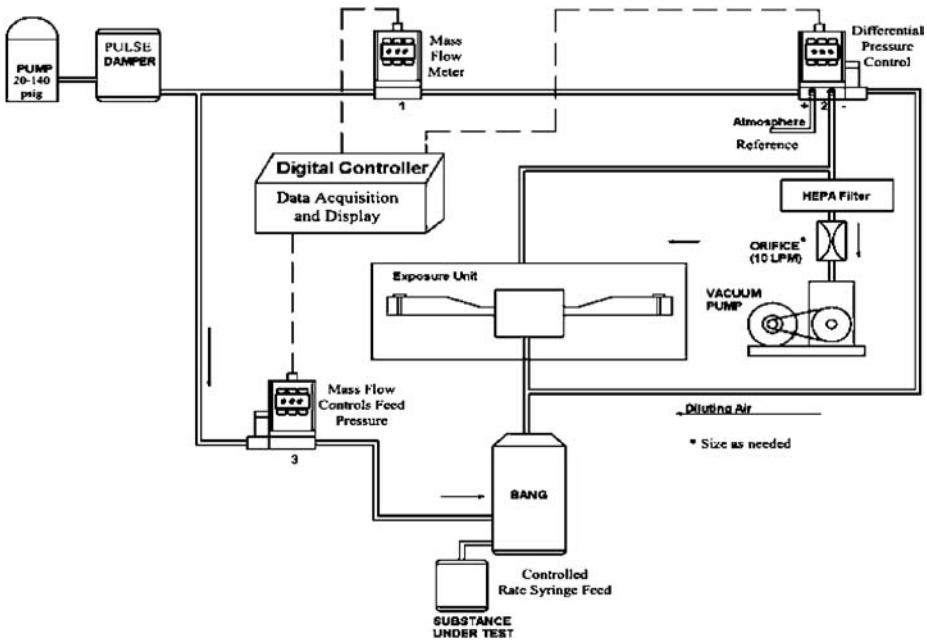


FIGURE 4.12 (Drawing courtesy of Neal Hartman, Alicat Scientific.)

- The system operates using a single-jet BioAerosol Nebulizing Generator (BANG) shown in Figure 4.16. In the same space, a standard one-, three-, or six-jet Collison will also work in this setting. The chosen aerosol generator unit with vertical discharge attaches directly to the bottom of the exposure system. Thus, any condensation that occurs within the central plenum can return to the aerosol generator.
- The bacterial suspension within the dose jar is drawn by aspiration through the aerosol generator.—This forms an aerosol that is stripped of large particles within the droplet filter/impactor that is built into the outlet.
- The generated aerosol meets an airstream (wet, dry, or mixed humidity) that then enters the system exposure unit at its base.

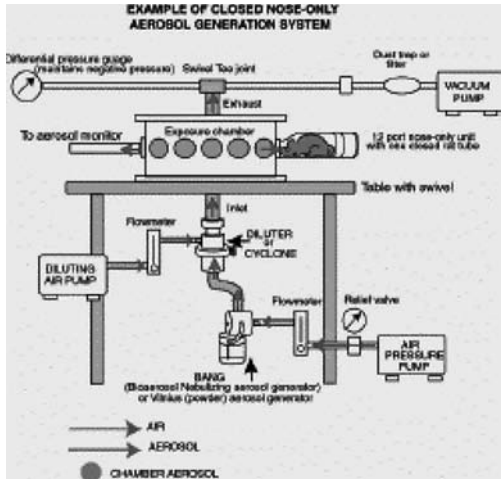


FIGURE 4.13 (Illustration by Susan Shami.)

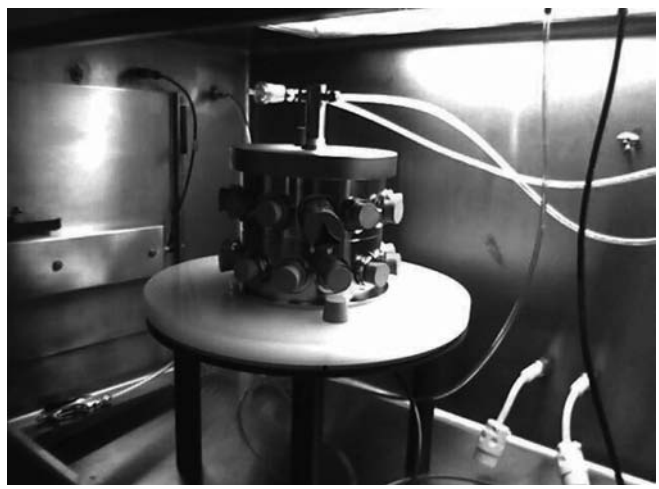


FIGURE 4.14 (University of Kentucky–Photo by R.J. Jaeger)

- After entering the inner plenum and being delivered outward, each animal inhales the delivered aerosol via directed flow from the central plenum.
- Exhaled gasses and the noninhaled aerosol exit the individual nose cones.
- The preceding step occurs via the outer exhaust plenum seen in Figure 4.16.
- The exhaust air is conducted through a high-efficiency particulate air (HEPA) filter to the vacuum line. An in-line sampler or impinger may be attached to the outflow line allowing collection of pathogen for subsequent analysis.

4.3.1 Start-Up and Operation

- The first task is to turn on the vacuum pump or initiate vacuum-controlled outflow. This causes the system to become negative.
- Verify that the entire exposure chamber is negative relative to the pressure within the hood or glove box. This can be done by opening one port in the system and observing the pressure gauge. Recheck all stoppers, plugs, tubing connections, etc. and confirm that there

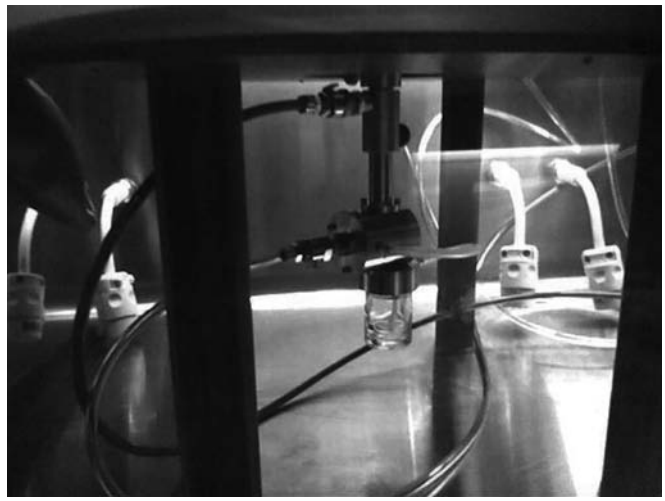


FIGURE 4.15 (University of Kentucky–Photo by R.J. Jaeger)

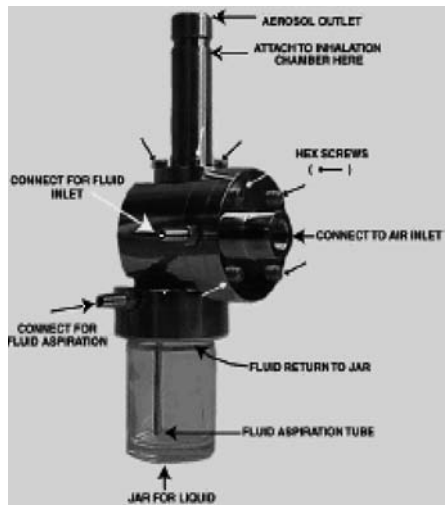


FIGURE 4.16 (Illustration by S. Shami)

are no obvious leaks. Test various locations by moving the connections and observe the pressure gauge.

- Turn on the air pump or activate the air supply. In a 24-port mouse system, set the dilution or breathing air to 4.5 l/min (90% of outflow rate if a 5 l/min flow restrictor [choke] is used). Note that the chamber becomes less negative as breathing air is added to the system.
- Verify that the system continues to be at least 0.1 inch of water negative at the desired airflow. Record chamber pressure relative to hood. You may wish to choose between wet and dry air sources for diluting air. Combine both such that the chamber remains slightly (0.1 inch H_2O) negative.
- Move animals from the housing room to the exposure facility.
- Insert each animal into a restraint tube. The animals are moved into the hood, BSL-3 cabinet, or the exposure location via the air lock. Carefully insert the test animals into ports,

one after another, removing plugs or stoppers and then setting them aside as each animal is inserted. The stoppers should be easily accessible for later use.
- Let the clean dilution air run for 15 min. This allows the animals to acclimate and for the investigator to confirm that connections are secure.
- Confirm and record the flow rate. Confirm that the gauges used have been calibrated with suitable instruments and the readings are reliable.
- Check all animal positions to be certain that none have turned around or are retracted within the tube.

4.3.2 Procedure, Assuming That a Pathogen Exposure Will Be Done

- Prepare a bacterial suspension (10–15 ml) in advance and place in the nebulizer cup or jar. Alternatively, a pump-fed delivery system may be used. This is covered in a separate SOP. The particle density of the suspension may vary and should be chosen based on the desired infectivity, e.g., desired number of colony-forming units per milliliter.
- Shut diluting air down, causing the chamber to swing abruptly negative. Quickly set the nebulizer flow switches or controls to on. These should have been set previously to the same or slightly less flow as the diluting air.
- Confirm or adjust flow of dilution air as required (or shut off if not used) and set aerosol flow to 4.9 l/min. This assumes a 5-l/min overall flow rate. Confirm that there is at least 0.1 inch of water negative within the exposure unit. A greater pressure difference may be preferred for reasons of safety. ***In any event, do not allow the system to become positive***.
- The aerosol exposure should continue for 20–30 min. Mice should be infected with a defined number of organisms, but the exact number remains to be determined. The exact number of organisms is determined by separate measurements, e.g., serial dilution of standard innoculum.
- When the desired exposure time is reached (end of exposure), shut down the nebulizer air flow and allow the system to swing negative, e.g., 1.5 inches of H_2O. Then initiate the dilution airflow. Allow a time, e.g., 15 min of clean air operation, to remove the residual aerosol from the system.
- Remove the infected animals one at a time from the exposure space. Insert the port stopper as each animal is removed. We suggest removing animals to a class 2 hood and using filtered transport cages. This may be subject to local biosafety rules that vary from laboratory to laboratory.
- Remove each animal from its restraint tube carefully. Place the subject into a transporting cage with a filter lid. Using a cart or sealed system, the animals are transported and relocated to the housing room.
- As each animal is removed, replace the stoppers in each exposure port to seal the system.
- Disconnect the nebulizer. Discard the remaining bacterial suspension after adding a suitable disinfectant such as sodium hypochlorite solution, 70% ethanol, or another approved decontamination solution. Use a clean nebulizer cup, shut off diluting air, and restart the aerosol unit.
- Allow the disinfecting aerosol to circulate through the tubing and exposure system for a **set** time, e.g., 20 min. The purpose of this step is to clean, disinfect, and sterilize the unit from within the chamber.
- After each exposure, the animal restraint tubes are placed in a container with a lid and disinfected in a suitable solution. After a set time, e.g., 20 min or more (q.s.) the restraint tubes are washed in a suitable solution such as hot water and detergent. The nebulizer is disinfected externally in a suitable solution.

- The various layers are rinsed with disinfectant, followed by warm tap water; then they may be sprayed with 70% ethanol as an additional safety factor. The entire chamber is disassembled as desired to inspect seals and to ensure proper drying. Residual sodium hypochlorite should be removed by the water rinse. A distilled water rinse can be used and then the system can be allowed to air dry.
- Note that aluminum-plated or anodized parts may corrode when left wet or kept in contact with alkaline solutions. Physical damage of aluminum parts that breach plating or anodized coatings can lead to leaks and/or corrosion. Thus, the integrity of system elements must be confirmed before the start of each experiment.
- As noted the exposure unit should always be rinsed with clear water followed by a distilled rinse and then allowed to air dry. A separate SOP is used for steps involving an autoclave.
- The bacterial challenge dose is determined by sacrificing one or more animals at a suitable time after exposure. Two hours is a standard sacrifice time. Remove the lungs and homogenize and plate them on appropriate agar medium. This should be the subject of SOPs that are presented in the following works: Israeli (1994) and Jensen et al. (1994).

4.4 CONCLUSION

This chapter seeks to inform the reader and the user of the inhalation system about the origin and application of methods suitable for use with pathogens or highly toxic agents that are delivered as aerosols. The information offered in this chapter should be considered general in nature but specific in intent. That is, case-by-case determinations of particle size, particle number, and infectivity may alter the methods that are applied in individual cases.

Individual toxicants vary and, thus, no generalities should be applied without a modicum of caution. There is no replacement for experience and cautious investigation. The local institutional rules must be factored into any work performed.

Finally, our greatest reliance is placed on individual investigators, acting in a competent manner and developing their methods and further qualifying their coinvestigators. We encourage readers to share their observations and methods through publication and meeting presentations.

ACKNOWLEDGMENTS

Daisy Margolin's photographic skills are gratefully acknowledged. R.J. Jaeger wishes to acknowledge the contribution of Mark Buller and his associates at St. Louis University Medical School who generously shared their experiences with inhalation exposure of animals with all who asked. The authors also thank Donald Gardner for his helpful assistance.

REFERENCES

Alexander, D.J., Mather, A., and Dines, G.D., A snout-only inhalation exposure system for use in rabbit teratology studies, *Inhalation Toxicol.* 9, 477–490, 1997.

Baumgartner, H. and Coggins, C.R.E., Description of a continuous-smoking inhalation machine for exposing small animals to tobacco smoke, *Beit. Tab. Int.,* 10, 169–174, 1980.

Bernstein, D.M., Mast, R., Anderson, R., Hesterberg, T.W., Musselman, R., Kamstrup, O., and Hadley, J., An experimental approach to the evaluation of the biopersistence of respirable synthetic fibers and minerals, *Environ. Health Perspect.,* 102 (Suppl. 5), 15–18, 1994.

Bernstein, D.M., Morscheidt, C., Grimm, H.G., Thevenaz, P., and Teichert, U., Evaluation of soluble fibers using the inhalation biopersistence model, a nine-fiber comparison, *Inhalation Toxicol.,* 8, 345, 1996.

Bernstein, D.M., Morscheidt, C., Tiesler, H., Grimm, H.G., Thevenaz, P., and Teichert, U., Evaluation of the biopersistence of commercial and experimental fibers following inhalation, *Inhalation Toxicol.,* 7, 1031–1058, 1995.

Cannon, W.C., Blanton, E.F., and Mcdonald, K.E., The flow-past chamber: an improved nose-only exposure system for rodents, *Am. Ind. Hyg. Assoc. J.,* 44, 923–928, 1983.

Dontenwill, W., Experimental investigations and effects of cigarette smoke inhalation on small laboratory animals, in *Inhalation Carcinogenesis*, Hanne, M.C.J., Nettesheim, P., and Gilbert, J.R., Eds., 1970.

Drew, R.T. and Laskin, S., Environmental inhalation chambers, *Methods of Animal Experimentation,* 4, 1–41, 1973.

Fox, E., Alexander, D.J., and Robinson, D.W., A Comparison of the Performance of Flow-Past and Conventional Inhalation Exposure Chambers Using Metered Dose Inhalers, Nebulisers or Dust Generators, unpublished work, 2001.

Fraser, D.A., Bales, R.E., Lippmann, M., and Stokinger, H.E., Exposure Chambers for Research in Animal Inhalation, Public Health Monograph 57, U.S. Government Printing Office, Washington, DC, 1959.

Gardner, D.E. and Kennedy, G., Jr., Methodologies and technology for animal inhalation toxicology studies, in *Toxicology of the Lung,* 2nd ed., Gardner, Ed., Raven Press, New York, 1993, pp. 1–30.

Henderson, D.W., An apparatus for the study of airborne infection, *J. Hyg.,* 50, 53–68, 1952.

Hickey, A.J., *Inhalation Aerosols: Physical and Biological Basis for Therapy,* Marcel Dekker, New York, 1996.

Homburger, F., Bernfeld, P., Bogdonoff, P., Kelley, T., and Walton, R., *Toxicol. Appl. Pharmacol.* 10, 382, 1967.

Ip, A.Y. and Niven, R.W., Prediction and experimental determination of solute output from a collison nebulizer, *J. Pharm. Sci.* 83, 1047–1051, 1994.

Israeli, E.E.A., Death mechanisms in microbial bioaerosols with special reference to the freeze dried analog, in *Atmospheric Microbial Aerosols: Theory and Application,* B. Lighthart, B. and A.J., Mohr, Eds., Chapman and Hall, New York, 1994, pp. 166–191.

Jaeger, R.J., *Modular Reversible-Flow-Past Nasopulmonary Respiratory Dosing System for Laboratory Animals,* U.S. Patent 5,297,502, 1994.

Jensen, P.A., Lighthart, B., and Mohr, A.J., Instrumentation used with microbial bioaerosols, in *Atmospheric Microbial Aerosols: Theory and Application,* Lighthart B. and Mohr, A.J., Eds., Chapman and Hall, New York, 1994, pp. 226–284.

Leong, B.K.J., *Inhalation Toxicology and Technology,* Ann Arbor Science, Ann Arbor, MI, 1981.

Lurie, M.B., Heppleston, A.G., Abramson, S., and Swartz, I.B., Evaluation of method of quantitative airborne infection and its use in study of pathogenesis of tuberculosis, *Am. Rev. Tuberc.,* 61, 765–797, 1950.

MacFarland, H., Designs and operational characteristics of inhalation exposure equipment, in *Inhalation Toxicology: Research Methods, Applications, and Evaluation,* Occupational Health Series, Vol. 12, hardcover ed., Salem, H., Ed., Marcel Dekker, New York, 1987, pp. 93–120.

MacFarland, H.N., Design and operational characteristics of inhalation exposure equipment—a review, *Fundam. Appl. Toxicol.,* 3, 603–613, 1983.

McClellan, R.O. and Henderson, R.F., *Concepts in Inhalation Toxicology,* 2nd ed., Taylor and Francis, London, 1995.

Middlebrook, G., An apparatus for airborne infection of mice, Proc. Soc. Exp. Biol. Med., 80, 105–110, 1952.

Moss, O.R., Animal exposure systems: conflicting goals between inhalation dosimetry and animal husbandry, 1997, pp. 51–73a.

Moss, O.R. and Asgharian, B., Precise inhalation dosimetry with minimun consumption of product: the challenge of operating inhalation exposure systems at their design limits, Byron, P.R., Dalby, R.N., and Farr, S.J., Eds., Respiratory and Drug Delivery IV, Interpharm Press, Buffalo Grove, NY, 1994, pp. 197–201.

Moss, O.R. and Cheng, Y.-S., Generalization and characterization of test atmospheres: particles and droplets, in *Concepts in Inhalation Toxicology,* 2 ed., McClellan, R.O., and Henderson, R.F., Eds., Taylor & Francis, Washington, DC, 1995, pp. 91–126.

Narciso, S.P., Nadziejko, E., Chen, L.C., Gordon, T., and Nadziejko, C., Adaptation to stress induced by restraining rats and mice in nose-only inhalation holders, *Inhalation Toxicol.,* 15, 1133–1143, 2003.

North, R.J., Mycobacterium tuberculosis is strikingly more virulent for mice when given via the respiratory than via the intravenous route, *J. Infect. Dis.,* 172, 1550–1553, 1995.

Pauluhn, J. and Mohr, U., Inhalation studies in laboratory animals—current concepts and alternatives, *Toxicol. Pathol.,* 28, 5, 734–753, 2000.

Phalen, R.F., *Methods in Inhalation Toxicology,* CRC Press, Boca Raton, FL, 1997.

Salem, H., Principles of inhalation toxicology, in *Inhalation Toxicology Research: Methods, Applications, and Evaluation,* Salem, H., Ed., Marcel.Dekker, New York, 1987, pp. 7–9.

Salem, H.G.D.E., Health aspects of bioaerosols, in *Atmospheric Microbial Aerosols: Theory and Application,* Lighthart, B., and Mohr, A.J., Eds., Chapman & Hall, London, 1994, pp. 304–330.

Tsenova, L., Moreira, A.D., Party, E., Freedman, V.H., and Kaplan, G., Aerosol infection of mice with mycobacteria using a nose-only exposure device, *J. Amer. Biological Safety Assoc.,* 2, 20–31, 1997.

Willeke, K., Generation of aerosols: exposure facilities, in *Generation of Aerosols and Facilities for Exposure Experiments,* Willeke, K., Ed., Ann Arbor Science, Ann Arbor, MI, 1980, pp. 443–458.

Wong, B.A., Inhalation exposure systems design, methods, and operation, in *Toxicology of the Lung,* 3rd ed., Gardner, D.E., C.J.D., and McClellan, R.O., Eds., Taylor & Francis, Philadelphia, 1999, pp. 1–53.

5 Low-Level Effects of VX Vapor Exposure on Pupil Size and Cholinesterase Levels in Rats

Bernard J. Benton, Douglas R. Sommerville, J. Steven Anthony, Ronald B. Crosier, Edward M. Jakubowski, Christopher E. Whalley, Paul A. Dabisch, Stanley W. Hulet, Kathy L. Matson, Charles L. Crouse, Robert J. Mioduszewski, and Sandra A. Thomson

CONTENTS

5.1 INTRODUCTION

O-Ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothiolate (VX) is an organophosphorous (OP) compound that has been the subject of much research for more than half a century. It is extremely

toxic with an equivalent dose of VX being substantially more toxic than related nerve agents such as sarin (GB), cyclosarin (GF), tabun (GA), and soman (GD). Most of what is known about the effects of VX on whole animals is derived from studies administering VX subcutaneously, percutaneously, intravenously, or as an inhaled aerosol (Craig et al., 1977; Rickett et al., 1986; Gupta et al., 1991; Bide and Risk, 2000). However, few studies exist in which reliable toxicity estimates in animals have been established for VX administered as a vapor (Hartman, 2002). Contributing to this lack of information is the difficulty in producing stable vapor concentrations in a controlled environment due to the extremely low vapor pressure of VX (0.00063 mmHg at 25°C compared with 2.9 mmHg at 25°C for sarin [GB]).

The available literature addressing the toxicity of VX vapor includes two studies that dealt with the toxic effects of chemically neutralized VX in rats (Manthei et al., 1990; Muse et al., 2002). Several studies have dealt with either aerosolized VX (Bide and Risk, 2000) or VX mixed with other compounds (Weimer and Ballard, 1960; Dimmick et al., 1979). One recent study examined the toxicity of VX vapor inhalation in rats using a "nose-only" exposure design (Bide et al., 1996), but did not address the issue of first noticeable effect (FNE) associated with very low concentrations of VX vapor. The concept of FNE is used to define the threshold concentrations of nerve agents below which there are no observable effects and above which more severe measurable effects are produced. Defining the FNE for a whole-body inhalation exposure to VX vapor is necessary for any comprehensive risk assessment dealing with exposure to VX vapor. Previous studies in our laboratory using GB (Mioduszewski et al., 2002) and GF (Whalley et al., 2004) vapor have shown miosis to be the FNE resulting from a whole-body inhalation exposure. In those two studies and the present study, miosis was used as the experimental end point. Miosis was defined as a 50% reduction in pupil diameter relative to preexposure baseline measurements.

Our objectives were to determine the median effective concentrations (EC_{50}s) of VX vapor that produced miosis in rats at three exposure durations of 10, 60, and 240 min; to determine the degree of cholinesterase inhibition in whole blood; to determine whether the miotic effects and cholinesterase depression of VX vapor exposure were gender dependent; and to develop an empirical toxic load

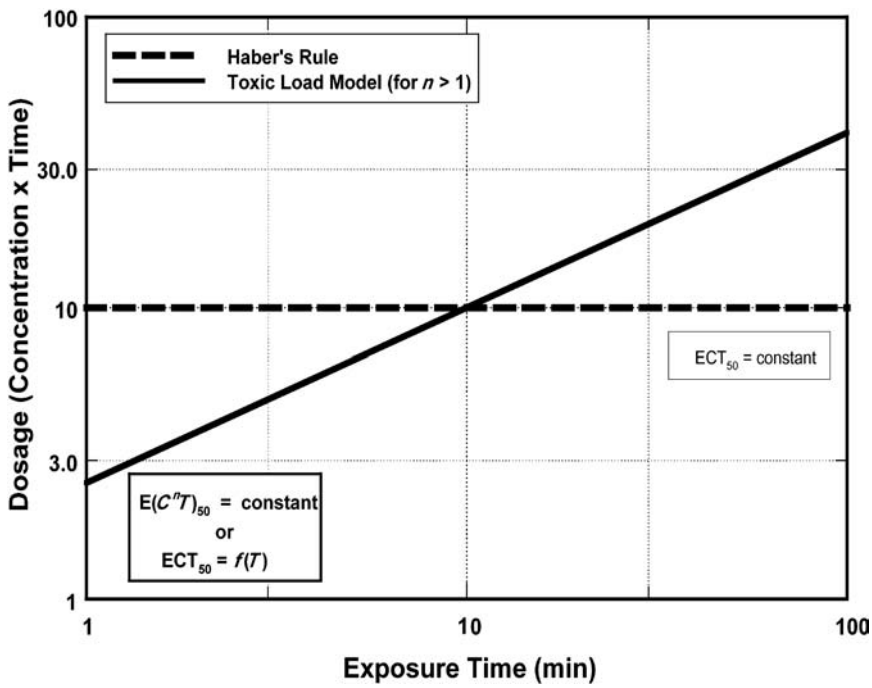


FIGURE 5.1 Comparison of Haber's rule and toxic load models for toxicity time dependence.

model for predicting VX vapor-induced miosis for duration times not tested directly. Our toxic load model is based on the initial work on dose–response relationships between concentrations of various chemicals and duration of exposure (Haber, 1924). The relationship, known as Haber's Rule, is described by the equation $C \times T = k$, where C is equal to the atmospheric concentration of the chemical being tested, T is equal to the duration of exposure, and k is a constant for some effect or response. This equation assigns equal importance to concentration and time in determining the response. Thus, the product of $C \times T$ would remain constant regardless of the concentration or exposure time. This assumption proved to be inadequate for many chemicals when attempting to describe cumulative toxicity effects. Thus, the equation was modified to better describe the relationship between concentration and exposure time for a given chemical (ten Berge et al., 1986). The equation $C^n \times T = k$ includes the exponent n, which is an experimentally determined, chemical specific value that helps describe the nonlinear relationship between concentration and duration of exposure (Figure 5.1). An integral part of our toxic load model was the estimation of this n value for miosis producing levels of VX vapor.

Whole-body vapor exposures were conducted in a 750-l dynamic airflow inhalation chamber. Rats were exposed for 10, 60, or 240 min. Five concentrations of VX were tested at each exposure duration. Baseline values for whole blood cholinesterase levels and pupil size were established in each rat prior to exposure.

Separate median effective dosage (ECT_{50}) values for miosis were established for male and female rats at each exposure duration. The values were derived from pupil measurements taken within 1 h after exposure. Table 5.1 shows that VX is approximately an order of magnitude more potent than GB and GF. There was significant acetylcholinesterase (AChE) depression at the highest concentrations

TABLE 5.1 Miosis Level EC_{50} and ECT_{50} Values for VX, GB, and GF

			95% Fiducial Interval			95% Fiducial Interval	
Sex	Time (min)	EC_{50} (mg/m^3)	Lower Limit	Upper Limit	ECT_{50} (mg-min/m^3)	Lower Limit	Upper Limit
				VX			
m	10	0.01	0.0085	0.0124	0.102	0.085	0.124
m	60	0.004	0.0030	0.00520	0.229	0.180	0.300
m	240	0.002	0.0015	0.0023	0.443	0.363	0.547
f	10	0.007	0.0060	0.0089	0.073	0.060	0.089
f	60	0.002	0.0014	0.0023	0.106	0.087	0.136
f	240	0.001	0.0009	0.0014	0.268	0.219	0.326
				GB			
m	10	0.087	0.076	0.099	0.87	0.76	0.99
m	60	0.030	0.022	0.043	1.80	1.34	2.58
m	240	0.024	0.016	0.044	5.76	3.84	10.56
f	10	0.068	0.059	0.078	0.68	0.59	0.78
f	60	0.020	0.014	0.027	1.20	0.84	1.62
f	240	0.012	0.006	0.019	2.88	1.44	4.56
				GF			
m	10	0.184	0.146	0.239	1.843	1.46	2.39
m	60	0.042	0.031	0.059	2.511	1.86	3.56
m	240	0.029	0.023	0.038	7.031	5.41	9.19
f	10	0.080	0.063	0.099	0.796	0.63	0.99
f	60	0.024	0.018	0.031	1.413	1.13	1.84
f	240	0.017	0.014	0.022	4.155	3.27	5.25

of each exposure duration. There were significant gender differences in the ECT_{50} values for miosis at each exposure duration. An empirical toxic load model was developed and the toxic exponent for miosis (n) in the equation $C^n \times T = k$ was determined to be $n = 1.65$.

The data provide important information regarding the relationship between exposure levels, gender sensitivity, miosis, and cholinesterase depression.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals

O-Ethyl-*S*-[2-(diisopropylamino) ethyl] methylphosphonothiolate (VX) was used for all vapor exposures. Seven iterations of a ^{31}P NMR analysis were performed according to an established method (Brickhouse et al., 1997) to certify the purity of the material as 93.6 ± 0.5 mol% pure.

5.2.2 Inhalation Chamber

Whole-body vapor exposures were conducted in a 750-l dynamic airflow inhalation chamber. The chamber was hexagonal and constructed of stainless steel with plexiglas windows on each of its six sides. The interior of the exposure chamber was maintained under negative pressure (0.25 in. H_2O), which was measured by a calibrated magnehelix (Dwyer, Michigan City, IN). Room air was` drawn through the exposure chamber (570–580 l/min) and measured at the chamber outlet with a calibrated thermoanemometer (Alnor model 8565, Skokie, IL). Temperature and humidity were recorded at the start of every exposure.

5.2.3 Vapor Generation

The vapor generation system was located at the chamber inlet and was contained within a stainless steel box maintained under negative pressure. Saturated VX vapor streams (0.00037–0.016 mg/m^3) were generated by a continuous flow of nitrogen carrier gas (8–202 ml/min) through a glass saturator cell (Glasssblowers Inc., Turnersville, NJ) containing 1 ml of liquid VX (Figure 5.2). The main body of the saturator cell contained a porous, hollow, ceramic cylinder, which increased the contact area between the liquid VX and the nitrogen carrier gas by absorbing the liquid VX. The saturator cell was fabricated to allow nitrogen gas to make three passes along the surface of the wetted ceramic cylinder (Alundum-fused alumina, Norton Co., Colorado Springs, CO) before exiting the outlet arm of the saturator cell. The saturator cell body was immersed in a constant temperature bath (Thermo NESLAB, Portsmouth, NH) containing mineral oil. Alterations in nitrogen gas flow rate and temperature were used to regulate the amount of VX vapor entering the inhalation chamber.

5.2.4 Sampling System Sorbent Tubes

The solid sorbent tube sampling system consisted of a 20:35 mesh Tenax-TA fast-flow sorbent tube (Dynatherm part AO-06–2717) and a thermal desorption unit (TDU) (ACEM-900, Dynatherm Analytical Instruments, Kelton, PA) coupled to a gas chromatograph with flame photometric detection (GC/FPD). Vapor samples were drawn from the middle of the exposure chamber by inserting a rod containing a sampling tube through small access ports located on the walls of the chamber. The rod was connected to a vacuum line that drew a sample through the tube at a rate of 3–5 l/min for 1–9 min depending on the chamber concentration. Sample flow rates were controlled with calibrated mass flow controllers (Matheson Gas Products, Montgomeryville, PA) and verified before and after sampling with a calibrated flowmeter (DryCal, Bios International, Pompton Plains, NJ) connected in-line with the sample stream. The sample tube was transferred to the TDU and prepared for injection onto a Restek RTX-5 column (15 m × 0.32 mm × 0.5 μm). Temperature and flow programming

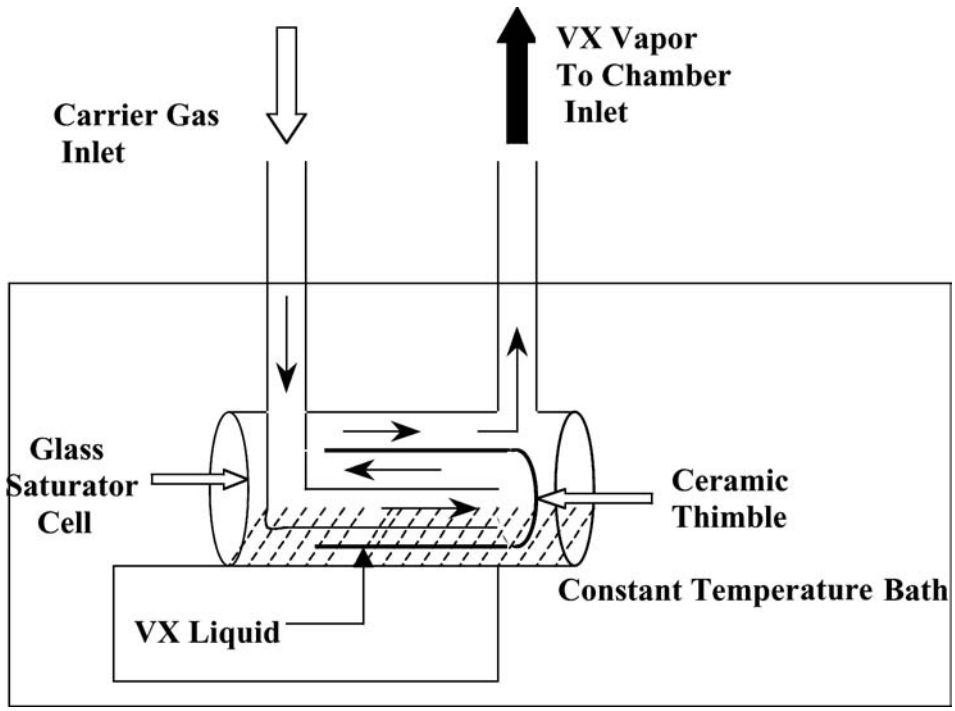


FIGURE 5.2 VX vapor generation using a saturator cell.

within the TDU desorbed VX from the sorbent tube directly onto the GC column. Detection was performed with FPD in the phosphorous mode.

Concentration uniformity was checked at several locations throughout the chamber, including areas directly above the animal cages. At higher generated agent concentrations, vacuum pumps were used to draw air through glass fiber filter pads at high flow rates to test for the presence of aerosols. Analysis of the glass fiber pads required isopropanol desorption and liquid extract injection onto a 20:35 mesh Tenax-TA fast-flow sorbent tube. The sorbent tube was thermally desorbed and analyzed by GC/FPD. All findings for the presence of aerosolized VX were negative.

5.2.5 Animal Model

Sexually mature male and female Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) weighing between 180 and 300 g were used in this study. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility. The animals were quarantined for a minimum of 5 days after their arrival. Ambient conditions were maintained at 70 ± 5°F, 30–70% relative humidity with a 12:12 hour light-dark cycle. Rats were provided with certified laboratory rat chow and filtered house water ad libitum, except during exposure. All experiments and procedures were approved by the U.S. Army Edgewood Chemical Biological Center Institutional Animal Care and Use Committee and conducted in accordance with the requirements of Army Regulation 70-18 and the National Research Council's *Guide for the Care and Use of Laboratory Animals*.

5.2.6 Blood Sample Collection

Blood samples were drawn from the tail vein of all test rats and used for the cholinesterase inhibition assays. Blood draws were done once before exposure, approximately 60 min. after exposure, and 7 days after exposure. Approximately 300 μl of blood was collected at each draw.

5.2.7 Cholinesterase (ChE) Inhibition Assays

The method used for measuring whole-blood acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities was a modification of the Ellman Reference Method (Ellman et al., 1961).

Approximately 300 μl of blood was collected for determination of whole-blood AChE and BChE activities. For each blood sample, a 10-μl aliquot of clot-free whole blood was added to 2 ml of distilled water in a 13 × 75 mm test tube followed by addition of 200 μl of 0.69 m*M* phosphate buffer at pH 7.4 (EQM Research, Cincinnati, OH). Each tube was then vortexed and 200 μl of the resulting solution from each tube was transferred to individual wells on a 96-well plate. Twenty-five microliters of 30 m*M* 5,5-dithiobis-2-nitrobenzoic acid (DTNB) was added to each well. For determination of AChE activity, 25 μl of a solution containing 10 m*M* of the substrate acetylthiocholine and 200 μ*M* 10-(α-diethylaminopropionyl)-phenothiazine, a specific inhibitor of butyrylcholinesterase (EQM Research), was added to the appropriate wells of the 96-well plate. For determination of BChE activity, 25 μl of a solution containing 20 m*M* butyrylthiocholine (EQM Research) was added to the appropriate wells of the 96-well plate. The plate was then read at 450 nm and 37°C using a SpectraMax Plus^{384} microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA) for 10 min, and analyzed using SoftMax Pro LS version 4.3 software (Molecular Devices Corp., Sunnyvale, CA).

BChE activity values in whole blood were expressed as units of activity per gram of total plasma protein (U/g TPP). To determine total plasma protein (TPP) concentration in grams per milliliter, whole blood was centrifuged to separate the plasma from the red blood cells (RBCs). Plasma was placed in a refractometer (American Optical Co., Keene, NH) and the total protein was read directly from the TPP scale.

AChE activity values in whole blood were expressed as units of activity per gram of hemoglobin (U/g HGB). Hemoglobin was measured by the Oshiro method (Oshiro et al., 1982). In brief, 225 μl of hemolysate was added to a 96-well (uncoated) Greiner microplate (Greiner BioOne, Longwood, FL) along with 25 μl of 2.08 m*M* sodium lauryl sulfate in a 30 m*M* phosphate buffer (pH 7.2). The plate was read at 536 nm and 37°C using a SpectraMax Plus^{384} microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA) for 10 min and analyzed by using SoftMax Pro LS version 4.3 software (Molecular Devices Corp.). All specimens were assayed in duplicate.

5.2.8 Photography

This study utilized a noninvasive method for assessing pupil size whereby projected infrared (IR) light (880 nm) was reflected off the animal's retina, producing an image of a bright pupil surrounded by a dark iris (Miller et al., 2002, 2003a, 2003b). The right eye of all rats in the study was digitally photographed on 3 different days before exposure to establish an average baseline pupil size. Pictures were also taken 60 min, 2 h, 24 h, 48 h, and 7 days after exposure. All photographs were taken under low-light conditions (< 10 foot-candles). All rats were temporarily restrained while photographed. Restraint lasted approximately 30 sec per rat and involved immobilizing the head of the rat in a yoke.

5.2.9 Experimental Design

Groups of rats were exposed for either 10, 60, or 240 min. Five concentrations of VX were tested at each exposure duration. For each exposure, 10 rats were placed in a compartmentalized stainless steel cage (20 × 14 × 4in.) with each rat occupying a separate compartment (4 × 7 × 4in.). The chamber could accommodate two of these steel cages, allowing for a maximum of 20 rats to be exposed at once. The cages were placed on the floor of the exposure chamber before the introduction of VX. Each exposure consisted of 10 male and/or 10 female rats exposed to VX vapor. An additional 5 male and/or 5 female control rats were placed in a separate control chamber and exposed to air only. The t_{99} (time to attain 99% of the equilibrium concentration within the chamber) ranged from 7.7 to 8.2 min. Physical parameters monitored during exposure included chamber airflow, nitrogen flow rate through the saturator cell, chamber room temperature, and relative humidity. Following the exposure,

the chamber was purged with air for 10 min before removing the rats. After removal from the chamber, the rats were observed for any overt toxic signs of exposure such as tremors, salivation, or lacrimation. Clinical signs of exposure were monitored twice daily for up to 7 days after exposure. After 7 days post-exposure, surviving rats were euthanized in accordance with the Report of the American Veterinary Medical Association (AVMA) Panel on Euthanasia (1993).

5.2.10 Calculation of Pupil Diameter

To determine the effect of the exposure on pupil size, changes in pupil diameter were assessed as follows. The ratio of pupil radius to iris radius was calculated for all rats ($\prod_{ind}$) and used as an indicator of pupil size. For each rat, the postexposure $\prod_{ind}$ was divided by the preexposure $\prod_{ind}$. For VX-exposed rats, this post- versus pre-$\prod_{ind}$ ratio was divided by the average post- versus pre-$\prod_{ind}$ ratio of the same-sex control animals. The result of this calculation was the pupil diameter ratio ($\prod_{ratio}$) of post- versus preexposure values for an individual rat adjusted for controls.

5.2.11 Calculation of Blood ChE Values

For each rat, the postexposure ChE (either AChE or BChE) activity level was divided by the pre-exposure ChE activity level. For VX-exposed rats, this value was then divided by the mean value for the same-sex control rats. The result of this calculation was the whole-blood ChE ratio of post- versus preexposure values for an individual rat adjusted for controls. The whole-blood AChE ratio is denoted by α_{ratio}, and the whole-blood BChE ratio is denoted by β_{ratio}.

5.2.12 Data Analysis

Minitab®, Version 13 (Minitab, Inc., State College, PA) was used for all statistical analyses.

Analysis of variance (ANOVA) (Fox, 1997) was used to determine statistically significant differences between control and exposed rats. A p-value of <0.05 was the criterion for statistical significance.

For statistically significant pupil constriction and blood ChE depression, a probit analysis (Finney, 1971) was conducted to calculate ECT_{50}s. The following equation was used to fit the experimental data:

$$Y_N = (Y_P - 5) = k_C \left(\log_{10} C\right) + \sum_{i}^{3} \sum_{j}^{2} k_{i,j} \left(\text{Time}\right)_i \left(\text{Gender}\right)_j \tag{5.1}$$

Where Y_N is a normit, Y_P is a probit, the k_S are fitted coefficients, C is vapor concentration, Time is the exposure durations (treated as a three-level factor), and Gender is a two-level factor. The fitted coefficient, k_C, is the estimate for the probit slope for concentration. Y_N equals −1, 0 and 1 at the 16, 50, and 84% response levels, respectively. The binary response modeled in Equation (5.1) was the presence of either miosis or blood ChE depression in an exposed rat. Miosis was defined as at least 50% pupil diameter constriction ($\prod_{ratio} \leq 0.50$), whereas ChE depression was defined as a 50% decrease in baseline ChE levels (α_{ratio} (AChE) or β_{ratio} (BChE) ≤ 0.50), respectively.

Binary and ordinal logistic regressions (with a normit link function) (Finney, 1971; Agresti, 1990; Fox, 1997) were used to fit the toxic load model for probability of effect to the blood ChE depression and pupil diameter data sets, respectively. The model used was:

$$Y_N = k_0 + k_C \left(\log_{10} C\right) + k_T \left(\log_{10} T\right) + k_S \,\text{Sex} \tag{5.2}$$

Where Sex was coded −1 for female rats and 1 for male rats, k_S is the fitted coefficient for the factor Sex, the constants k_C and k_T are the probit slopes for concentration and time, respectively. The exposure duration, T, is treated as a covariate in Equation (5.2) (in contrast to duration as a factor in Equation [5.1]). For ordinal regression, k_0 is replaced by $k_1, k_2, \ldots, k_N$, which act as the intercepts for

the N levels of response used in the analysis. The significance of the interaction between $\log_{10} T$ and Sex was investigated for both pupil constriction and ChE depression, and in both cases, this interaction was not statistically significant.

The ratio (k_C / k_T) equals the toxic load exponent, n. If this ratio is not different (with statistical significance) from one, then Haber's Rule (Haber, 1924) is appropriate for modeling the toxicity. Otherwise, the classic toxic load model ($C^n \times T = k$) is the proper approach (ten Berge et al., 1986; Sommerville et al., 2004) assuming no significant curvature exists in the experimental data used to fit the model.

5.3 RESULTS

This study focused on estimating ECT_{50}s for miosis in rats exposed to low levels of VX vapor for 10, 60, or 240 min. By exposing groups of rats to five different concentrations of VX vapor per exposure duration, we were able to establish ECT_{50}s for miosis and ChE depression for both male and female rats at each of the exposure durations. Subsequently, these data were used to formulate multifactor models to predict dose–response relationships and the probability of incurring VX vapor-induced miosis and ChE depression as a function of exposure concentration and duration.

5.3.1 Pupil Response as a Function of Observation Period

Of the five postexposure observation periods (1 h, 2 h, 24 h, 48 h, and 7 days), the lowest $\prod_{ratio}$ values (greatest miotic response) occurred 1 h postexposure (Figure 5.3). ANOVAs were done for each observation period. For 1-h, 2-h, and 24-h postexposure periods, statistically significant differences occurred between the ratio values ($\prod_{ratio}$) of exposed and control rats. However, for the 48-h and 7-day postexposure periods, $\prod_{ratio}$ values for exposed and control rats were not significantly different. Thus, for the range of vapor concentrations and dosages investigated, complete recovery from a miotic response to VX vapor exposure in male and female rats occurred between 24 h and 48 h postexposure.

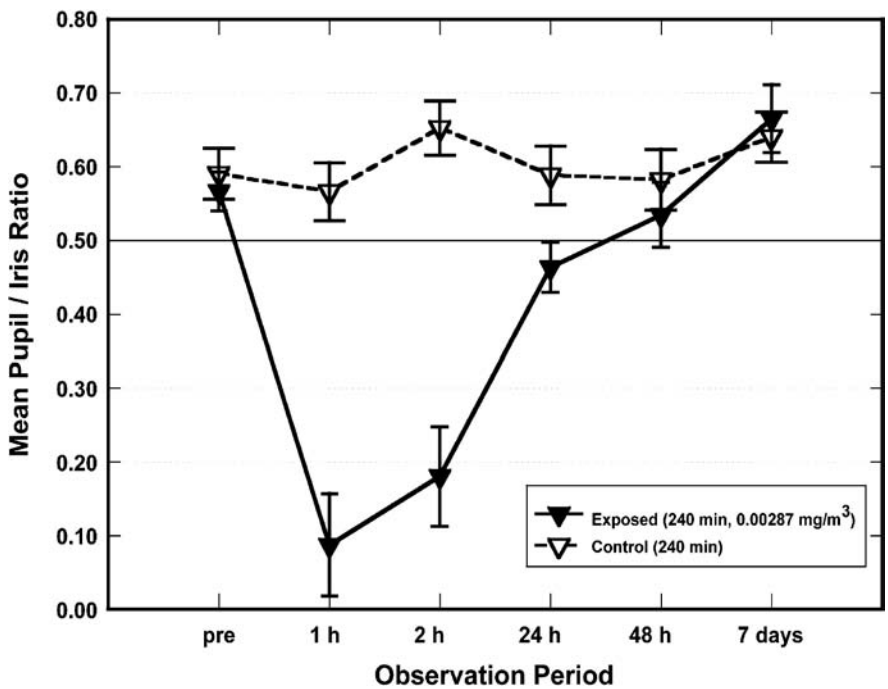


FIGURE 5.3 Representative graph showing mean pupil-to-iris ratio values for male rats exposed to VX vapor for 240 min.

5.3.2 Probit Analysis of Miotic Response

The $\prod_{ratio}$ values for each rat (1 h postexposure) were converted into binary data, with ($\prod_{ratio} \leq 0.50$) being the criterion for miosis. A probit analysis was then performed (with Equation [5.1]) on the binary data, and median effective concentrations (EC_{50}) and dosages (ECT_{50}) for miosis were calculated for each gender-exposure duration combination (Table 5.1). For comparison, the values for GB (Mioduszewski et al., 2002a) and GF (Whalley et al., 2004) are also included in Table 5.1 and Figure 5.4.

The probit slope for concentration (k_C), was found to equal 5.2, with a 95% confidence interval of 3.9 to 6.5. The ECT_{50}s of the male and female rats were statistically significant from each other at all exposure durations, with the female rats being more sensitive.

5.3.3 Analysis of Time Dependence of ECT_{50} (Miosis)

The effect of exposure duration on the miotic response was investigated via ordinal logistic regression by using Equation (5.2). Ternary miosis data (1-h postexposure) was generated for the analysis by using the following categories:

Score of 0: $\prod_{ratio} \leq 0.50$	number of exposed rats in group: 90
Score of 1: $0.50 < \prod_{ratio} \leq 0.84$	number of exposed rats in group: 135
Score of 2: $0.84 < \prod_{ratio}$	number of exposed rats in group: 75

A ternary scoring system was found to give the best regression fit to the quantal data. The following normit fits (see Equation [5.2]) were obtained for the boundaries between scores 0 and 1 (Equation [5.3]) and scores 1 to 2 (Equation [5.4]), respectively.

$$Y_N\{0\} = (5.1107) + (3.5946)(\log_{10} C) + (2.1772)(\log_{10} T) + (-0.2984)\,\text{Sex} \tag{5.3}$$

$$Y_N\{1\} = (6.8141) + (3.5946)(\log_{10} C) + (2.1772)(\log_{10} T) + (-0.2984)\,\text{Sex} \tag{5.4}$$

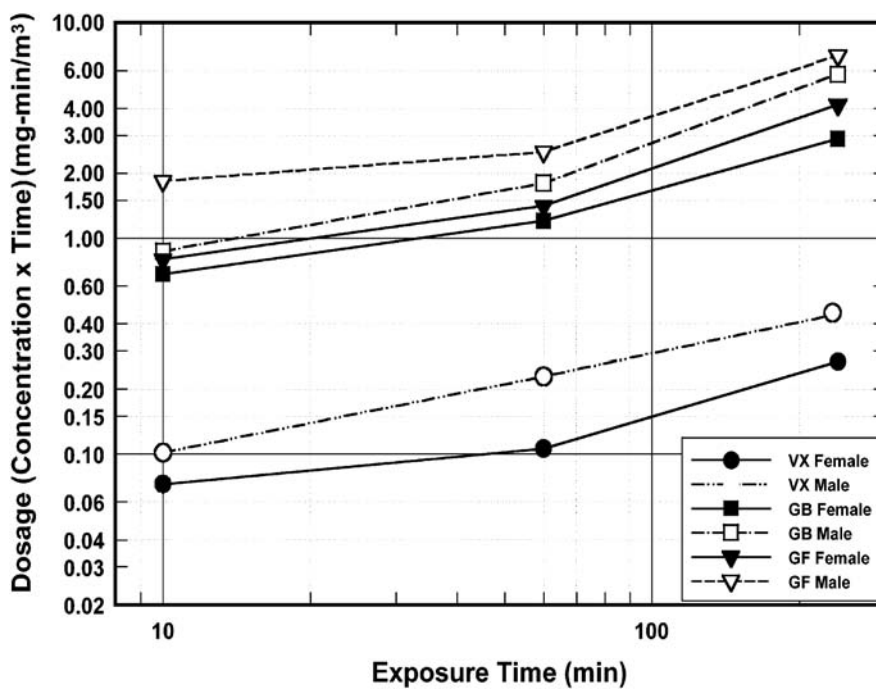


FIGURE 5.4 Comparison of ECT_{50} estimates for miosis in rats for VX, GB (Mioduszewski et al., 2002a) and GF (Whalley et al., 2004) as a function of exposure duration.

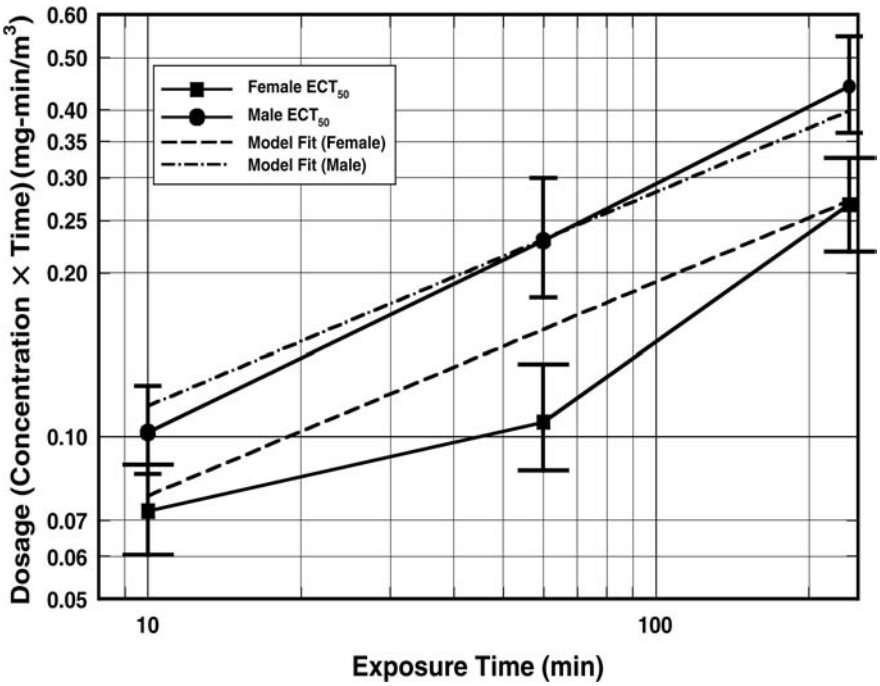


FIGURE 5.5 Comparison of toxic load model fit (Equation [5.3]) with VX ECT_{50} estimates for miosis for male and female rats (from Equation [5.1]) as a function of exposure duration.

A plot of Equation (5.3) is compared with the ECT_{50}s plotted in Figure 5.5. The toxic load exponent value (identical for both Equations [5.3] and [5.4]) equals (3.5946/2.1772) or 1.65 ± 0.092 SE (Table 5.2). The 95% confidence interval for the exponent value is 1.47 to 1.83. Because this interval does not overlap 1, the toxic load exponent (n) for miosis is different from 1 (with at least 95% statistical significance). Therefore, a toxic load model better describes the time dependence of the probability of miosis than does Haber's Rule. Also, gender (Sex) was found to be statistically significant because the female rats were more sensitive by a factor of 1.46.

Potential lack of fit for the toxic load model was tested by adding the term $(\log_{10} T)^2$ to equation (5.2) to test for curvature. It was found that this term was statistically significant. Thus, there is significant curvature, and the toxic load model does not adequately explain the time dependence of the miotic response relationship. However, the toxic load model is still a better alternative for explaining the data than Haber's Rule.

5.3.4 Analysis of Variance for Blood ChE Response

ANOVAs were performed separately on both the AChE and BChE 1-h postexposure data. In the BChE data, no statistically significant difference was found between the ratio values (β_{ratio}) of the exposed and control rats at any of the exposure durations and dosages. For the AChE data, there were statistically significant differences between the ratio values (α_{ratio}) of exposed and control rats at each of the three exposure durations (10, 60, and 240 min). For 10- and 240-min exposures, significant depression occurred at the two highest vapor concentrations of each duration, whereas for the 60-min exposures significant depression occurred at only the highest vapor concentration (Table 5.3).

5.3.5 Probit Analysis of AChE Response

The α_{ratio} values (1-h postexposure) were converted into binary data, with ($\alpha_{ratio} \leq 0.50$) being the criteria for AChE depression. A probit analysis was performed on the binary data (using Equation [5.1]),

TABLE 5.2 Probit Slopes and Toxic Load Exponents (*n*) Determined from Toxic Load Modeling

End Point	Terms in Model	κ_c	SE (*C*)	κ_t	SE (*T*)	*n*	SE (*n*)
Miosis	Log C (Time)(Sex)	5.24	0.63	—	—	—	—
Miosis	Log C Log *T* Sex	3.59	0.32	2.18	0.23	1.65	0.09
AChE depression	Log C (Time)(Sex)	3.72	0.71	—	—	—	—
AChE depression	Log C Log *T* Sex	3.24	0.58	2.06	0.35	1.57	0.14

Values obtained from various ordinal (miosis) and binary (AChE) logistic regression model fits.

and median effective concentrations (EC_{50}) and dosages (ECT_{50}) for AChE depression were calculated for each exposure duration (Table 5.4).

The probit slope for concentration (k_C) equaled 3.7, with a 95% confidence interval of 2.3 to 5.1. The ECT_{50}s of the male and female rats were not statistically different from each other at each of the three exposure durations (10, 60, and 240 min).

5.3.6 Analysis of Time Dependence of ECT_{50} (AChE Depression)

The effect of exposure duration on the AChE depression response in rats exposed to VX vapor was investigated via binary logistic regression with Equation (5.2). The following normit fit was obtained:

$$Y_N = (3.5240) + (3.2388)(\log_{10} C) + (2.0605)(\log_{10} T) + (-0.2140)\,\text{Sex} \tag{5.5}$$

Equation (5.5) has been plotted in Figure 5.6 along with the ECT_{50}s for AChE depression. The toxic load exponent value equals (3.2388 / 2.0605) or 1.57, with a standard error of 0.14 (Table 5.2). Thus, the 95% confidence interval for the exponent value is the range from 1.29 to 1.85. Because this interval does not overlap 1, the toxic load exponent for AChE depression is significantly different from 1. Therefore, the toxic load model better describes the time dependence of the probability of AChE depression than Haber's Rule.

Potential lack of fit for the toxic load model was tested by adding the term $(\log_{10} T)^2$ to Equation (5.2) to test for curvature. It was found that this term and its interaction with Sex were not statistically significant. Thus, there is no significant curvature and the toxic load model adequately explains the time dependence of the AChE response relationship.

5.3.7 Gender Differences in AChE Depression

The *p* value for Sex in Equation (5.5) equals 0.066, or in other words, the null hypothesis (i.e., no difference in AChE depression exists between the genders) can be rejected with 93.4% confidence. If exposure duration is treated as a factor instead of a covariate in Equation (5.5), the *p*-value for Sex drops to 0.049 (or 95.1% confidence for rejecting the null hypothesis). From the probit analysis results, the *p*-value was not close to being less than 0.05. So, the statistical significance of the Sex term, in general, is marginal, but it is enough to support the reporting of separate ECT_{50}s for the two genders.

5.3.8 Comparison of Miotic and AChE Responses

The toxic load exponents 1.65 (miosis) and 1.57 (AChE depression) from Equations (5.3) and (5.5), respectively, are not significantly different from each other. In addition, the ECT_{50}s for miosis in both male and female rats are lower in value than those for AChE depression (Tables 5.1 and 5.4). To illustrate miosis as the first noticeable effect (FNE) of VX exposure, each of the 300 exposed rats were

TABLE 5.3 Concentrations and Dosages for VX Vapor-Exposed Rats at Each Exposure Duration

Time (min)	Conc. (mg/m^3)	Dose (mg-min/m^3)	Sex	Time (min)	Conc. (mg/m^3)	Dose (mg-min/m^3)	Sex	Time (min)	Conc. (mg/m^3)	Dose (mg-min/m^3)	Sex
10	0.00318	0.0318	f	60	0.00045	0.0270	f	240	0.00037	0.0888	f
10	0.00410	0.0410	f	60	0.00058	0.0348	m	240	0.00067	0.161	mf
10	0.00560	0.0560	mf	60	0.00080	0.0480	mf	240	0.00105	0.252	mf
10	0.00710	0.0710	m	60	0.00108	0.0648	f	240	0.00137	0.329	m
10	0.00830	0.0830	m	60	0.00137	0.0822	f	240	0.00183	0.439	mf
10	0.00832	0.0832	mf	60	0.00160	0.0960	mf	240	0.00201	0.482	f
10	0.00870	0.0870	f	60	0.00270	0.162	m	240	0.00287	0.689	m
10	0.01600	0.1600	m	60	0.00364	0.218	m	—	—	—	—

TABLE 5.4 Blood AChE Depression EC_{50} and ECT_{50} Values for Rats Exposed to VX Vapor

Gender	Time (min)	EC_{50} (mg/m^3)	95% Fiducial Limits Lower	95% Fiducial Limits Upper	ECT_{50} (mg-min/m^3)	95% Fiducial Limits Lower	95% Fiducial Limits Upper
Male	10	0.0200	0.0143	0.0346	0.200	0.143	0.346
	60	0.00634	0.00413	0.0124	0.380	0.248	0.744
	240	0.00270	0.00204	0.00414	0.648	0.490	0.994
Female	10	0.0153	0.0106	0.0285	0.153	0.106	0.285
	60	0.00340	0.00216	0.00737	0.204	0.130	0.442
	240	0.00217	0.00163	0.00324	0.521	0.391	0.778

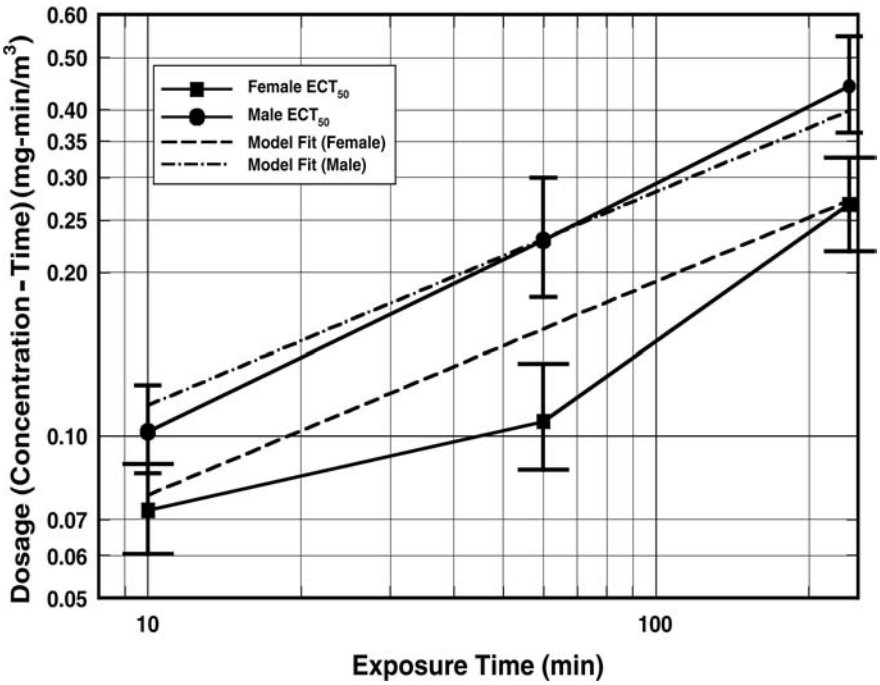


FIGURE 5.6 Comparison of toxic load model fit (Equation [5.5]) with VX ECT_{50} estimates for AChE depression for male and female rats (from Equation [5.1]) as a function of exposure duration.

placed into one of four categories based on their paired binary responses for miosis ($\prod_{ratio} \leq 0.50$) and AChE depression ($\alpha_{ratio} \leq 0.50$):

1. Rat shows neither miosis nor AChE depression (203 rats)
2. Rat shows miosis but not AChE depression (62 rats)
3. Rat does not show miosis but does show AChE depression (7 rats)
4. Rat shows both miosis and AChE depression (28 rats)

Of particular interest are categories 2 and 3 containing 69 rats total—rats only showing one of the two possible signs. Sixty-two rats showed miosis without AChE depression and 7 rats had AChE depression without miosis. Therefore, miosis occurred without AChE depression 90% of the time in those rats showing only one sign or the other.

5.4 DISCUSSION

During the past several years much of the work in our laboratory has focused on establishing ECT_{50}s for miosis as a function of exposure duration for GB (Mioduszewski et al., 2002a; Hulet et al., in preparation) and GF (Whalley et al., 2004) vapor. The results of the current study on low-level VX vapor exposures add to this database and directly establish ECT_{50}s for VX-induced miosis in rats (Table 5.1). These newly established ECT_{50}s for VX will reduce the need for relative potency analysis using other nerve agents such as GB to establish toxicity levels for VX inhalation exposures (Hartmann, 2002). Based on the results of the present study, VX potency for male and female rats at all exposure durations, ranges between 7.9 and 13.0 times greater than GB. Similarly, VX is 10.9 to 18.1 times more potent than GF (Table 5.1 and Figure 5.4). However, note that the method used for the determination of pupil diameter in the GF and present study differed from that used in the GB study by Mioduszewski et al. (2002a).

In addition to potency comparisons based on similar exposure duration, comparison of the toxic load exponents offers insight into whether relative potency between two agents is constant with respect to duration. If there is no significant difference between a pair of exponent values, then the relative potency is constant. Among the rat miosis studies, the exponent values (n_{all}) of GB and VX are significantly different from each other with the n for GB larger than the n for VX. Therefore, VX is increasingly more potent than GB as exposure duration increases. The exponent value for GF lies in between those of GB and VX and its value is not significantly different from either GB or VX.

The need to develop toxic load exponents in the equation $C^n \times T = k$ arose because Haber's Rule failed to adequately describe the relationship between concentration and time, and the net effect this interaction had on cumulative toxicity levels. It should be recognized that the toxic load relationship is based more on empirical observations than on basic biological theories (Griffiths, 1991; Fairhurst and Turner, 1993; Sommerville et al., 2004). It is not surprising that an empirical model would provide a poor fit when extended over too wide a range. In miosis studies performed to date (Mioduszewski et al., 2002a; Whalley et al., 2004; the present study), significant upward curvature was found in the relationship between effective median dosages (ECT_{50}s) and exposure duration (Figure 5.4). Thus, the toxic load model does not adequately explain the time dependence of the miotic response relationship. Several possible explanations exist for the curvature in the ECT_{50} versus exposure duration relationships found in the GB, GF, and VX rat miosis studies. First, the curvature is the natural relationship for the species-agent-end point systems under consideration. The next two categories presuppose that the toxic load model is the proper model and that any observed curvature must be due to factors whose effect on miosis levels is not constant with respect to exposure duration. For instance, certain behavioral and physiological factors (i.e., changes in activity, sleep, closed eyelids, etc.) are likely to have more pronounced effects on the observed levels of miosis in longer duration exposures. Also, the potential existed for recovery from miosis during the time after the conclusion of the exposures until the rats' eyes were photographed for the presence of miosis. This potential for recovery was greatest following the 240-min exposures because of the greater influence of concentration in determining miosis when the toxic load exponent is greater than 1 in the equation $C^n \times T = k$. Thus, greater concentrations of VX at shorter times have a more pronounced effect on pupil size than lesser concentrations over longer exposure durations. The net effect is that more rats were borderline miotic at the longer exposure durations, allowing for a faster recovery from VX-induced miosis before assessment of pupil diameter (unpublished observations).

These last two possibilities have important implications for risk assessment applications of the determined ECT_{50}s. Figure 5.7 illustrates this point with plots of two ECT_{50} extrapolations based on the female rat VX miosis data. The extrapolation based on the 10- and 60-min ECT_{50} values produces lower (more conservative) ECT_{50} estimates at longer durations than a similar extrapolation that is based on the 60- and 240-min ECT_{50} values. From a risk assessment perspective, the former has an implicit built-in safety factor. Expanding this approach, a second set of toxic load exponents (n_{short}) was calculated for GB, GF, and VX miosis based on a reanalysis of the original data with exposure durations greater than 60 min excluded. Thus, with the uncertainty about why the curvature exists, more weight should be given to the more conservative n_{short} values rather than the n_{all} values when

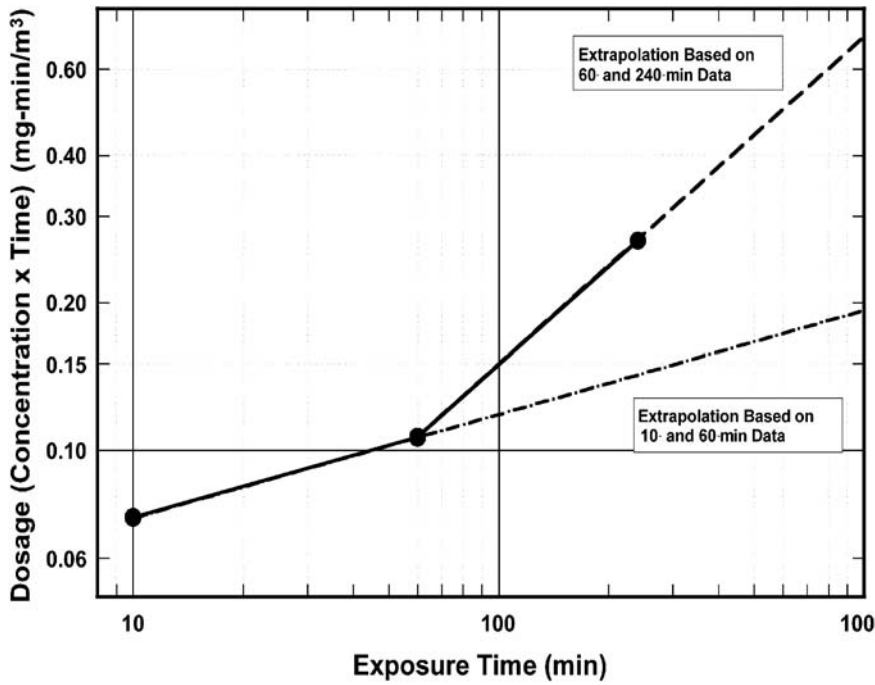


FIGURE 5.7 Comparison of different extrapolations of the median effective dosage—exposure duration relationship using the female rat VX miosis ECT_{50} data.

TABLE 5.5 Miosis Toxic Load Exponents (*n*) for GB, GF, and VX

		Toxic Load Exponents (*n*)				
Agent	**Species**	**Whole Dataset**		$T \leq 60$ **min**	$(n_{all} - n_{short})$	**Source**
		n_{all}	**95% CI**	n_{short}		
GB	Rat	2.33	1.85 to 2.81	1.62	0.71	Mioduszewski et al. (2002a)
GF	Rat	1.98	1.70 to 2.26	1.30	0.68	Whalley et al. (2004)
VX	Rat	1.65	1.47 to 1.83	1.35	0.30	Present study

attempting to establish a human toxic load exponent for miosis. Moreover, estimates for a lethality toxic load exponent value should not be based on the results of experimental miosis studies, because it can be expected that differing degrees of curvature would come with different end points.

The need to establish separate ECT_{50}s for males and females at each exposure duration is because female rats are significantly more sensitive to VX vapor. The differential sensitivity to this OP was consistent with similar findings from recent GB (Mioduszewski et al., 2002a, 2002b) and GF (Whalley et al., 2004) rat studies. In addition, numerous studies show that the actions of a variety of other drugs such as amobarbital, nicotine (Holck et al., 1937), strychnine, and picrotoxin (Kato et al., 1962), to name a few, are more pronounced and/or persist longer in female rats than in male rats (Kato, 1974). In many instances, gender differences in drug sensitivity appear to be mediated at least in part by androgens present in male rats that can increase two to three times the activities of drug-metabolizing enzymes in liver microsomes (Booth and Gillette, 1962). The present study was tracking low-level VX vapor-induced miosis, considered the result of a localized depression of AChE. This localized effect might preclude involvement of liver microsome-mediated changes in VX toxicity, but other gender differences related to the eye also may indirectly alter the local response to CW agents. There is evidence of structural dimorphism of the lacrimal gland (Sullivan et al., 1990) and gender differences in the quantities and activity levels of various enzymes associated with the

lens of the rat eye (Bours et al., 1988). These gender differences in rats are well documented and emphasize the point that males and females cannot be assumed to have the same thresholds for response. Whatever the reasons for the increased sensitivity of female rats to VX vapor, the larger issue is whether these gender differences in the rat are relevant to humans.

The finding that exposure to the highest concentrations of VX vapor at the three exposure durations produced significant whole-blood AChE depression differed from previous results obtained with miosis-producing levels of GB (Mioduszewski et al., 2002a) and GF (Whalley et al., 2004) in rats. In those studies, there was no significant depression of AChE, carboxylesterase (CaE), or BChE after exposure to GB or GF. The difference in AChE depression after exposure to miosis producing levels of VX vapor may be due to its higher binding affinity for AChE. In a study looking at the effectiveness of CaE protection against the toxicity of OP compounds such as VX, soman, sarin and tabun, Maxwell (1992) found that VX was the most specific in vitro inhibitor of AChE but showed very little affinity for CaE. In the present study, gender differences in the degree of AChE depression were minimal but may have been masked by the large variability in baseline AChE activity levels within and between individual rats. Also, the fact that significant depression only occurred at the highest dosages of VX used in this study limited the number of groups available for statistical comparison.

Because this study used whole-body inhalation exposures, a potential confounding effect of a low-volatility, lipophilic compound such as VX was the possibility of delayed toxicity effects due to percutaneous absorption. To account for agent deposition on the surface of the animal, Jakubowski et al., (in preparation) performed a study in which two groups of rats (eight to nine rats per group) were exposed to whole-body VX vapor concentrations of 0.08–0.09 mg/m^3 for 240 min. These conditions represented dosages far exceeding what was necessary to produce miosis (Table 5.3). After exposure, surviving rats were euthanized and VX was extracted from the surface of the rat by using whole-body immersion into isopropyl alcohol. The average amount of VX recovered per rat from the combined results of groups 1 and 2 for whole-body extraction was 13.8 + 3.2 μg as determined by GC-mass spectrometry. This amount of surface deposition of VX represents less than one tenth of 1% of the entire exposure dose, and this exposure dose represented a "worst case" scenario. The highest exposure dose actually used in this study was 0.00287 mg/m^3 for 240 min (0.689 mg-min/m^3). From postexposure observations of all exposed rats in this study, we have concluded that there were no observable delayed toxicity effects such as tremors, salivation, or convulsions. Figure 5.3 illustrates our findings that any delayed miotic effects were minimal. In addition, statistical analysis of all five postexposure observation periods revealed that the greatest miotic response occurred at the 1-h postexposure observation period. Also, for the range of vapor concentrations and dosages investigated, complete recovery from a miotic response to VX vapor exposure in male and female rats occurred between 1 and 2 days postexposure.

Previous studies in our laboratory with GB (Mioduszewski et al., 2002a) and GF (Whalley, et al., 2004) have shown that miosis is the FNE resulting from a whole-body inhalation exposure. In these studies, there was no significant ChE depression in either the plasma or RBC components of the blood. In contrast, the current study with VX has identified significant whole-blood AChE depression at the higher dosage levels of each exposure duration. To determine whether AChE depression could be expected to occur before miosis, probit analysis was used to determine ECT_{50}s for AChE depression of whole blood. Tables 5.1 and 5.4 list the ECT_{50}s for both miosis and AChE depression, respectively. ECT_{50} values for miosis are approximately 1.5 to 2 times lower than those for AChE depression. Also, of the 300 VX-exposed rats, 21% of all the exposed rats had miosis without AChE depression whereas 2% of exposed rats had AChE depression without miosis. Miosis, therefore, would be expected to occur first at these low dosages of VX vapor.

5.5 CONCLUSIONS

This study filled some of the gaps in our understanding of the threshold effects of low-level VX vapor exposure. In contrast to that predicted by using Haber's Rule, the ECT_{50}s associated with miosis and AChE inhibition were not constant over time. Toxic load exponents ($C^{1.65} \times T = k$ for

miosis and $C^{1.57} \times T = k$ for AChE inhibition) describing the threshold effects of VX vapor dosage over time were developed. Although female rats were more sensitive to the miotic effects of VX vapor than males in this study, the applicability of gender differences in the sensitivity to OPs in human populations is unresolved. Insofar as effects such as miosis may have an impact on both military and worker operational effectiveness, the results of the current study are critical to operational risk management. Data derived from this study are also essential for determining appropriate threshold levels for detection and determination of when it is safe to come out of protective posture for any operations involving VX. It must be emphasized that the results of this study pertain only to miosis levels of VX exposure. Estimates for a lethality toxic load exponent value should not be based on the results of experimental miosis studies, because it is possible that, with lethal concentrations of VX vapor, percutaneous effects may have a greater influence on the dose–response relationship.

REFERENCES

Agresti, A., *Categorical Data Analysis,* John Wiley & Sons, New York, 1990.

American Veterinary Medical Association, Report of the AVMA Panel on Euthanasia, *JAVMA*, 202, ... 1993.

Bide, R.W. and Risk, D.J., Inhalation Toxicity of Aerosolized Nerve Agents. 1. VX Revisited. Defence Research Establishment Suffield, Technical Report *DRES TR 2000–063* (Unclassified Report), Sept. 2000.

Bide, R.W., Risk, D.J., and Beifus, D.G., A Functional Small Volume Inhalation System for Highly Toxic Materials, in *Proceedings of the 1996 ERDEC Scientific Conference on Chemical and Biological Defense Research*, 19–22 November 1996, AD-A334 105, pp. 25–32.

Booth, J. and Gillette, J.R., The effect of anabolic steroids on drug metabolism by microsomal enzymes in rat liver, *J. Pharmacol. Exp. Ther*., 137, 374–379, 1962.

Bours, J., Fink, H., and Hockwin, O., The quantification of eight enzymes from the ageing rat lens, with respect to sex differences and special reference to aldolase, *Curr. Eye Res.,* 7, 449–455, 1988.

Brickhouse, M.D., Rees, M.S., O'Connor, R.J., and Durst, H.D., Nuclear Magnetic Resonance (NMR) Analysis of G-class and VX Nerve Agents and Reaction Masses Produced by Their Chemical Neutralization, *ERDEC-TR-449,* U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, UNCLASSIFIED Report (AD-A339 308), 1997.

Craig, F.N., Cummings, E.G., and Sim, V.M., Environmental temperature and the percutaneous absorption of a cholinesterase inhibitor, VX, *J. Invest. Dermatol.,* 68, 357–361, 1977.

Dimmick, R.L., Jr., Owens, E.J., and Farrand, R.L., The Acute Inhalation Toxicity of QL in the Rat and Guinea Pig. *ARCSL-TR-79031* (Unclassified), 1979.

Ellman, G.L., Courtney, K.D., Andres, V., Jr., and Featherstone, R.M., A new rapid colormetric determination of acetylcholinesterase activity, *Biochem. Pharm.,* 7, 88–95, 1961.

Fairhurst, S. and Turner, R.M., Toxicological assessments in relation to major hazards, *Haz. Mater. 33,* 215–227, 1993.

Finney, D.J., *Probit Analysis,* 3rd ed., Cambridge University Press, London, 1971.

Fox, J., *Applied Regression Analysis, Linear Models, and Related Methods,* Sage, Thousand Oaks, CA, 1997.

Griffiths, R.F., The use of probit expressions in the assessment of acute population impact of toxic releases, *J. Loss Prev. Process Ind.*, 4 49–57, 1991.

Gupta, R.C., Patterson, G.T., and Dettbarn, W.D., Comparison of cholinergic and neuromuscular toxicity following acute exposure to sarin and VX in rat, *Fundam. App Toxico.,* 16, 449–458, 1991.

Haber, F.R., Zur geschichte des gaskrieges, in *Funf Vortrage aus Jahren 1920–1923*, Springer, Berlin, 1924.

Hartmann, H.M., Evaluation of risk assessment guideline levels for the chemical warfare agents mustard, GB, and VX, *Regul. Toxicol.Pharmacol.,* 35, 347–356, 2002.

Holck, H.G.O., Kanan, M.A., Mills, L.M., and Smith, E.L., Studies upon the sex-difference in rats in tolerance to certain barbiturates and to nicotine, *J. Pharmacol. Exp. Ther.,* 60, 323, 1937.

Hulet, S.W., Sommerville, D.R., Benton, B.J., Forster, J.S., Manthei, J.H., Miller, D.B., Scotto, J.A., Jarvis, J.R., Way, R.A., Muse, W.T., Gaviola, B., Burnett, D., Crosier, R.B., Mioduszewski, R.J., and Thomson, S.A., Low-level sarin vapor exposure in the Gottingen minipig: effect of exposure concentration and duration on pupil size (In preparation).

Jakubowski, E.M., Benton, B.J., Whalley, C.E., Anthony, J.S., Haley, M.V., Manthei, J.H., Way, R.A., Burnett, D.C., Gaviola, B.P., Scotto, J.A., Sommerville, D.R., Crosier, R.B., Edwards, J.L., Evans, R.A., McGuire, J.M.,

Crouse, C.L., Matson, K.L., Mioduszewski, R.J., and Thomson, S.A., The inhalation toxicity testing of VX vapor in rats at miosis levels: VX surface contamination analysis and fluoride ion generated product determination (in preparation).

Kato, R., Sex-related differences in drug metabolism, *Drug Metab. Rev.,* 3, 1–32, 1974.

Kato, R., Chiesara, E., and Vassanelli, P., Metabolic differences of strychnine in the rat in relation to sex, *Jpn. J. Pharmacol.,* 12, 26–33, 1962a.

Kato, R., Chiesara, E., and Vassanelli, P., Increased activity of microsomal strychnine- metabolizing enzyme induced by phenobarbital and other drugs, *Biochem. Pharmacol.,* 11, 913–922, 1962b.

Manthei, J.H., Beckett, E.L., Heitkamp, D.H., James, J.T., Cameron, K.P., Bona, D.M., Moore, R.D., and Vickers, E.L., Toxic Hazard Evaluation of Decontaminated/Neutralized Chemical Surety Materials Waste at the U.S. Army Chemical Research, Development and Engineering Center, *CRDEC-TR-146* (Unclassified), Nov. 1990.

Maxwell, D.M., The specificity of carboxylesterase protection against the toxicity of organophosphorus compounds, *Toxicol. Appl. Pharmacol.*, 114, 306–312, 1992.

Miller, D.B., Benton, B.J., Hulet, S.W., Mioduszewski, R.J., Whalley, C.E., Carpin, J.C., and Thomson, S.A., An infrared image acquisition and analysis method for quantifying optical responses to chemical agent vapor exposure, in *2002 Joint Services Scientific Conference on Chemical & Biological Defense,* 19–21 November 2002, Hunt Valley, MD, 2002.

Miller, D.B., Benton, B.J., Hulet, S.W., Mioduszewski, R.J., Whalley, C.E., Carpin, J.C., and Thomson, S.A., An automated infrared image acquisition and analysis method for quantifying optical responses to chemical agent vapor exposure, in *29th Annual IEEE Northeast Bioengineering Conference,* 22–23 March 2003, New Jersey Institute of Technology, Newark, NJ, 2003a.

Miller, D.B., Benton, B.J., Hulet, S.W., Mioduszewski, R.J., Whalley, C.E., Carpin, J.C., and Thomson, S.A., An image analysis method for quantifying elliptical and partially obstructed pupil areas in response to chemical agent vapor exposure, in *29th Annual IEEE Northeast Bioengineering Conference,* 22–23 March 2003, New Jersey Institute of Technology, Newark, NJ, 2003b.

Mioduszewski, R.J., Manthei, J.H., Way, R.A., Burnett, D.C., Gaviola, B.I., Muse, W.T., Jr., Matson, K.L., Sommerville, D.R., Crosier, R.B., Scotto, J.A., McCaskey, D.A., Crouse, C.L., and Thomson, S.A., Low-Level Sarin Vapor Exposure in Rats: Effect of Exposure Concentration and Duration on Pupil Size, *ECBC-TR-235,* U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD (Unclassified Report), May 2002a.

Mioduszewski, R.J., Manthei, J.H., Way, R.A., Burnett, D.C., Gaviola, B.P., Muse, W.T., Thomson, S.A., Sommerville, D.R., and Crosier, R.B., Interaction of exposure concentration and duration in determining acute toxic effects of sarin vapor in rats, *Toxicol. Sci.,* 66, 176–184, 2002b.

Muse, W.T., Jr., Anthony, J.S., Thomson, S.A., Crouse, C.L., and Crouse, L.C.B., Acute Inhalation Toxicity of Chemically Neutralized/Hydrolyzed VX in Rats, SCWO Effluent Prior to Evaporation SCWO Effluent Post-Evaporation, *ECBC-TR-219* (Unclassified Report), Apr. 2002.

Oshiro, I., Takenaka, T.,and Maeda, J., New method for hemoglobin determination by using sodium lauryl sulfate (SLS), *Clin. Biochem,* 15, 83–88, 1982.

Rickett, D.L., Glenn, J.F., and Beers, E.T., Central respiratory effects VX. Neuromuscular actions of nerve agents, *Neurotoxicology,* 7, 225–236, 1986.

Sommerville, D.R., Park, K.H., Kierzewski, M.O., Dunkel, M.D., Hutton, M.I., and Pinto, N.A., Toxic Lad modeling, in *Inhalation Toxicology,* H. Salem, Ed., Marcel Dekker, New York, (in press).

Sullivan, D.A., Hann, L.E., Yee, L., and Allansmith, M.R., Age- and gender-related influence on the lacrimal gland and tears, *Acta Ophthalmol (Copenh)*, 68, 188–194, 1990.

ten Berge, W.F., Zwart, A., and Appelman, L.M., Concentration-time mortality response relationship of irritant and systemically acting vapours and gases,. J. Haz. Mater., 13, 301–309, 1986.

Weimer, J.T. and Ballard, T.A., Note on the Influence of Inhaled SNA on the Inhalation Toxicity of VX to Rats, *CWL-TM-24–36,* (Unclassified), 1960.

Whalley, C.E., Benton, B.J., Manthei, J.H., Way, R.A., Jakubowski, E.M., Jr., Burnett, D.C., Gaviola, B.I., Crosier, R.B., Sommerville, D.R., Muse, W.T., Forster, J.S., Mioduszewski, R.J., Thomson, S.A., Scotto, J.A., Miller, D.B., Crouse, C.L., Matson, K.L., and Edwards, J.L., Low-Level Cycolosarin (GF) Vapor Exposure in Rats: Effect of Exposure Concentration and Duration on Pupil Size, U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, *ECBC-TR-407* (Unclassified Report), September 2004.

6 Application of Intratracheal Instillation Exposure to the Etiological Determination of a Pulmonary Disease Outbreak: Nylon Flock as an Example

Dale W. Porter and Ann F. Hubbs

CONTENTS

6.1 INTRODUCTION

Pulmonary toxicology studies require exposure of the lungs to a chemical or particle. The two basic approaches to accomplish such pulmonary exposures are intratracheal (IT) instillation or inhalation. It is widely considered that IT exposures can be applied to comparative pulmonary toxicology studies (Driscoll et al., 2000). The premise of this chapter is that appropriately conducted IT exposures also provide a useful and powerful tool for determining disease etiology. To demonstrate this point and to highlight important considerations in the experimental design of such experiments, a specific example in which the application of IT exposures was crucial to determining the etiology of a pulmonary disease outbreak of unknown origin will be discussed.

6.2 IDENTIFICATION AND CHARACTERIZATION OF A PULMONARY DISEASE OUTBREAK OF UNKNOWN ORIGIN

Nylon flock is finely cut nylon and is used for the manufacture of upholstery used in a variety of products. As early as 1990, cases of interstitial lung disease were noted in nylon flock workers and

were described in a 1995 case series (Lougheed et al., 1995). In this report, 5 of 88 workers developed interstitial lung disease (ILD). In addition, disease recurred in the workers that returned to the workplace (Lougheed et al., 1995). Biopsies from three of the patients demonstrated important tissue alterations, including infiltration of alveolar spaces by macrophages (desquamative interstitial pneumonia), hypertrophy and hyperplasia of alveolar type II cells, and a predominantly lymphoplasmacytic interstitial infiltrate. The latter observation would become increasingly important as additional cases were reported.

An occupationally associated inhaled agent was the suspected cause of the ILD outbreak, although the exact etiological agent was not known. Initially, nylon flock produced at the plant was believed to be above the respirable size range (Lougheed et al., 1995) and thus was not considered a causative factor in disease development. *Fusarium* sp. was cultured from adhesives used at one plant, leading to the hypothesis that mycotoxins were a potential source of the disease. However, ILD continued to be reported in workplaces producing nylon flock, including worksites without mold contamination, as well as in the worksite where the ILD was first described. These observations suggested that biological contaminants were not responsible for the ILD outbreak.

Other occupational agents, such as crystalline silica, asbestos, coal dust, some metals (e.g., beryllium, cobalt), and some chemicals (e.g., anhydrides, isocyanates) were also considered as causative factors, because exposure to these agents can also result in the development of occupational ILD (Lopez and Salvaggio, 1994; Schwartz et al., 1994; Redlich, 1996; Rose, 1996). However, none of these materials were present in the plants with affected nylon flock workers and consequently could not explain the ILD outbreak. Thus, the etiological agent for the disease outbreak at the nylon flock plants appeared to be new and unknown.

By 1998, it was known that ILD affected nylon flock workers in five separate worksites (Eschenbacher et al., 1999). Morphological changes in lung tissues from affected workers varied, but one morphological pattern was unique to the nylon flock workers: the infiltration of lymphocytes into and around bronchioles with the associated appearance of lymphoid aggregates. This morphological alteration was termed lymphocytic bronchiolitis and peribronchiolitis with lymphoid hyperplasia (Boag et al., 1999). However, not all nylon flock workers with ILD had lymphocytic infiltrates. The spectrum of pulmonary pathological alternations in nylon flock workers also included diffuse alveolar damage, chronic bronchiolocentric interstitial pneumonia with foci of bronchiolitis obliterans organizing pneumonia, nonspecific interstitial pneumonia, and desquamative interstitial pneumonia (Lougheed et al., 1995; Boag et al., 1999; Kern et al., 2000).

6.3 MEDICAL AND ENVIRONMENTAL FIELD STUDIES

Case studies indicated that nylon flock workers who developed ILD, and returned to or remained in the workplace, relapsed or failed to improve (Boag et al., 1999; Eschenbacher et al., 1999). This suggested the possibility of a susceptible population and a persistent airborne etiological agent.

Medical and industrial hygiene cross-sectional surveys were conducted to quantify respiratory morbidity and to identify work-related factors that could be causally related to the ILD outbreak (NIOSH, 1998; Burkhart et al., 1999). The medical survey, which included a review of medical records of workers who were diagnosed with ILD, revealed a high prevalence of respiratory symptoms and respiratory diagnoses among workers from three departments. The dust present in one of the nylon flock plants included respirable samples that had a length to width ratio of >3 consistent with respirable fibers of irregular shape and appeared to arise as shreds from the larger nylon flock fibers (Burkhart et al., 1999). The finding of respirable-size nylon flock fibers contradicted an earlier report (Lougheed et al., 1995) and caused the nylon flock fibers to be reconsidered as a possible etiological agent for the ILD in nylon flock plant workers. Further evidence indicating that the nylon flock fibers might be a possible etiological agent came from observations of industrial processes, in conjunction with the medical survey and industrial hygiene results that indicated that the airborne dust or a chemical additive component of the dust might be responsible for lung disease in nylon

flock workers. To investigate this hypothesis, *in vivo* toxicological studies were initiated to investigate the pulmonary toxicity potential of airborne dust and of the components of the airborne dust.

6.4 TOXICOLOGICAL STUDIES

6.4.1 Important Considerations

The IT exposure route was chosen to investigate the hypothesis that airborne dust collected at a flock plant, or a component of the dust, may be responsible for the reported ILD outbreak. The IT exposure technique was selected for these studies primarily because of the limited amount of material available for study. Further, shreds of nylon flock often were in the form of irregularly-shaped nylon fibers in the respirable but polydispersed size range. Longer fibers tend to be more toxic than shorter fibers (Oberdorster, 2000; Bernstein et al., 2001). Some of the longer fibers that are deposited in the human lung are filtered out in the rodent nose (Oberdorster, 2000). IT instillation of such fibers allows exposure of the rat lung to long respirable fibers, which are more efficiently deposited in the human than rat lung (Driscoll et al., 2000). In addition, IT exposures allowed the studies to be completed in a much shorter time frame than an inhalation exposure, because an inhalation exposure would have required the development and validation of an exposure system. In the absence of any laboratory evidence that the dust was responsible for the ILD outbreak, and the urgency with which the information was needed, the expenditure of time and resources to develop such an inhalation system seemed imprudent.

Once the IT exposure route was selected for use, several other factors needed to be considered. These considerations regarded the anesthetic and vehicle to be used, as well as the physical characteristics and dose of test material to be given to the animal. All these factors contribute to the success of a study employing IT exposures.

An anesthetic that is chosen should suppress the animal's reflexes for a minimal period to allow IT instillation, but it should be short acting, thus allowing the animal to quickly recover and resume normal respiration. Methohexital (Brevital, Eli Lilly and Company, Indianapolis, IN) has been used routinely in this laboratory for this purpose in rats, although other alternatives are available. With regard to the use of methohexital, we routinely lightly anesthetized rats with an intraperitoneal injection of sodium methohexital (28 mg methohexital/kg body weight). Rats thus anesthetized are easily handled for IT instillation but recover within minutes after the administration of anesthetic.

Another important factor that should be considered is the choice of vehicle. To administer a sample by IT instillation, it is usually necessary to suspend the sample in some liquid, because the suspension of the sample in liquid facilitates delivery and distribution in the lung. We have found that sterile phosphate-buffered saline (PBS) causes little to no response in the lung when IT instilled. The same is also true for normal saline (0.9% w/v), although in this case any buffering capacity is lacking. However, it is important that the vehicle be free of endotoxin contamination, because endotoxin can cause pulmonary inflammation and damage, and thus can produce false positives if not controlled.

Regarding dose, materials that have not been previously studied are problematic, because no literature exists on which to base a decision. In such cases, a dose similar to doses of other occupational dusts that are known to cause pulmonary responses is an appropriate dose to begin testing. For example, it has been reported that a number of occupationally relevant dusts, exposed by IT instillation at 2.5- and 10-mg doses, resulted in pulmonary inflammation and damage only at the 10-mg dose (Hubbs et al., 2001). Fibers with known occupational hazards, such as chrysotile and glass, have been demonstrated to cause pulmonary alterations after exposure by IT instillation to 5 and 20 mg of fibers, respectively (Bernstein et al., 1980; Lemaire et al., 1985). Thus, a dose of 10 mg/kg body weight was chosen for the studies of this new, potentially toxic occupational dust.

The last, and perhaps most important consideration, is the particle size of the material that will be instilled. Although particles that normally would not be respirable can be instilled into the lung

by using IT techniques, the results of such studies may not provide data that are comparable with an inhalation exposure. Thus, particles that are respirable in humans should be used in IT studies.

These general considerations are highlighted in the following example, in which an *in vivo* pulmonary toxicology study of nylon flock dust using IT instillation exposures was used to determine the etiological agent responsible for a pulmonary disease outbreak (Porter et al., 1999).

6.4.2 Test Samples

Four different samples were examined in these studies: airborne dust, washed airborne dust, soluble fraction, and nylon tow. Airborne dust was collected at the nylon flock plant from air samples. The largest particles observed were the flock itself, usually being approximately 1 mm long and 10–15 μm in diameter. Because of their size, these particles would not be respirable and thus were excluded from the samples used for IT instillation by application of a vertical elutriator, which has been previously described in detail (Burkhart et al., 1999). Thus, the dust sample used was a respirable fraction in humans. Washed airborne dust and soluble fraction were prepared by extracting a portion of the airborne dust with water and isolating the resulting dust (washed airborne dust) and the water extract (soluble fraction) by centrifugation. The last material tested was dust prepared in the laboratory by cutting nylon tow with a rotary knife mill and isolating a respirable fiber fraction. This nylon tow contained finish materials used in its manufacture, but it did not contain any of the finish or dye components commonly used at the flock plant. The milled nylon tow was fiberlike and in the respirable size range with an average diameter of approximately 2 μm and an average length of approximately 14 μm (Porter et al., 1999).

6.4.3 Dosage and IT Instillation

Because these dusts had not been studied previously, we used a dose that is known to cause pulmonary inflammation in response to other well-known occupational dusts. Rats received either a 10 mg/kg body weight dose of a dust or an equivalent dose (1 ml/kg body weight) of PBS (vehicle control). For studies of the soluble fraction, rats received either a 1.3 ml/kg body weight dose of the soluble fraction or a 1.3 ml/kg body weight dose of PBS (vehicle control). This dose of the soluble fraction contained approximately the same amount of endotoxin present in a 10 mg/kg dose of the airborne dust.

6.4.4 Bronchoalveolar Lavage and Histological Sample Collection

In this study, bronchoalveolar lavage (BAL) and tissue samples for histological studies were obtained from the same rat, a design that provides advantages in comparison with having separate animals for BAL and histology. First, this approach allows BAL and histology end points to be conducted when the number of animals that can be exposed is limited by the amount of sample available for study. Second, it allows the distribution of instilled material to be qualitatively assessed. For example, if the IT administration resulted in a relatively diffuse deposition, then inflammation should be observed in both lung lobes examined histologically and in lung lobes used for BAL.

Rats were euthanized 1 day after IT instillation of all test materials and 29 days after IT exposure for airborne dust. A tracheal cannula was inserted, the left lung lobe was clamped off with a hemostat, ligated, and removed below the ligature for histopathology. The right lung was lavaged through the cannula with ice-cold PBS, the first lavage used 3 ml and subsequent lavages used 4 ml until a total of 40 ml of lavage fluid was collected. The first BAL was kept separate from the rest of the lavage fluid. BAL cells were isolated by centrifugation ($500 \times g$, 10 min, 4°C). The acellular supernate from the first lavage (BAL fluid) was decanted and saved for later analysis. The acellular supernate from the rest of the lavage was decanted and discarded. BAL cells from all lavages were resuspended in HEPES buffer (10 m*M* *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 145 m*M* NaCl, 5.0 m*M* KCl, 1.0 m*M* $CaCl_2$, 5.5 m*M* D-glucose; pH 7.4) and centrifuged ($500 \times g$, 10 min, 4°C), and the

supernate was decanted and discarded. The BAL cell pellet was then resuspended in HEPES buffer and placed on ice. Cell counts and differentials were obtained by using a Coulter Multisizer II and AccuComp software (Coulter Electronincs, Hialeah, FL).

6.4.5 End Points

Examination of the effect of the test agents on alveolar macrophage (AM) function and pulmonary inflammation and damage at 1-day post-IT are summarized in Table 6.1. AM function was assessed by measuring zymosan-stimulated AM chemiluminescence, which is a measure of reactive oxygen species production. To determine pulmonary inflammation and damage, BAL polymorphonuclear leukocytes (PMNs) and BAL fluid albumin concentrations were quantified, respectively. Versus vehicle controls, zymosan-stimulated AM chemiluminescence was significantly increased by all three dusts and the soluble fraction. In addition, all three dusts and the soluble fraction caused a significant increase in BAL PMNs and BAL fluid albumin as compared with vehicle control; however, the soluble fraction was less active than any dust fraction.

Table 6.1 AM Function and Pulmonary Inflammation 1 Day Post-IT Exposure [a]

Agent	AM Chemiluminescence [b] (cpm × 10^5/0.5 × 10^6 AM/15 min)	BAL PMN (10^6 cells/rat)	BAL Fluid Albumin(mg/ml)
Vehicle	2.27 ± 0.45	0.75 ± 0.11	0.20 ± 0.01
Airborne dust	259.95 ± 24.39*	8.89 ± 1.48*	0.35 ± 0.04*
Washed dust	66.77 ± 12.32*	3.94 ± 0.55*	0.34 ± 0.02*
Nylon tow	21.01 ± 6.20*	5.59 ± 1.32*	0.37 ± 0.03*
Soluble fraction	7.73 ± 3.11*	1.43 ± 0.19*	0.33 ± 0.05*

[a] An asterisk (*) indicates a significant difference versus vehicle ($p < 0.05$).

[b] Zymosan-stimulated chemiluminescence measured in the presence of 1 mg unopsonized zymosan and 0.04 mg% (w/v) luminol.

Table 6.2 Histopathology Scores[a] in Rats Exposed to Agents Present in Nylon Flocking at 1 Day Postexposure

Exposure	Suppurative or Histiocytic and Suppurative Pneumonia [a, b]
Vehicle	0.7 ± 0.3
Airborne dust	5.8 ± 0.2*
Washed dust	6.4 ± 0.9*
Nylon tow	5.4 ± 0.5*
Soluble fraction	0.8 ± 0.8

[a] The pneumonia pathology score is the sum of the scores for severity (intensity) and distribution of inflammation in the lung where each were scored on a scale of 0 to 5 as previously described (Hubbs et al., 2002).

[b] An asterisk (*) indicates significant different from control value (Kruskal–Wallis One-Way Analysis of Variance on Ranks, Dunn's Intergroup Comparison).

TABLE 6.3 AM Function and Pulmonary Inflammation 1 and 29 Days Post-IT Exposure [a]

	1 Day Post-IT		29 Days Post-IT	
	Vehicle	Airborne Dust	Vehicle	Airborne Dust
AM chemiluminescence [b] (cpm $\times$ 10^5/0.5 $\times$ 10^6 AM/15 min)	2.52 ± 0.43	259.95 ± 24.39*	1.96 ± 0.41	1.17 ± 0.19
BAL PMNs (10^6 cells/rat)	0.56 ± 0.06	8.89 ± 1.48*	0.66 ± 0.04	0.80 ± 0.97
BAL fluid albumin (mg/ml)	0.18 ± 0.01	0.35 ± 0.04*	0.20 ± 0.02	0.20 ± 0.01

[a] An asterisk (*) indicates a significant difference versus vehicle ($p < 0.05$).

[b] Zymosan-stimulated chemiluminescence measured in the presence of 1 mg unopsonized zymosan and 0.04 mg% (w/v) luminol.

Table 6.4 Histopathology Scores in Rats Exposed to Airborne Dust or Vehicle at 29 Days Postexposure

Exposure	Alveolar Histiocytosis with Intralesional Birefringent Fibers [a]
Vehicle	0.0 ± 0.0
Airborne dust	4.8 ± 0.2*

[a] An asterisk (*) indicates significant different from control ($p = 0.008$, Mann–Whitney Rank Sum Test).

Histopathological assessment of rat lungs exposed to airborne dust at 1 day post-IT exhibited suppurative (neutrophilic) inflammation, consistent with the BAL cell differentials. Similar histopathological assessments also were made for washed airborne dust, soluble fraction, and nylon tow dust. These histological assessments indicated that airborne, washed airborne, and nylon tow dusts all caused suppurative or suppurative and histiocytic pneumonia, whereas the soluble fraction was far less active (Table 6.2).

Examination of the effect of airborne dust on AM function and pulmonary inflammation and damage at 1 and 29 days post-IT is summarized in Table 6.3. At 1 day post-IT, airborne dust caused significant increases in zymosan-stimulated AM chemiluminescence, BAL PMNs, and BAL fluid albumin concentrations versus vehicle control, but by 29 days these parameters had returned to vehicle control levels. The histological results were consistent with the BAL end points but revealed that some pulmonary alterations persisted. At 29 days post-IT exposure, each of the five rats that received airborne dust had multiple foci of minimal to mild alveolar histiocytosis (proliferation of AMs) associated with birefringent fibers localized within macrophages at sites of alveolar histiocytosis (Table 6.4).

6.4.6 Using IT Instillations of Nylon Flock as a Tool for Understanding the ILD Outbreak

Several major conclusions from this study, as they relate to the ILD outbreak at the nylon flock plant, can be made. First, the airborne dust collected at the nylon flock plant was inflammatory. Second, nylon tow, which has fiber properties similar to those observed in the airborne dust but was not treated or exposed to chemical or biological contaminants added during the flocking process, was also inflammatory. Third, the washed airborne dust was inflammatory, but BAL data suggest that the intensity of inflammation was less than that produced by the airborne dust. Last, respirable nylon fibers persisted in the lungs of rats 29 days after IT instillation, and these persistent fibers were associated with aggregates of macrophages.

6.5 HUMAN RELEVANCE

When we instilled the airborne dusts present within the nylon flock industry into rats, we found that these dusts were acutely inflammatory; we further found that inflammation was also produced by the washed airborne dust and the respirable nylon fibers derived from cut tow (nylon fiber shreds), a component of the airborne dust. The soluble fraction produced less acute inflammation by BAL and no significant histopathologically detectable inflammation. Twenty-nine days after instillation, foci of alveolar histiocytosis were associated with retained respirable fibers that were birefringent and morphologically consistent with retained nylon fiber shreds. Each of these findings implicated the respirable nylon fibers in the airborne dust as being initially inflammatory, biologically persistent for at least 4 weeks in the rodent lung, and associated with the alveolar histiocytosis that remained more than 4 weeks after exposure.

For mineral fibers, long, nonphagocytizable fibers tend to be removed by dissolution (Bernstein et al., 2001). If this is also true for manufactured organic fibers, biologically persistent fibers of low solubility would be expected to slowly release their components at the site of deposition. The morphological changes in the human patients exposed to nylon flock are diverse but are frequently characterized by infiltration of lymphocytes or macrophages (Lougheed et al., 1995; Eschenbacher et al., 1999; Kern et al., 2000). This is a cellular response which suggests up-regulation of cellular immunity, a process that usually involves T-helper 1 (T_H1) pattern responses in the lung as opposed to allergic or T-helper 2 (T_H2) patterns of response.

Although processes that control the immune response in the lung are incompletely investigated, several recent findings may be relevant to ILD seen in the nylon flock industry. Antigen exposure in the lung frequently leads to the development of antigenic tolerance (Steinman et al., 2003). When sensitization occurs, peripheral sensitization and pulmonary challenge tend to cause a predominantly T_H1 (cellular) response, whereas pulmonary sensitization and pulmonary challenge tend to cause a T_H2 (allergic) type of response (Constant et al., 2002). However, in chronic silicosis, which is a highly investigated occupational lung disease caused by exposure to crystalline silica, dust exposure and challenge are pulmonary, but both T_H1 and T_H2 activation occur with a predominance of T_H1 activation (Weissman et al., 2001). Recently, antigen presentation by alveolar macrophages has been suggested to stimulate T_H1-type immune responses in the lung (Tang et al., 2001). In rats, the ability of alveolar macrophages to release T_H1- or T_H2-type cytokines is strain dependent, suggesting a genetic basis for variable lung immunopathological responses (Sirois and Bissonnette, 2001).

Our IT instillations with nylon flocking materials provided useful insights into important toxicological differences between different occupational agents, namely silica and nylon flock. These studies are even more interesting in view of recent advances in pulmonary immunology. The studies indicated that, of the agents investigated, the airborne dust collected from the worksite was highly inflammatory and was also more inflammatory than the components of the dust that were tested. Retrospective comparisons with other IT instillation experiments of occupationally important agents previously conducted in our laboratory supported the highly inflammatory nature of these dusts (Porter et al., 1999). In general, such comparisons are accepted as a valid use of data obtained from studies using the IT exposure technique (Driscoll et al., 2000) and could not have been conducted in a timely fashion by inhalation exposures. In addition, this study showed that the respirable nylon fibers that were a component of the workplace dusts were highly inflammatory and that some fibers persisted in AMs in the lungs of rats for at least 4 weeks and were associated with focal histocytosis. In view of recent evidence regarding the role of AMs and inhaled dusts in altered pulmonary immune response, the persistence of these workplace fibers in the lungs of exposed rats indicated that these fibers played a potentially important role in the etiology of this disease outbreak.

6.6 SUMMARY

The example above indicates that when appropriate consideration is given to the design of an experiment utilizing IT instillation exposures, the data obtained can provide information relevant to human

inhalation exposures. In this case, the study provided crucial evidence that suggested that the etiological agent causing an outbreak of ILD at an industrial plant was a respirable fraction of nylon shreds produced during nylon flock production.

REFERENCES

Bernstein, D.M., Drew, R.T., and Kuschner, M., Experimental approaches for exposure to sized glass fibers, *Environ. Health Perspect.,* 34, 47–57, 1980.

Bernstein, D.M., Riego Sintes, J.M., Ersboell, B.K., and Kunert, J., Biopersistence of synthetic mineral fibers as a predictor of chronic inhalation toxicity in rats, *Inhalation Toxicol.,* 13, 823–849, 2001.

Boag, A.H., Colby, T.V., Fraire, A.E., Kuhn, C., III, Roggli, V.L., Travis, W.D., and Vallyathan, V., The pathology of interstitial lung disease in nylon flock workers, Am J. Surg. Pathol., 23, 1539–1545, 1999.

Burkhart, J., Piacitelli, C., Schwegler-Berry, D., and Jones, W., Environmental study of nylon flocking process, *J. Toxicol. Environ. Health Part A,* 57, 1–23, 1999.

Constant, S.L., Brogdon, J.L., Piggott, D.A., Herrick, C.A., Visintin, I., Ruddle, N.H., and Bottomly, K., Resident lung antigen-presenting cells have the capacity to promote Th2 T cell differentiation in situ, J. Clin. Invest., 110, 1441–1448, 2002.

Driscoll, K.E., Costa, D.L., Hatch, G., Henderson, R., Oberdorster, G., Salem, H., and Schlesinger, R.B., Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: uses and limitations, Toxicol. Sci., 55, 24–35, 2000.

Eschenbacher, W.L., Kreiss, K., Lougheed, M.D., Pransky, G.S., Day, B., and Castellan, R.M., Nylon flock-associated interstitial lung disease, Am. J. Respir. Crit. Care Med., 159, 2003–2008, 1999.

Hubbs, A.F., Battelli, L.A., Goldsmith, W.T., Porter, D.W., Frazer, D., Friend, S., Schwegler-Berry, D., Mercer, R.R., Reynolds, J.S., Grote, A., Castranova, V., Kullman, G., Fedan, J.S., Dowdy, J., and Jones, W.G., Necrosis of nasal and airway epithelium in rats inhaling vapors of artificial butter flavoring, Toxicol. Appl. Pharmacol., 185, 128–135, 2002.

Hubbs, A.F., Minhas, N.S., Jones, W., Greskevitch, M., Battelli, L.A., Porter, D.W., Goldsmith, W.T., Frazer, D., Landsittel, D.P., Ma, J.Y., Barger, M., Hill, K., Schwegler-Berry, D., Robinson, V.A., and Castranova, V., Comparative pulmonary toxicity of 6 abrasive blasting agents, Toxicol. Sci., 61, 135–143, 2001.

Kern, D.G., Kuhn, C., III, Ely, E.W., Pransky, G.S., Mello, C.J., Fraire, A.E., and Muller, J., Flock worker's lung: broadening the spectrum of clinicopathology, narrowing the spectrum of suspected etiologies, *Chest,* 117, 251–259, 2000.

Lemaire, I., Nadeau, D., Dunnigan, J., and Masse, S., An assessment of the fibrogenic potential of very short 4T30 chrysotile by intratracheal instillation in rats, Environ. Res., 36, 314–326, 1985.

Lopez, M. and Salvaggio, J.E., Hypersensitivity pneumonitis, in *Textbook of Respiratory Medicine*, Murray, J.F., and Nadel, J.A., Eds., W.B. Saunders, Philadelphia, 1994, pp. 2018–2031.

Lougheed, M.D., Roos, J.O., Waddell, W.R., and Munt, P.W., Desquamative interstitial pneumonitis and diffuse alveolar damage in textile workers. Potential role of mycotoxins, *Chest,* 108, 1196–1200, 1995.

National Institute for Occupational Safety and Health (NIOSH), Hazard evaluation and technical assistance report: Microfibres, Inc., in NIOSH Report HETA 96-0093-2685, Washko, R., Burkhart, J., and Piacitelli, C., Eds., U.S. Department of Health and Human Services, Public Health Service, Centers of Disease Control, NIOSH, Cincinnati, 1998, pp. 1–37.

Oberdorster, G., Determinants of the pathogenicity of man-made vitreous fibers (MMVF), Int. Arch. Occup. Environ. Health., 73 (Suppl.), S60–S68, 2000.

Porter, D.W., Castranova, V., Robinson, V.A., Hubbs, A.F., Mercer, R.R., Scabilloni, J., Goldsmith, T., Schwegler-Berry, D., Battelli, L., Washko, R., Burkhart, J., Piacitelli, C., Whitmer, M., and Jones, W., Acute inflammatory reaction in rats after intratracheal instillation of material collected from a nylon flocking plant, *J. Toxicol. Environ. Health Part A,* 57, 25–45, 1999.

Redlich, C.A., Pulmonary fibrosis and interstitial lung diseases, in *Occupational and Environmental Respiratory Disease,* Harber, P., Schenker, M.B., and Balmes, J.R., Eds., Mosby-Yearbook, St. Louis, 1996, pp. 216–227.

Rose, C., Hypersensitivity pneumonitis, in *Occupational and Environmental Respiratory Disease,* Harber, P., Schenker, M.B., and Balmes, J.R., Eds., Mosby-Yearbook, St. Louis, 1996, pp. 201–215.

Schwartz, M.I., King, T.E., and Cherniak, R.M., Infiltrative and interstitial lung diseases, in *Textbook of Respiratory Medicine,* JMurray, J.F., and Nadel, J.A., Eds., W.B. Saunders, Philadelphia, 1994, pp. 1803–1849.

Sirois, J. and Bissonnette, E.Y., Alveolar macrophages of allergic resistant and susceptible strains of rats show distinct cytokine profiles, Clin. Exp. Immunol., 126, 9–15, 2001.

Steinman, R.M., Hawiger, D., and Nussenzweig, M.C., Tolerogenic dendritic cells, Ann. Rev. Immunol., 21, 685–711, 2003.

Tang, C., Inman, M.D., van Rooijen, N., Yang, P., Shen, H., Matsumoto, K., and O'Byrne, P.M., Th type 1-stimulating activity of lung macrophages inhibits Th2-mediated allergic airway inflammation by an IFN-gamma-dependent mechanism, J. Immunol., 166, 1471–1481, 2001.

Weissman, D.N., Hubbs, A.F., Huang, S.H., Stanley, C.F., Rojanasakul, Y., and Ma, J.K., IgG subclass responses in experimental silicosis, J. Environ. Pathol. Toxicol. Oncol., 20 (Suppl. 1), 67–74, 2001.

7 The Use of Large Animals in Inhalation Toxicology

R.F.R. Brown, B.J.A. Jugg, and J. Jenner

CONTENTS

7.1 INTRODUCTION

The choice of the most appropriate animal to predict the toxic effects of chemicals in humans is a critical question to all branches of biological and biochemical research. In the past 100 years probably every animal on earth has been considered as possibly predictive of the human response to chemical injury. This chapter considers the use of large farm animals as models for the response of humans to the inhalation toxicity of chemicals. Most of the common laboratory animal species provide useful information in predicting the effects of inhaled toxicants and the treatment of their toxic effects in humans. Small animals, such as rats, mice, and guinea pigs, offer the advantage of larger numbers in experimental groups, thus allowing a better prediction of the variation of biological response. Small animals, however, do not easily allow the investigation of multiple pathophysiological changes without using multiple groups. Moreover the therapeutic or toxicological investigations

often require the design and construction of specialized equipment to account for the small size of the experimental subject. Some difficulties are involved in housing and handling larger animals, but where these can be overcome, the use of larger animals allows the investigation of multiple physiological changes resulting from toxic insult and the use of conventional equipment designed for clinical use in humans.

Large animals provide different problems in exposure to verifiable concentrations of inhaled chemicals than smaller animals.

Over the past ten years our laboratory has been using the pig to investigate the effects of lung-damaging agents, specifically phosgene, and how these can be treated in an intensive care environment. This chapter uses the methods and results of some of that work to illustrate the use of large animals in studying inhalation toxicology. Before considering these details however, it is necessary to review the two large farm animal species, the sheep and the pig, that have been used extensively in inhalation research and their anatomical and physiological similarities to humans.

7.2 COMPARATIVE ANATOMY AND PHYSIOLOGY

7.2.1 The Lungs

The anatomy and physiology of the immediate air–blood interface is very similar in all species. The main body of the lungs, or parenchyma, is composed of many alveoli that terminate the airways and bring the inspired air into close contact with the blood to enable gas exchange. At this point the only tissue separating the air and the blood is the thin squamous epithelium of the alveoli, a basement membrane and the endothelium of the capillaries.

Macroscopically the lungs are organized into a number of lobes, which, together with the heart, virtually fill the thoracic cavity. When the lungs are inflated within the thoracic cavity the lobes can be discerned by fissures but separated from each other when the lungs are removed from the thorax to show the lobes distinctly (Figure 7.1). The surface of the lungs shows many distinct hexagonal lobules.

In humans the lungs are of similar size, though the right is slightly heavier than the left. The left lung comprises two lobes, upper and lower (or superior and inferior), whereas the right lung has three, the upper, middle, and lower (superior, middle, and inferior). Each lobe is supplied with air from the trachea via a bronchus and with blood by a branch of the pulmonary artery arising from the right ventricle of the heart (Figure 7.2).

In the pig the right lung is a little larger than the left and has four lobes, the apical, middle, intermediate, and diaphragmatic lobes (Figure 7.1). The intermediate lobe is not present in humans. The left lung has only two lobes, though it may appear that it has three, the middle and apical lobes are not anatomically separate and are supplied by the same bronchus. Unlike in humans the apical lobe of the left lung is supplied by an accessory bronchus that branches off the trachea at a sharp angle about 2 to 3 cm above the bronchial bifurcation.

In sheep the right lung is almost twice the size of the left with lobules similar to the pig (Figure 7.1). The right lung has four lobes and the left has two. The apical lobe of the left lung is supplied by an ancillary bronchus similar to that in the pig.

Other than the greater lobulation of the right lung, the most marked difference between sheep, pigs, and humans may result from humans walking upright and pigs and sheep walking on all fours. The greater angle of the diaphragm in the quadrupeds means that more of the diaphragmatic or lower lobes of both lungs protrude over the abdominal space. This has the effect of placing the weight of the liver, spleen, and some of the gut onto the lower part of the diaphragmatic lobe when a pig or a sheep is placed on its back, as it may be when anesthetized for surgery. Thus, a degree of hemorrhagic change is observed in pigs anesthetized and placed on their backs for any period and can be mistaken for pathology related to a toxic insult.

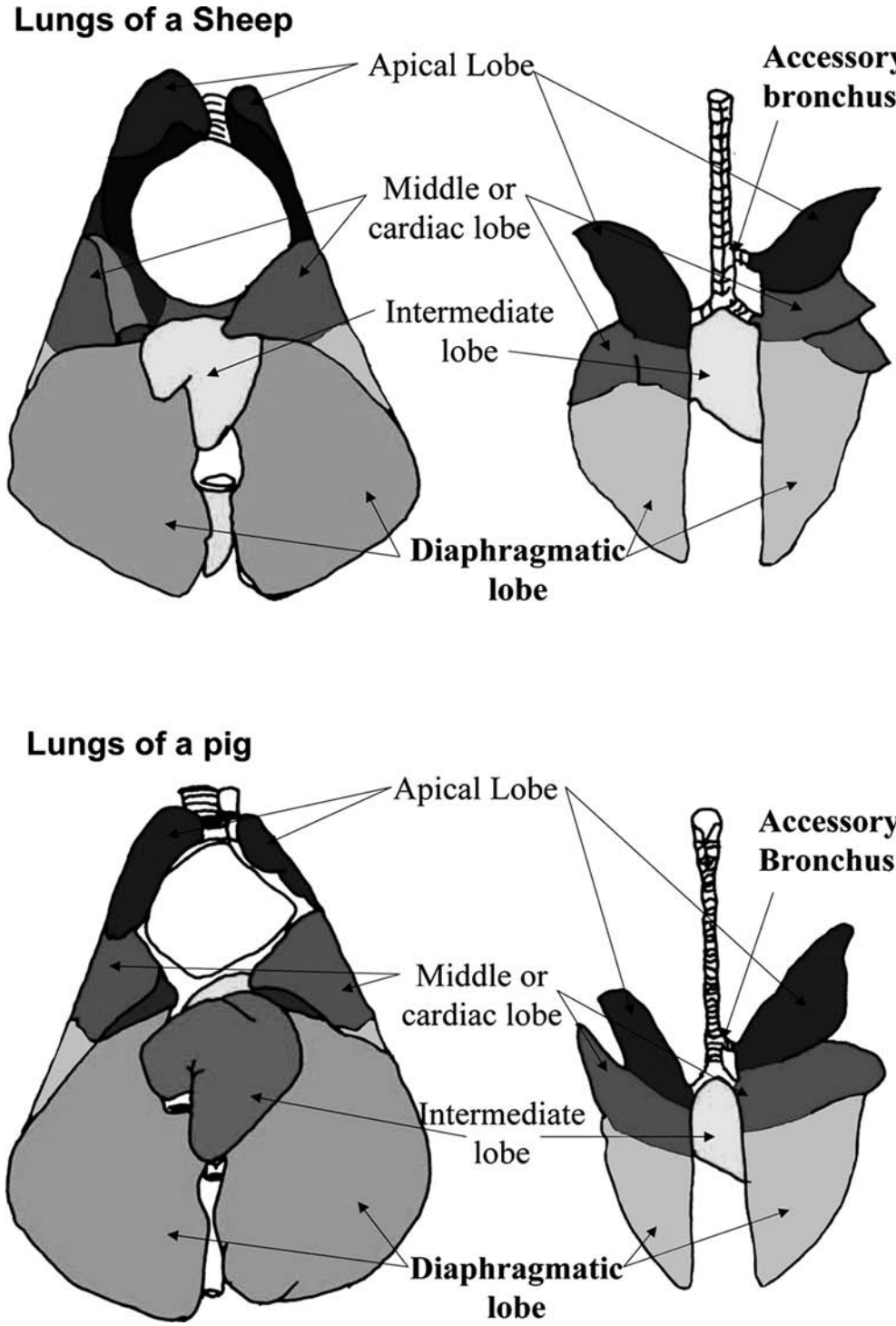


FIGURE 7.1 Diagrammatic representation of the lungs of sheep and pigs showing the appearance of the thoracic cavity (left) and when dissected (right). Note the accessory bronchus and intermediate lobes not present in humans.

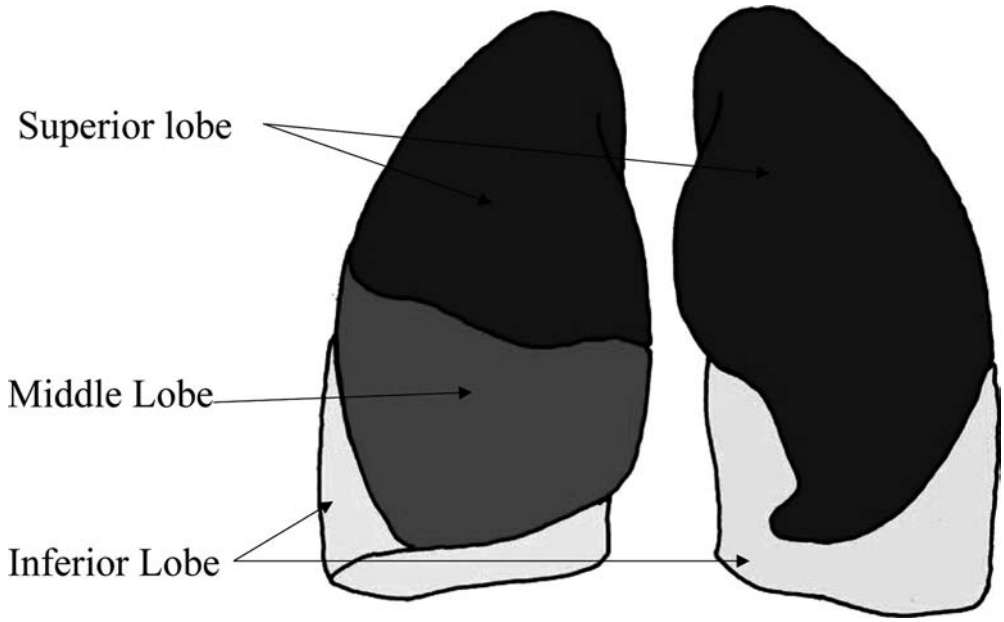


FIGURE 7.2 Diagrammatic representation of human lungs, showing the positions of the lobes as they would be in the chest cavity.

Despite these differences the lungs of sheep, pigs, and humans are very similar in form and function; provided the researcher is aware of the anatomical differences when designing studies, results from studying these animals should be predictive of what will happen to humans exposed to similar conditions.

7.2.2 Upper Airways

Probably the greatest interspecies difference with a direct impact on the use of animals to model human inhalation is in the anatomy of the upper airways. In common with many primates, humans have developed a reliance on their visual sense at the expense of the olfactory sense. Thus humans have lost the well-developed "nose" of lower animals, which presents a smaller surface area for the absorption of inhaled material present in the pig or the sheep. Moreover, the reliance of the other species on their olfactory sense makes them more reliant on breathing through their nose, only breathing through their mouths in extremis. Because toxic vapors, gases, and particulates are absorbed in the nasal cavities, the use of other species to model the effects of toxicants on humans requires some method of either bypassing the nasal cavities or taking their effects on pulmonary absorption into account.

Figure 7.3 shows the differences in the size of the nasal turbinates relative to the heads for humans and pigs, together with the degree of convolution of the turbinates. Pigs and sheep clearly have a much larger surface area compared to the size of their heads than humans. The effect of this on using animals to model effects in humans is that a larger percentage of the inhaled dose would potentially be absorbed in the upper respiratory tract of sheep and pigs than would be in humans. Similarly, because animals rely more heavily on their olfactory sense to find food and drink than humans do, damage to the olfactory epithelium may have an impact on the very survival of animals when it would not in humans.

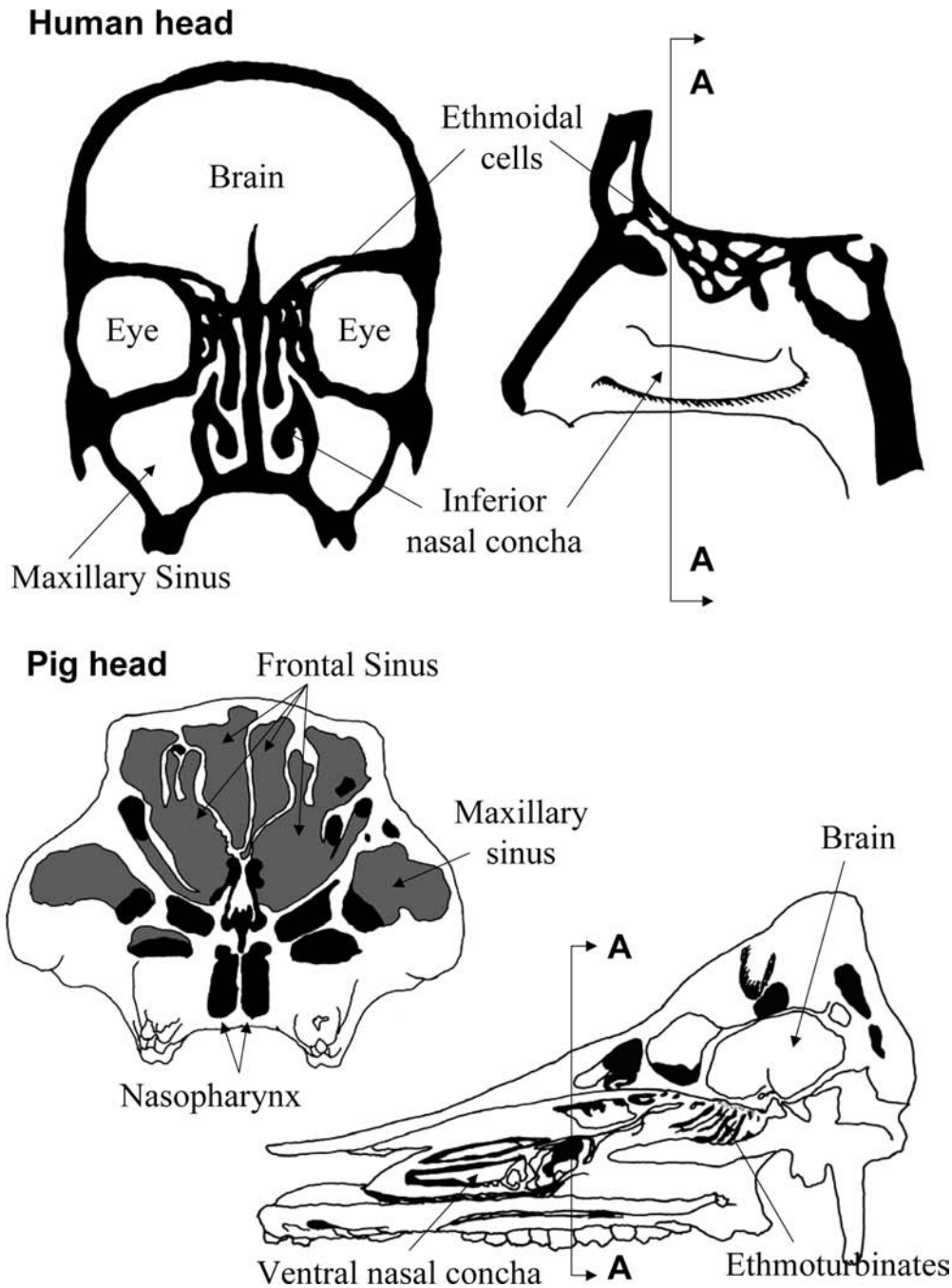


FIGURE 7.3 A comparison of the anatomy of the human and porcine head. Note the greater complexity and hence area available for absorption of the nasal cavities of the pig compared with the human. The transverse section is taken at the point indicated by the plane AA and is viewed in the direction of the arrows.

7.3 THE USE OF SHEEP AS A MODEL IN INHALATION TOXICOLOGY

The sheep has been used as a model to aid investigation of inhalation injury caused by smoke inhalation. Smoke inhalation is a major cause of death in domestic and industrial fires. In addition to the carbon monoxide and hydrogen cyanide present in smoke, which can kill by preventing the body from using and transporting oxygen, smoke can contain a number of gases that cause direct damage to the respiratory tract. These include gases like acrolein, phosgene (and other reactive halogenated hydrocarbons), and hydrogen chloride.

Like porcine models, in general, sheep are exposed to toxic gases via a face mask or tracheal intubation while anesthetized. During exposure animals are often laid on their backs or on their sides, which is not a natural posture for either species; the position the animal is placed in should be carefully considered during experimental design.

Sheep are commonly prepared by instrumentation. This involves the introduction of an arterial or venous cannula via the femoral or jugular vessels. Sometimes a Swan–Ganz type catheter is introduced to measure pulmonary arterial pressure. An advantage of using the sheep as a model of lung injury is the ease with which a fistula can be introduced into the efferent lymphatic duct from the medial mediastinal lymph node, enabling good lymph samples to be acquired (Staub et al., 1975).

This kind of preparation was described by Alpard et al. (2000), who reported the development of a model for clinically relevant smoke inhalation injury combined with a 40% cutaneous flame burn in sheep. Animals were anesthetized for surgery 24 h before injury was induced and prepared by the insertion of cannulas into the right femoral artery and vein, externalized through the subinguinal fold. A pulmonary arterial thermodilution catheter was introduced via a percutaneous access catheter into the right external jugular vein. These catheters enabled routine measurement of heart rate, arterial and venous pressure, central venous pressure, cardiac output, pulmonary artery occlusion pressure, and arterial and mixed venous blood gases.

Traber et al. (1988) described a method of exposing sheep to smoke by using a modified bee smoker that burned cotton toweling. They found a close correlation between duration of exposure and lung lymph formation and showed polymorphonucleocyte recruitment and release of chemotactic factors produced by the cells damaged by the chemicals in the smoke. They reported the stimulation of pulmonary macrophages and showed an increase in many enzyme activities (i.e., glucuronidase, trypsin, and elastase). Tasaki et al. (1997, 2002) also investigated the relationship between inhalation injury by smoke and heat and cutaneous burns. They instrumented sheep with a jugular catheter and a Swan–Ganz catheter for measuring pulmonary arterial pressure. The sheep were anesthetized with sodium pentobarbital for exposure to smoke from wood chips. A subjective scoring system was used to assess histological lung damage. Sheep were allowed to recover consciousness for 48 h (Tasaki et al., 1997) or 5 days (Tasaki et al., 2002). The addition of a 40% cutaneous burn to an inhalation smoke injury increased the postinjury hemodynamic changes and hypoproteinemia but not the hypoxic changes, pulmonary hypertension, or pulmonary edema.

Soejima et al. (2000) used sheep to investigate the role of nitric oxide (NO) in pulmonary injury. These workers were able to show that NO produced by inducible nitric oxide synthase plays an important role in combined smoke inhalation/third degree burn injury but does not affect the vascular permeability of third-degree-burned tissue.

Sheep have been used extensively to investigate the role of various mediators of inflammation in lung injury. Sakurai et al. (1999) used sheep to investigate the role of L-selectin in the development of lung injury following smoke inhalation injury combined with 40% cutaneous burn. Janssens et al. (1994) showed that Leucotriene B_4 might be involved in the development of edema caused by acrolein-containing smoke by using instrumented sheep pretreated with several different selective inhibitors of cyclo-oxygenase and lipoxygenase, whereas Brizio-Molteni et al. (1995) showed

that a thromboxane antagonist could decrease pulmonary lipid peroxidation. Demling et al. (1993) also demonstrated that airway damage caused by smoke inhalation increased parenchymal lipid peroxidation.

7.4 THE PIG AS A MODEL OF PHOSGENE-INDUCED LUNG INJURY

Though sheep have been used extensively for investigating lung injury, when our laboratory needed to test treatments of lung injury induced by phosgene, the pig was investigated as more representative of humans. Investigation of the sensitivities of ruminants (sheep and goats) to phosgene is greater than all other species investigated to date. Boyland et al. (1946) showed that the LCt_{50} for rats, mice, and guinea pigs was in the range of 1500 to 2000 $mg \cdot min \cdot m^{-3}$ and primates are significantly less sensitive than rats (Weston and Karel, 1947). Keeler et al., 1990, however, showed that the LCt_{50} for sheep was 13,300 $mg \cdot min \cdot m^{-3}$ and other studies have shown that the goat is similarly insensitive (Greeson and Walker, 1932; Karel and Weston, 1947). Small-animal models have allowed the definition of a lethal dosage and the relationship between that dosage and exposure time (Boyland et al., 1946). The use of a large animal fulfills the need to model phosgene-induced lung injury in a clinically appropriate environment and investigate candidate therapies. The pig has been used widely as a model of acute lung injury caused either by chemical agents, e.g., inhalation of chlorine (Gunnarsson et al., 2000), or intravenous administration of oleic acid (Lange et al., 2000) or physical trauma, e.g., repeated bronchiolar lavage (Sison et al., 2000). These models have been used to investigate a wide variety of therapies including the use of nitric oxide and surfactant (Sison et al., 2000) or the use of specific drug regimens, e.g., corticosteroids (Gunnarsson et al., 2000). Additionally, much of the instrumentation found within an intensive care unit (ICU) setting can be applied readily to a pig model because the trachiobroncheal and vascular architectures are similar in size to humans.

Research was carried out in our laboratories to develop a reproducible model of chemically induced lung injury, using phosgene as a model toxicant, in the anesthetized pig that could form the basis for the scientific study of a therapeutic regimen. The experimental details of this work are included as an example of the use of the pig in inhalation toxicology research.

7.5 THE ANIMAL MODEL

Juvenile female large white pigs (mean weight, 50 kg; range, 47 to 55 kg), obtained from commercial sources, were fed a standard pig diet and housed in pairs in a purpose-built animal-holding facility for 7 days before the start of the experiment. Animals were allowed access to food until 12 h prior to the study period and water intake was not restricted. Animals were premedicated with midazolam hydrochloride (Hypnovel) by intramuscular injection and anesthesia was induced by inhalation of Fluothane in oxygen and nitrous oxide. Animals were intubated with a cuffed endotracheal tube and inhalational anesthesia was maintained until venous access had been obtained. Anesthesia was subsequently maintained throughout the experiment by intravenous infusion of ketamine (Ketaset), midazolam hydrochloride (Hypnovel), and alfentanil hydrochloride (Rapifen) (Parker et al., 2000) administered at a dose rate of approximately 1 $ml \cdot kg^{-1} \cdot h^{-1}$.

Animals were maintained supine and allowed to breathe spontaneously with a F_{IO_2} of 0.3. Under aseptic conditions, the cervical vessels were surgically exposed and size 8 French Gauge (FG) catheters were introduced into each of the left internal jugular veins and the left common carotid arteries. A pulmonary artery thermodilution catheter was introduced via the right internal jugular vein. A 14FG Foley catheter was introduced into the bladder via an open cystotomy. Three standard electrocardiogram electrodes were attached to the skin to allow recording of the electrocardiogram. All monitoring devices were attached to a Propaq 106EL monitor. The pulmonary

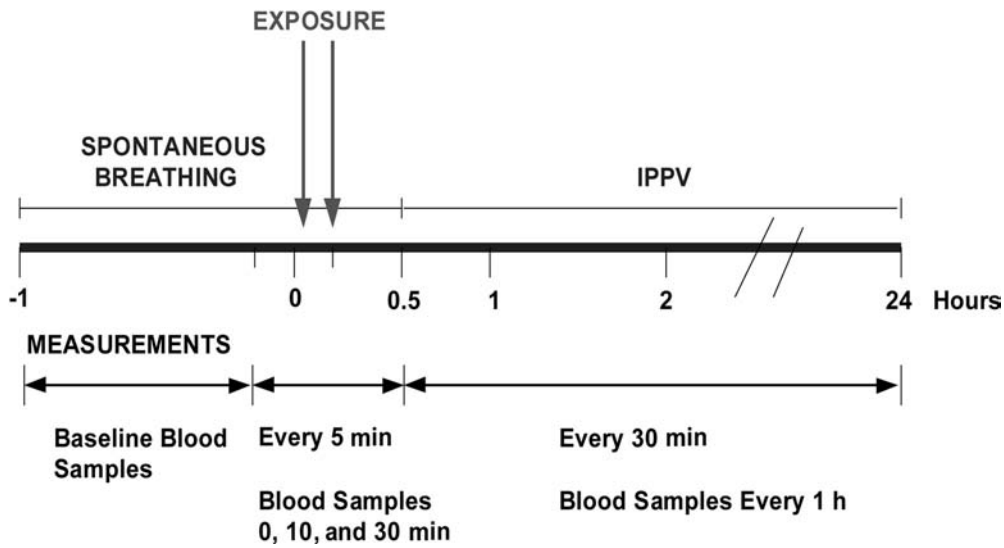


FIGURE 7.4 Experimental time line showing frequency of vital signs, cardiovascular measurements, and blood samples.

artery catheter was attached to a Baxter Vigilance monitor, which allowed continuous measurement of cardiac output, mixed venous oxygen saturation, and core temperature. Insensible losses were corrected by intravenous infusion of sodium chloride (0.18%) and glucose (4%) solution at a dose rate of 2.5 $ml \cdot kg^{-1} \cdot hr^{-1}$.

Following the completion of surgery, baseline measurements were taken for 1 h. The animal was then transferred to the exposure facility (Figure 7.4).

7.5.1 Phosgene Exposure

The exposure system consisted of a delivery apparatus, an exposure tube, and effluent and analysis equipment (Figure 7.5). The delivery system was a 10-l lamofoil bag mounted within a rigid Perspex box and filled with phosgene diluted with nitrogen. Delivery of phosgene was achieved by admitting nitrogen to the outer Perspex chamber, thereby subjecting the lamofoil bag to a positive pressure and directing the outflow to the exposure tube inlet to produce the required concentration. The outflow from the bag ($\approx$250 $ml \cdot min^{-1}$) is further diluted into a carrier flow of laboratory air (50 $l \cdot min^{-1}$), which carries the phosgene past the distal end of the endotracheal tube. The concentration of phosgene in the carrier flow is adjusted by changing the rate of flow out of the bag.

The animal was attached to the exposure tube, via the endotracheal tube, and allowed to equilibrate for 10 min before being exposed to phosgene for 10 min. Carrier air was drawn through the exposure tube at a flow rate 50 $l \cdot min^{-1}$. The concentration of phosgene within the exposure tube was monitored continuously by using a Miran infrared gas analyzer. Phosgene concentrations were recorded every 30 sec and were integrated over time to calculate the Ct (the product of concentration [$mg \cdot m^{-3}$] $\times$ time [min]) delivered to the animal. After exposure the animal remained attached to the exposure apparatus for 30 min and monitoring continued.

7.5.2 Ventilation

Thirty minutes after exposure the level of anesthesia was deepened sufficiently to permit intermittent positive pressure ventilation (IPPV) using an Evita 2 ventilator. The ventilator strategy was 20 $breaths \cdot min^{-1}$, F_{IO_2} of 0.3 and a tidal volume of 10 $ml \cdot kg^{-1}$. No additional positive end-expiratory

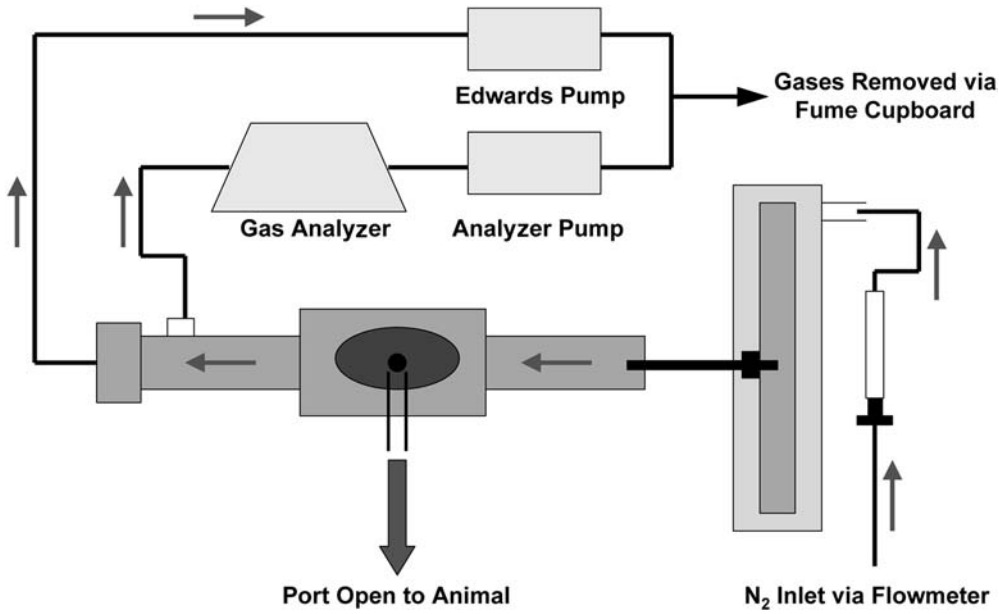


FIGURE 7.5 Schematic diagram of the apparatus used for phosgene exposure.

pressure (PEEP) was set on the ventilator. These settings remained constant for the duration of the experiment.

7.5.3 Physiological Measurements

Cardiac and pulmonary physiological parameters were recorded every 5 min for the first 30 min after exposure and then every 30 min thereafter throughout the course of the experiment (see Figure 7.4 and Table 7.1). Arterial and mixed venous blood samples were taken at regular intervals and immediately analyzed using a GEM blood-gas analyzer. Lung compliance was measured from the Evita 2 ventilator every 30 min during IPPV. Derived variables were calculated by using standard formulas (Edwards et al., 1993).

7.5.4 Postmortem and Histopathology

At the end of the 24-h monitoring period, or when the animal became moribund as evidenced by asystole and a mixed venous oxygen saturation of less than 15%, it was killed by an intravenous overdose of sodium pentabarbitone (200 $mg \cdot ml^{-1}$ Euthatal), and a full postmortem examination was carried out. After clamping of the trachea distal to the end of the endotracheal tube, the thorax was opened and the lungs and heart were removed following exsanguination by severing the major arteries. The lungs were then weighed for wet weight/body weight determination. Samples from each of the lobes, together with samples from other major organs, were taken, fixed in neutral buffered formalin, and processed for histopathological examination using routine methods.

7.5.5 Control Values and Statistical Analysis

Baseline values were used as controls during the first 0.5 h when the animal was spontaneously breathing (see Figure 7.4). When the animal was ventilated, the 1 h after exposure time point (first measurement under IPPV) was used as the "control" value (see Figure 7.4). Statistical analyses

TABLE 7.1 Physiological Measurements Recorded Throughout the Course of the Experiment

Measurement	Parameters
Vital signs	Core temperature
	Expired carbon dioxide
Cardiac physiology	Heart rate
	Mean arterial pressure
	Central venous pressure
	Mean pulmonary artery pressure
	Pulmonary artery wedge pressure
	Cardiac output
Pulmonary physiology (IPPV only)	Pulmonary resistance
	Pulmonary compliance
	Breathing rate
Blood chemistry (arterial and venous)	pH
	Po_2
	Pco_2
	Hco_3
	Tco_2
	Base excess
	Hematocrit
	O_2 saturation
	Mixed venous O_2 saturation
Derived cardiovascular variables	Systemic vascular resistance (indexed)
	Pulmonary vascular resistance (indexed)
	Stroke volume (indexed)
	Left and right ventricular stroke work (indexed)
Derived O_2 transport variables	Oxygen delivery and consumption
	Arterial O_2 content
	Mixed venous O_2 content
	Shunt fraction
	Oxygen extraction ratio

were made by two-factor (group and time) analysis of variance (ANOVA) followed by Duncan's test of significance between groups. Data were expressed as mean ($\pm$ SE) and p values < 0.05 were accepted as significant.

7.6 RESULTS

7.6.1 Preliminary Dose–Response Experiments

In these experiments animals were exposed to Cts of phosgene between 250 and 4000 mg·min·m^{-3}. The results may be summarized as follows:

- Animals not challenged with phosgene could be maintained in a stable condition for 24 h under anesthesia.

- At concentrations above Ct 500 mg·min·m^{-3} lung wet weight/body weight ratios increased in relation to the increase in Ct of phosgene.
- The pathology observed was similar to that observed in other animal species with areas of severe congestion, petechial hemorrhages, and consolidation. Histopathology showed areas of both interstitial and intra-alveolar edema and inflammatory cell infiltration.
- Animals exposed to a Ct of 2500 mg·min·m^{-3} or above and maintained either breathing spontaneously or supported by an assisted spontaneous breathing strategy died within 14 h of exposure.
- A Ct of 2500 mg·min·m^{-3} resulted in a reproducible lung injury and this dose was used in all future treatment studies.

7.6.2 The Effects of Exposure to a Ct of 2500 mg·min·m^{-3}

7.6.2.1 Survival

All control animals survived to the end of the 24-h monitoring period with stable hemodynamic and cardiovascular parameters. In the phosgene-exposed group three animals survived to 24 h, and the remainder (7) died or became moribund between 16.5 and 23 h (mean, 20 h).

7.6.2.2 Lung Wet Weight/Body Weight Ratio

Exposure to phosgene resulted in a significant increase in lung wet weight/body weight ratio (8.84 ± 1.03) when compared with controls (17.53 ± 3.35; $p < 0.005$) (Table 7.2).

7.6.2.3 Changes Observed either during or in the First 30 Min after Exposure

A transient but significant ($p < 0.05$) decrease in stroke volume index occurred immediately following the end of the phosgene exposure, which returned to normal by 20 min. A significant decrease in arterial oxygen saturation ($p < 0.05$) was recorded at 30 min, which returned to normal by 1 h after exposure. By this stage IPPV had been initiated (see Figure 7.4).

7.6.2.4 Changes Observed during Intermittent Positive Pressure Ventilation

Phosgene exposure resulted in a significant decrease in arterial pH from 6 h following exposure when compared with controls as well as decreases in PaO_2 (from 1 h postexposure) (Figures 7.6 and 7.7) and increases in $PaCO_2$ (from 12 h postexposure). Oxygen saturation was maintained at levels greater than 90% for up to 16 h and then fell gradually from 18 h onward to 67% in surviving

TABLE 7.2 Changes in Wet Weight/Body Weight Ratio in Animals Exposed to Phosgene (Ct 2500 mg·min·m^{-3}) and Ventilated with IPPV Ventilation

Group	Lung Wet Weight/Body Weight Ratio (± SD)
Control	8.84 ± 1.03
Phosgene conventional ventilation	17.53 ± 3.35**

** $p < 0.005$ when compared with control.

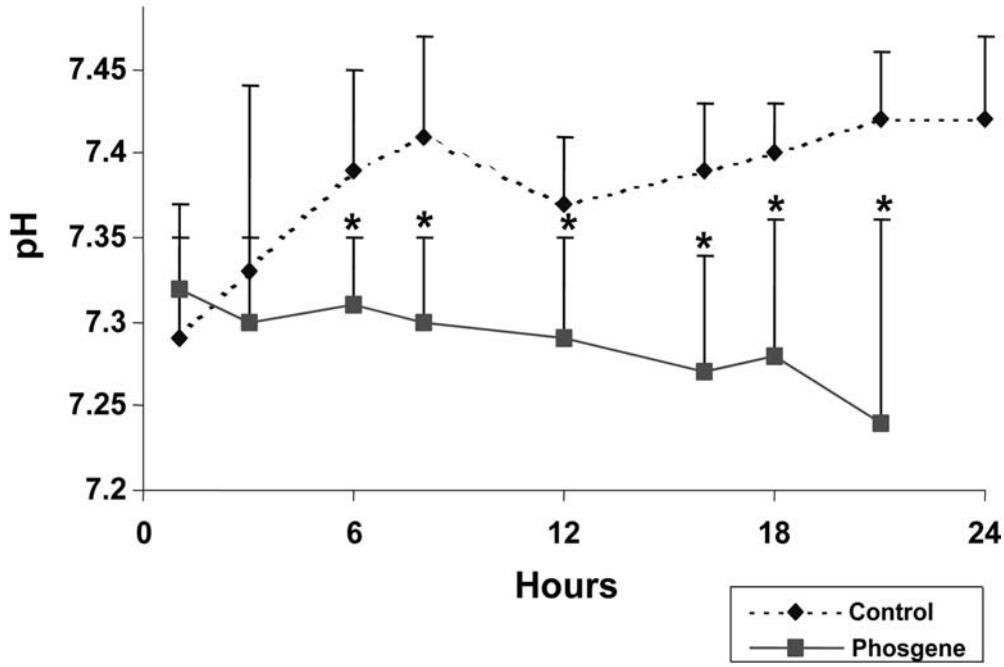


FIGURE 7.6 Changes in arterial pH after exposure to phosgene. * $p < 0.05$.

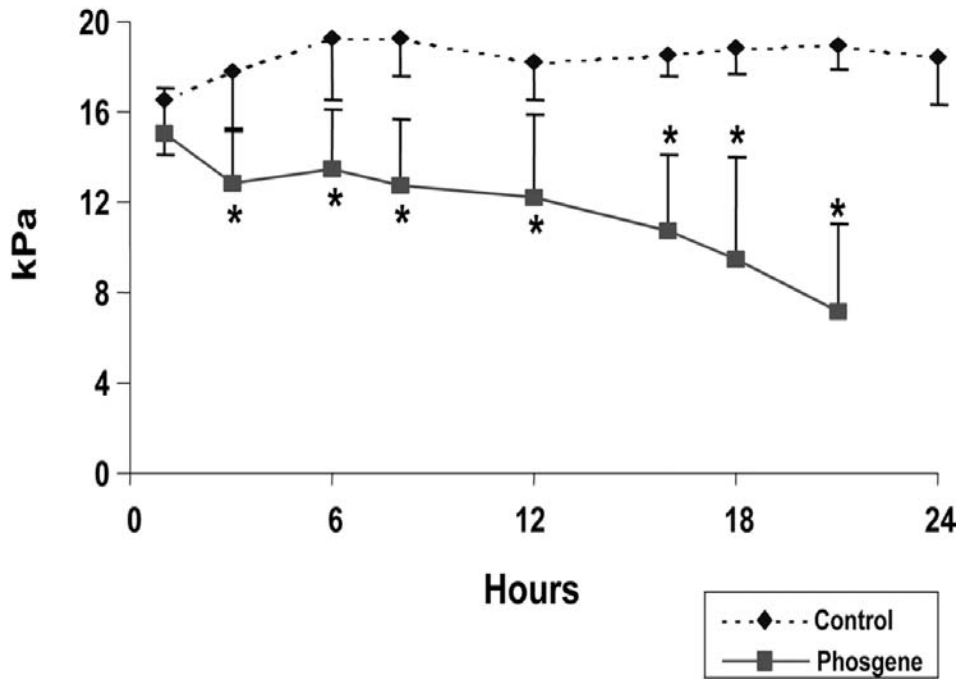


FIGURE 7.7 Changes in arterial PO_2 after exposure to phosgene. * $p < 0.05$.

animals at 21 h. No significant changes in mixed venous pH were observed. However, significant decreases in PvO_2 ($p = 0.04$) and saturation ($p = 0.01$) were noted from 12 h accompanied by an increase in $PvCO_2$. All other hemodynamic parameters remained unchanged.

7.6.2.5 Lung Compliance

In the phosgene-exposed group a significant ($p < 0.02$) reduction in compliance was noted from 6 h after exposure (Figure 7.8).

7.6.2.6 Circulatory Status

Pulmonary hypertension was noted with increased mean pulmonary artery pressure from 15 h post-phosgene exposure when compared with the 1 h control time point ($p = 0.04$) and the control group ($p < 0.001$ (Figure 7.9). Pulmonary vascular resistance index (PVRI) was elevated throughout the experimental period but reached statistical significance by 9 h postexposure (Figure 7.10). All other cardiovascular parameters, including cardiac index and systemic vascular resistance index (SVI), showed no significant changes.

7.6.2.7 Oxygen Transport Variables

Oxygen delivery was reduced in the phosgene-exposed group compared with the control group with significance ($p < 0.05$) at 21 h postexposure. In addition, a significant ($p < 0.05$) reduction occurred in oxygen consumption at this time. Arterial and mixed venous oxygen content also showed significant decreases both between control values and over time from 18 h postexposure ($p < 0.01$ and $p < 0.05$, respectively). Shunt fraction was significantly increased in the phosgene-exposed group ($p < 0.05$) both with time and when compared with control values (Table 7.3).

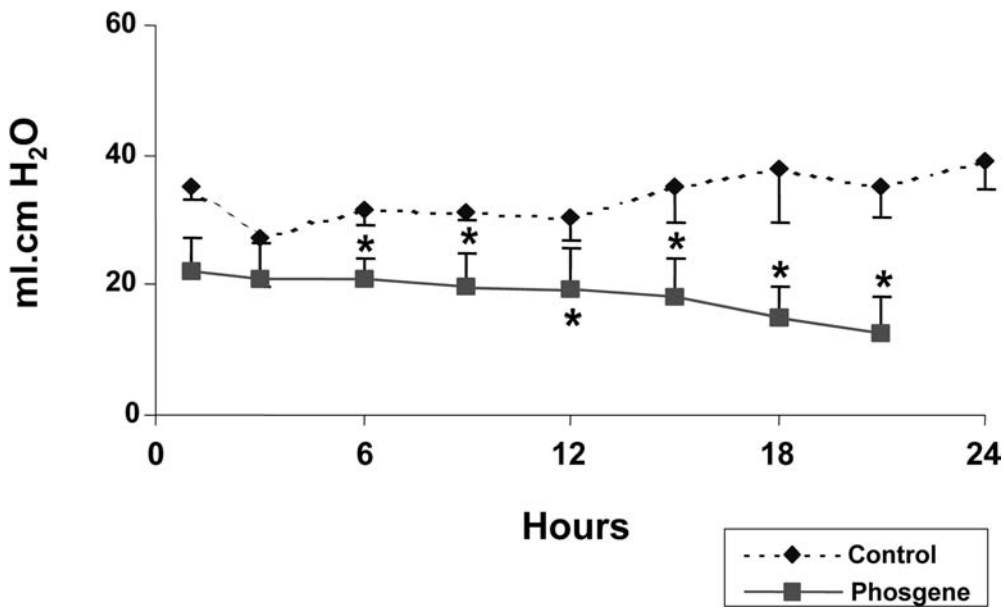


FIGURE 7.8 Changes in lung compliance after exposure to phosgene. * $p < 0.05$.

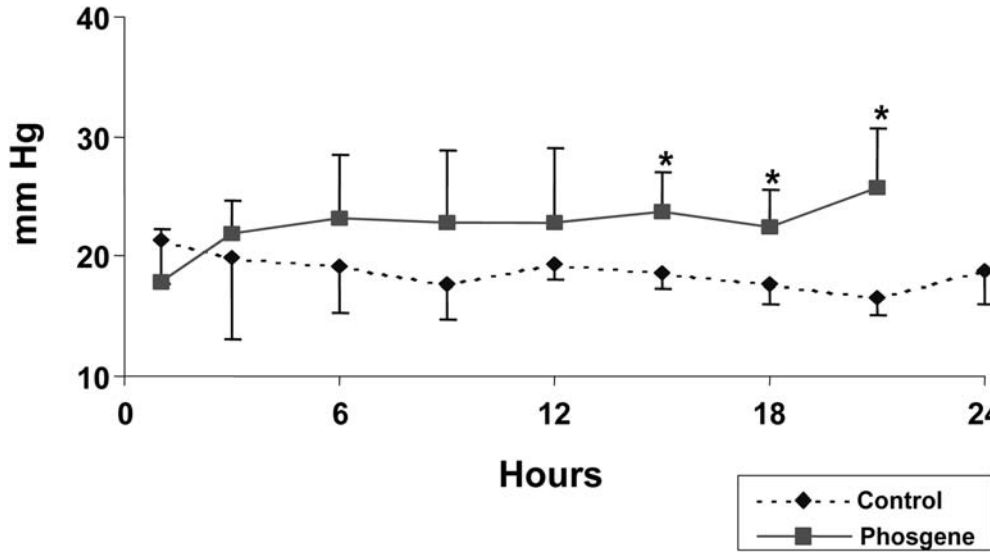


FIGURE 7.9 Changes in mean pulmonary artery pressure after exposure to phosgene. $*p < 0.05$.

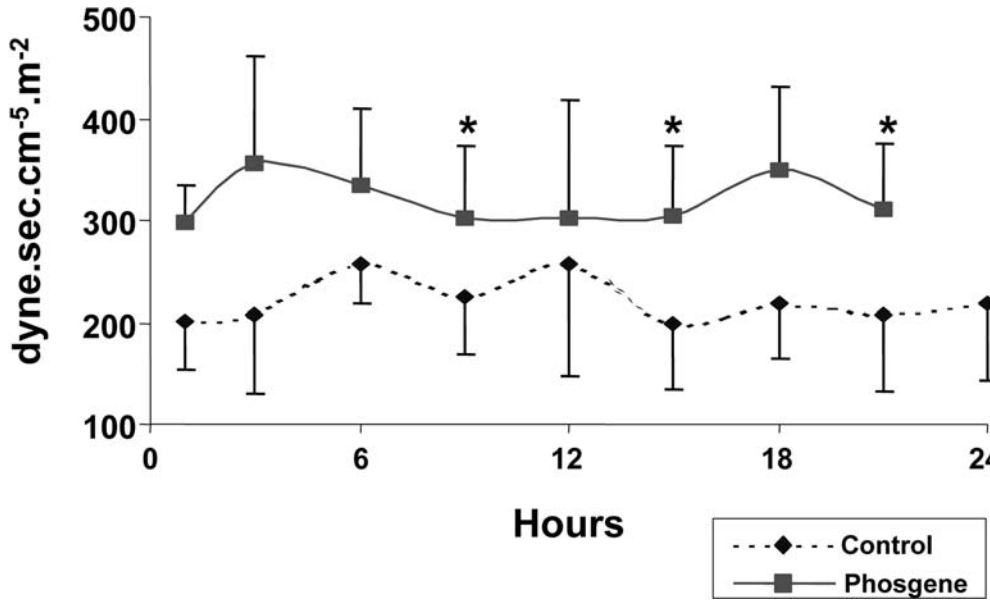


FIGURE 7.10 Changes in pulmonary vascular resistance (indexed) after exposure to phosgene. $*p < 0.05$.

7.6.2.8 Histopathology

In the decedents and the animals surviving 24 h the pattern of histopathological changes in the lungs were broadly similar to those reported in other animal species (Diller and Zante, 1982; Diller, 1985). There was moderate to severe interstitial, perivascular edema and congestion of the alveolar capillary network. Widespread alveolar flooding also occurred, in particular, in the dependent regions of the lung (Figures 7.11 and 7.12). The lower trachea and major bronchi showed severe focal epithelial necrosis

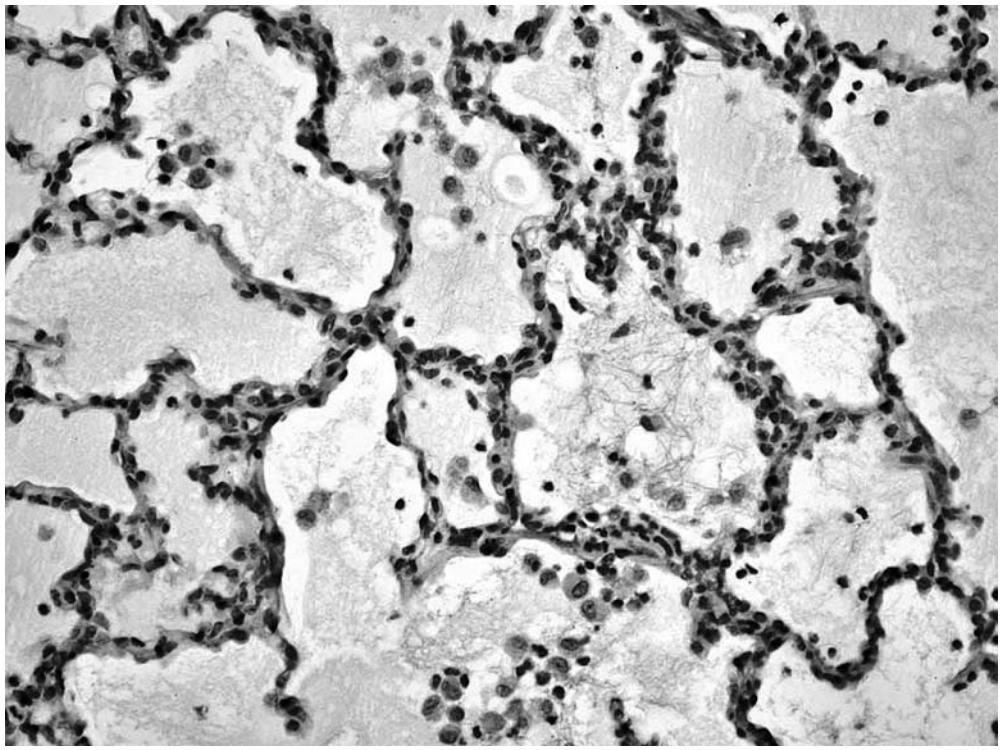


FIGURE 7.11 Lung micrograph from an animal exposed to phosgene (mean Ct 2500 mg·min·m^{-3}) and treated with a conventional ventilation strategy. There are extensive intra-alveolar edema and inflammatory cell infiltration (original magnification, ×250).

and widespread accumulation of proteinaceous fluid within the lumen. All other solid peripheral organs showed evidence of moderate to severe passive venous congestion.

Histopathology of the lungs from control animals showed mild atelectasis and minimal passive venous congestion, which was present mainly in the dorsal aspects of the caudal lobes. All other organs showed no significant abnormalities.

7.7 DISCUSSION

It is clear from the results of studies in large animals that a great deal of information can be gathered from individual animals if a large animal model is used. The ability to collect multiple physiological, biochemical, and pathological data from the same animal allows the construction of a very complete picture of the effects of an insult on the respiratory system. The results in pigs used as an illustration above have shown the effects of phosgene on the physiology of the lung in the form of compliance and resistance changes, on the vascular system as vascular resistance changes, and on lung pathology as measured by increased wet weight/body weight ratios and histological changes. This is made possible because the size of the airways and blood vessels allow easy intubation and multiple cannulations, and the body size permits multiple samples of blood and urine to be collected for analysis. Using large animals for inhalation research has some clear disadvantages compared with the use of small rodent species. The experiments are equipment and manpower intensive and they are difficult to carry out with as many replicates as are possible with smaller animals. These disadvantages are more than compensated for, however, by the ability to use equipment identical with that used clinically in humans and to obtain a large amount of information from each individual.

TABLE 7.3 Changes in Oxygen Transport Variables Observed in Anesthetized Large White Pigs Exposed to Phosgene (Ct 2500 mg·min·m^{-3}) and Ventilated with IPPV Ventilation

	Hours								
	1 (CG *n* = 5)	**3**	**6**	**9**	**12**	**15**	**18**	**21 (CG *n* = 3)**	**24 (CG *n* = 1)**
Oxygen consumption (ml·min^{-1}·m^{-2})	845 ± 405	545 ± 282	492 ± 240	502 ± 195	599 ± 268	456 ± 242	523 ± 222	439 ± 169	452
	484 ± 155	519 ± 138	425 ± 51	420 ± 56	388 ± 65	340 ± 86	275 ± 11	138 ± 42*	168.74
Oxygen delivery (ml·min^{-1}·m^{-2})	897 ± 345	567 ± 98	566 ± 75	565 ± 35	645 ± 123	536 ± 80	586 ± 118	512 ± 126	535
	642 ± 159	629 ± 125	536 ± 71	508 ± 18	581 ± 110	523 ± 28	553 ± 172	328 ± 44*	406
Arterial oxygen content (ml·dl^{-1})	13.6 ± 1.4	13 ± 0.9	12.4 ± 0.9	12.4 ± 0.8	12 ± 1	11.7 ± 0.6	11.8 ± 0.7	11.2 ± 0.3	11.2
	13.9 ± 1.7	13.3 ± 1.7	13.2 ± 1.2	12.9 ± 1	12.5 ± 1.4	11.8 ± 1.7	11.3 ± 1.6	8.3 ± 1.3**	10.8
Mixed venous oxygen content (ml·dl^{-1})	0.8 ± 0.13	0.79 ± 0.11	0.67 ± 0.12	0.7 ± 0.09	0.72 ± 0.03	0.69 ± 0.14	0.72 ± 0.08	0.67 ± 0.04	0.65
	0.75 ± 0.16	0.83 ± 0.09	0.81 ± 0.15	0.83 ± 0.1	0.68 ± 0.07	0.66 ± 0.16	0.51 ± 0.2**	0.4 ± 0.1**	0.4
Shunt fraction (%)	11.1 ± 4.09	6.48 ± 1.35	6.48 ± 1.34	5.87 ± 0.49	5.8 ± 0.29	5.92 ± 0.27	6.03 ± 0.26	6.26 ± 0.08	6.25
	7.51 ± 2.2	9.04 ± 3.4	8.3 ± 3.3	9.4 ± 4.5	14 ± 1.41*	19 ± 15.8	22.5 ± 16**	37.1 ± 26.7**	27.8

[a] Mean (± SD). For each variable the top line is the Control Data.

* $p < 0.05$; ** $p < 0.01$.

CG = phosgene

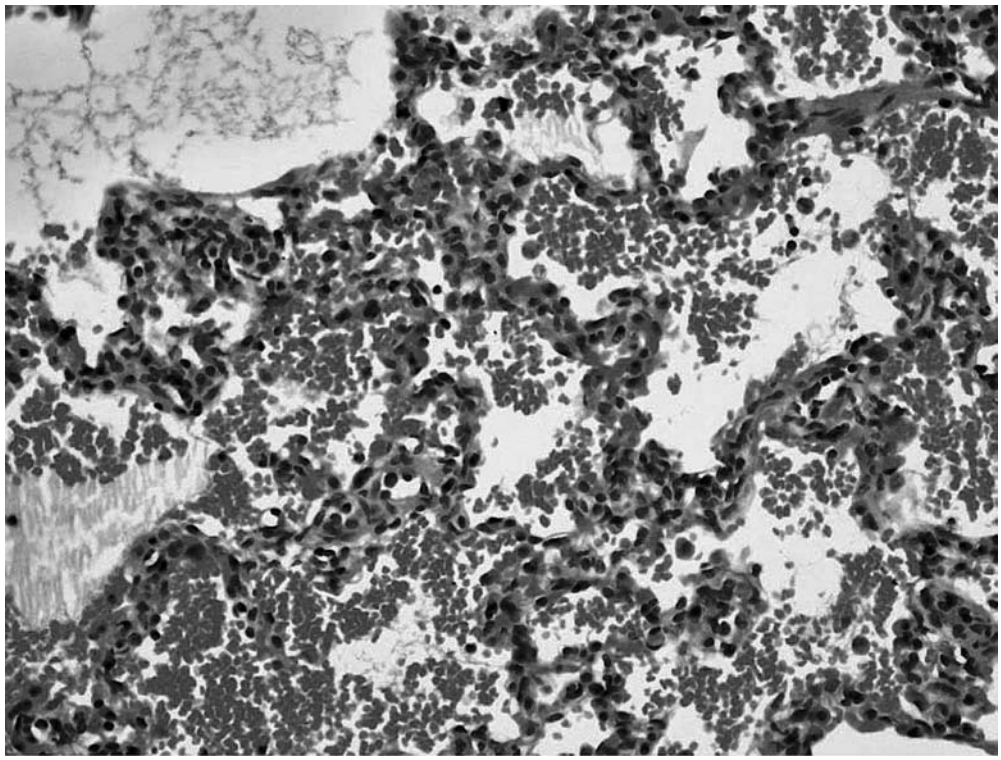


FIGURE 7.12 Lung micrograph from an animal exposed to phosgene (mean Ct 2500 mg·min·m^{-3}) and treated with a conventional ventilation strategy. There are extensive intra-alveolar hemorrhage, edema, and inflammatory cell infiltration (original magnification, ×250).

REFERENCES

Alpard, S.K., Zwischenberger, J.B., Tao, W., Deyo, D.J., Traber, D.L., and Bidani, A., New clinically relevant sheep model of severe respiratory failure secondary to combined smoke inhalation/cutaneous flame burn injury, *Crit. Care Med.*, 28, 1469–1476, 2000.

Boyland, E., McDonald, F.F., and Rumens, M.J., The variation in the toxicity of phosgene for small animals with the duration of exposure, *Br. J. Pharmacol*., 1, 81–89, 1946.

Brizio-Molteni, L., Loick, H.M., Traber, L.D., Traber, D.L., and Molteni, A., Plasma copper and iron changes in sheep after left lung inhalation injury: effect of the thromboxane antagonist BM 13.177 (Solutroban), *J. Burn Care Rehabil.,*16, 16–22, (1995).

Demling, R., Picard, L., Campbell, C., and Lalonde, C., Relationship of burn-induced lung lipid peroxidation on the degree of injury after smoke inhalation and a body burn, *Crit. Care Med.,* 21, 1935–1943, 1993.

Diller, W.F., Pathogenesis of phosgene poisoning, *Toxicol, Ind. Health*, 1, 7–15, 1985.

Diller, W.F. and Zante, R., Dose effect relationships for phosgene applications to humans and animals, *Zentralbl. Arbmed. Arbshutz. Prophyl. Ergon*., 32, 360–368, 1982.

Greeson, C.E. and Walker, W.J.G., Report on experiments carried out with goats at rest to determine the concentration of phosgene which with (1) two minutes exposure (2) a ten minutes exposure will produce a 50% death rate within a period of 72 hours, Porton Report 976, U.K., 1932.

Gunnarsson, M., Walther, S.M., Seidal, T., and Lennquist, S., Effects of inhalation of corticosteroids immediately after experimental chlorine gas lung injury, *J. Trauma,* 48, 101–107, 2000.

Janssens, S.P., Musto, S.W., Hutchison, W.G., Spence, C., Witten, M., Jung, W., and Hales, C.A., Cyclooxygenase and lipoxygenase inhibition by BW-755C reduces acrolein smoke-induced acute lung injury, *J.Appl. Physiol.,* 77, 888–895, 1994.

Karel, L. and Weston, R.E., A biological assay of inhaled substances by the dosimetric method. The retained median lethal dose and the respiratory response in unanesthetised normal goats exposed to different concentrations of phosgene, *J. Ind. Hyg. Toxicol*., 29, 23–28, 1947.

Keeler, J.R., Hurt., H.H., Nold, J.B., and Lennox, W.J., Estimation of the LCTSO of phosgene in sheep, *Drug Chem. Toxicol.,* 13, 229–239, 1990.

Keller, J.R., Hurt, H.H., Nold, J.B., Corcoran, K.D., and Tezak-Reid, T.M., Phosgene-induced injury in sheep, *Inhalation Toxicol*., 2, 391–406, 1990.

Lange, N.R., Kozlowski, J.K., Gust, R., Shapiro, S.D., and Schuster, D.P., Effect of partial liquid ventilation on pulmonary vascular permeability and edema after experimental acute lung injury, *Am. J Respir. Crit. Care Med*., 162, 271–277, 2000.

Parker, S.J., Hill, P.F., Brown, D., Kenward, C.E., and Watkins, P.E., A porcine model of sepsis resulting from the combined insults of hemorrhage and peritonitis, *Shock*, 13, 291–296, 2000.

Sakurai, H., Schmalstieg, F.C., Traber, L.D., Hawkins, H.K., and Traber, D.L., Role of L-selectin in physiological manifestations after burn and smoke inhalation injury in sheep, *J. Appl. Physiol.*, 86, 1151–1159, 1999.

Sison, C., Bry, K., Sephus, J., and Hallman, M., Effects of inhaled nitric oxide and surfactant treatment on lung function and pulmonary hemodynamics in bronchoalveolar-lavage-induced respiratory failure, *Pediatr. Pulmonol*., 29, 202–209, 2000.

Soejima, K., McGuire, R., Snyder, N., Uchida, T., Szabo, C., Salzman, A., Traber, L.D., and Traber, D.L., The effect of inducible nitric oxide synthase (iNOS) inhibition on smoke inhalation injury in sheep, *Shock*, 13, 261–266, 2000.

Staub, N.C., Bland, R.D., Brigham, K.L., Demling, R., Erdmann, A.J., III, and Woolverton, W.C., Preparation of chronic lung lymph fistulas in sheep, *J. Surg Res.,* 19, 315–320, 1975.

Tasaki, O., Dubick, M.A., Goodwin, C.W., and Pruitt, B.A., Effects of burns on inhalation injury in sheep: a 5-day study, *J. Trauma Inj. Infect. Crit. Care,* 52, 351–357, 2002.

Tasaki, O., Goodwin, C.W., Saitoh, D., Mozingo, D.W., Ishihara, S., Brinkley, W.W., Cioffi, W.G., Jr., and Pruitt, B.A., Jr., Effects of burns on inhalation injury, *J. Trauma*, 43, 603–607, 1997.

Traber, D.L., Linares, H.A., Herndon, D.N., and Prien, T., The pathophysiology of inhalation injury—a review, *Burns Including Thermal Inj*., 14, 357–364, 1988.

Weston, R.E. and Karel, L., An adaption of the dosimetric method for use in smaller animals. The retained median lethal dose and the respiratory response in normal, unanesthetized rhesus monkeys (Maccaca mulatta), *J. Ind. Hyg. Toxicol*., 29, 29–33, 1947.

8 Toxic Load Modeling

Douglas R. Sommerville, Kyong H. Park, Michael O. Kierzewski, Michael D. Dunkel, Matthew I. Hutton, and Nirmala A. Pinto

CONTENTS

8.1 INTRODUCTION

For each individual, there is a dose or dosage[1] that is just sufficient to produce a specified biological response. These just-sufficient dosages are called effective dosages to distinguish them from administered dosages. The statistical properties of this response distribution have been reviewed extensively (Finney, 1971, 1978; Neumann and Kimmel, 1998) and summarized (Salem, 1987; Crosier and Sommerville, 2002). One of the more important aspects of the effective dosages is that the distribution of effective dosages for a homogeneous population is usually lognormal. The severity and probability of effects experienced depend on several factors but primarily on the amount of toxic

[1] The terms dose and dosage are often used interchangeably, but they do have different definitions. Dose is the total amount of a substance that is administered, while dosage is an amount administered relative to some other quantity (e.g., body mass, body surface, and/or time). For IH and PC vapor exposures, dosage is the term used (from Salem, 1987).

material received. For inhalation (IH) and percutaneous (PC) vapor toxicology, when the just-sufficient dosage for an effect is relatively insensitive to exposure duration, the calculation of the probability of effect is straightforward (as explained in greater detail later in this chapter). However, when the effective vapor/aerosol IH or PC dosages are a function of exposure duration, a different approach is required. Instead of quantifying the amount of toxic material in terms of dosage, a new term, toxic load (TL), has been developed and extensively used to predict the probability and severity of the response (Fairhurst and Turner, 1993; Ride, 1995; Yee, 1996a, 1996b; Yee and Ye, 1996; Mannan, 2005). TL is normally expressed as some function of vapor concentration (C) and exposure duration (T), with TL equaling C^nT being a typical form. Unfortunately, the introduction of the toxic load to solve the problem of duration-dependent effective dosages has led to other complications and issues.

In this chapter, the application of the toxic load in hazard assessment modeling and the complications associated with its use are reviewed and discussed for acute IH and PC vapor exposures. Some of the key issues that are discussed include: (1) the disconnect between the exposure scenarios used in IH and PC vapor toxicology chamber studies and real-life exposure scenarios; (2) the best way to calculate the toxic load; (3) the determination and quantification of the influence of vapor concentration fluctuations on the toxicological effect of a particular TL; and (4) the sensitivity of hazard area predictions to how the TL is modeled and to changes in parameter values found in the TL model being used. For the latter, a sensitivity analysis was conducted for this chapter with TL values calculated based on actual data from outdoor field trials with tracer gas (Biltoft, 1997; Yee et al., 1998).

8.2 DOSE–RESPONSE STATISTICS AND THE TOXIC LOAD

The same statistical theory used for measures of dose and dosages (Finney, 1971) has also been adopted for use with toxic load (Withers and Lees, 1985; Griffiths, 1991; Fairhurst and Turner, 1993; Schubach, 1995, 1997; Yee, 1996a, 1996b; Ferguson and Hendershot, 2000). The following discussion has been largely extracted from a brief review by Crosier and Sommerville (2002) of the statistical theory behind dose–response. Where the substitution of TL for dosage is not straightforward it is specifically noted.

A plot of the density function, Φ, for the normal distribution of log(effective dosage), produces the well-known bell-shaped curve (Box et al., 1978; Hattis et al., 1999). The two parameters most often used to characterize a normal distribution are its mean μ and variance σ^2 (or standard deviation σ) (Hines and Montgomery,1990). By using these two parameters, every normally distributed random variable X can be standardized to a standard normal random variable Z:

$$Z = \frac{(X - \mu)}{\sigma} \tag{8.1}$$

In toxicology, X is usually the logarithm of the dosage.

Although statisticians typically describe the lognormal distribution of effective dosages by the mean and variance of log(effective dosage), toxicologists usually describe the distribution by the median effective dosage, ED_{50}, and the probit (or Bliss) slope, m:

$$ED_{50} = \text{antilog}(\eta) \tag{8.2}$$

$$m = 1/\sigma \tag{8.3}$$

where η is the median of log(effective dosage). The median effective dosage, $ED_{50,}$ is the dosage at which 50% of the exposed individuals will exhibit a specified biological response. For vapor exposures, the equivalent dosage term is the 50% effective concentration (EC_{50}) or the product of the exposure concentration and exposure time (ECT_{50}) (Salem, 1987). Median effective dosages are in the original units, not in logarithms of the original units, and hence are easier to interpret than μ. Although the mean μ and median η of a normal distribution are the same ($\mu = \eta$), this property does not hold for a lognormal distribution.

Effective dosages for response levels other than 50% can be calculated from μ and σ by solving for X in Equation (8.1) and using the Z value corresponding to the cumulative probability of interest. The 50% response level corresponds to a Z value of zero. Tables of cumulative probabilities and their corresponding Z values are found in standard statistical textbooks (Box et al., 1978; Hines and Montgomery, 1990) or obtained by using statistical software (MINITAB®, 2001).

Toxicologists traditionally use base 10 logarithms to calculate the probit slope (Bliss, 1934; Finney, 1971, 1978), and engineers often use natural logarithms (Griffiths, 1991; Schubach, 1995; Yee, 1996a, 1996b). Probit slopes based on both natural and base 10 logarithms are found in the literature. Care must be exercised when comparing probit slopes from different sources.

The probit slope equals the number of standard deviations (ΔZ) corresponding to a factor of either 10 or e (= 2.718 . .) change in effective dosage (ED) (Bakshi et al., 1997). Thus, a probit slope (base 10) of six means that a factor of 10 change in ED corresponds to six standard deviations ($\Delta Z = 6$). For the normal distribution, a range of Z from negative four (very sensitive individuals) to four (highly tolerant individuals) (or $\Delta Z = 8$) encompasses more than 99.99% of the total population. If the toxicant has a probit slope of eight, a factor of 10 separates the effective dosages for these two Z values. The higher the probit slope the closer the two tails of the distribution are in terms of ED (in other words, there is less variance in the effective dosages of the population).

Though the normal distribution is continuous, quantal data (response versus no response) are used to estimate the parameters (median and probit slope) of the distribution of effective dosages (Finney, 1971, 1978). Probit analysis and maximum likelihood estimation (MLE) are used to estimate these parameters from experimental data (Finney, 1971; Fairhurst and Turner, 1993; Fox, 1997). The following equation is fitted via probit analysis/MLE for vapor toxicity studies (Finney, 1971; Fairhurst and Turner, 1993; Yee, 1996a, 1996b):

$$Y_N = \left(Y_P - 5\right) = k_0 + k_1 \log C + k_2 \log T \tag{8.4}$$

where Y_N is a normit, Y_P is a probit, and the k values are fitted coefficients, C is vapor concentration, and T is exposure time. The constants k_1 and k_2 are the probit slopes for concentration and time, respectively. Experiments are often conducted with exposure time held constant, which reduces Equation (8.4) to the traditional probit equation (Finney, 1971). Thus, the probit slope for a vapor exposure usually refers to the slope on vapor concentration ($m = k_1$) instead of the slope on exposure duration. Some studies report a probit slope for the toxic load (ten Berge and van Heemst, 1983; Poblete and Lees, 1984; Withers and Lee, 1985; Marshall, 1989; Fairhurst and Turner, 1993; Franks et al., 1996). Thus, when comparing probit slope values from various studies, a common basis has to be used.

When fitting Equation (8.4), all variability in the data will contribute to the estimate for m, be it from variance due to individual susceptibilities, batch effects, experimental error, etc. Probit analysis performed on a compilation of data from many sources will not produce an accurate measure of variance among individuals because of the heterogeneity introduced by differences among the studies (e.g., experiment procedures, type of animals used, etc.) (Franks et al., 1996). The effect of such heterogeneity will be to lower the probit slope. Also, using a compilation of data from many sources can complicate the accurate estimation of the toxic load exponent (Fairhurst and Turner, 1993).

When TL is being used as the measure of toxicant amount received by an individual, Equation (8.4) is usually written in the following form:

$$Y_N = a + b \log TL \tag{8.5}$$

with b being the probit slope on a TL basis.

It should be recognized that the TL relationship (in Equation [8.5]) is based more on empirical observations than on basic biological theories (Griffiths, 1991; Fairhurst and Turner, 1993; Yee, 1996a, 1996b). As a result, Equation (8.5) needs to be derived empirically on an individual toxicant basis from acute toxicity experiments where both vapor concentration and exposure duration are varied (ten Berge et al., 1986). However, the value for the TL exponent can offer some insight into

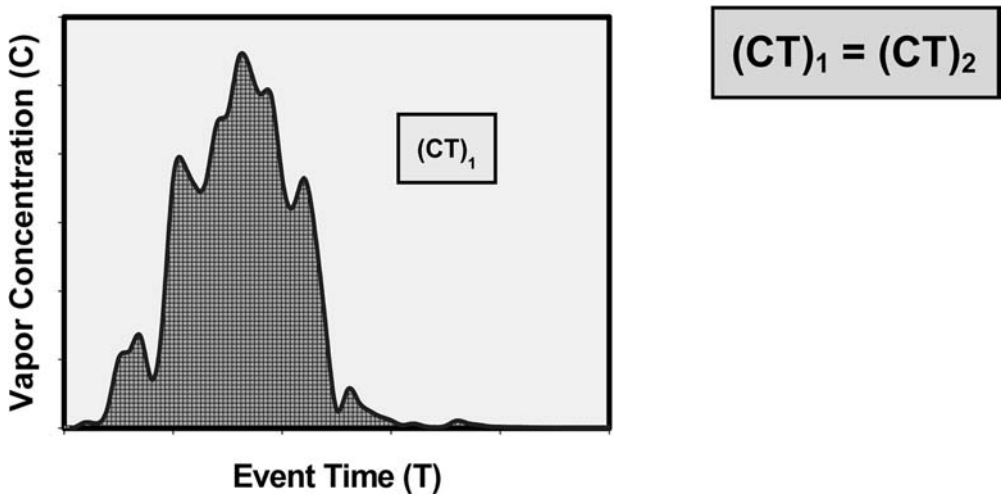


FIGURE 8.1a Example of real-world vapor concentration–time profile.

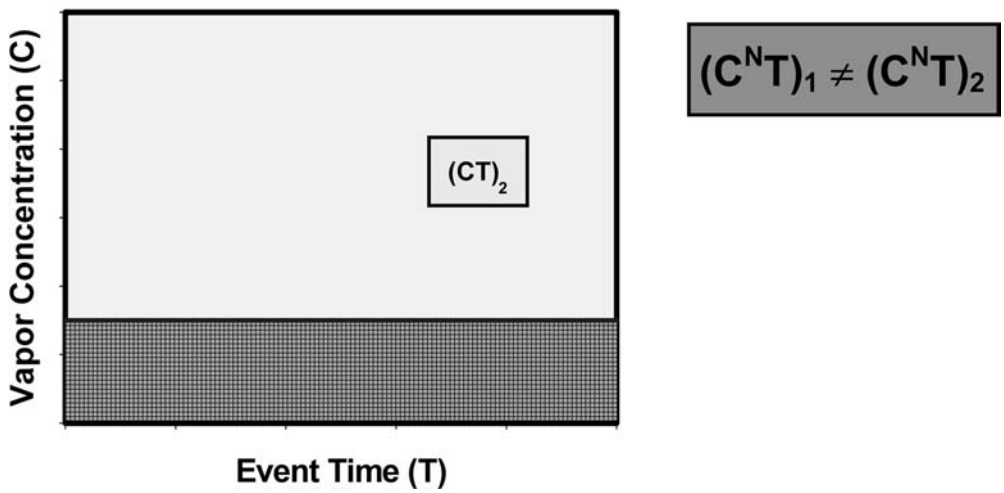


FIGURE 8.1b Example of idealized vapor concentration–time profile in animal exposure chamber.

the influence of underlying toxicological mechanisms (i.e., metabolic detoxification) involved in a particular toxicant exposure (Griffiths, 1991). For instance, it can be argued that if $n > 1$ for a particular chemical exposure, then significant detoxification may be occurring, because in this situation the required effective dosage is increasing as the dosage is spread over a longer exposure duration. Slowing the rate of dosage administration gives any existing detoxification mechanism a chance to function, thereby causing an increase in the ECT_{50} as a function of time.

The substitution of TL for dosage into the dose–response equation produces a paradox. If TL is defined as C^nT (more on this in the next section), then Equation (8.5) can be written in the following algebraically equivalent forms (which are also equivalent to Equation [8.4]):

$$Y_N = k_0 + k_2 \log(C^{k_1/k_2}T) = k_0 + k_2 \log(TL_1) \tag{8.6}$$

$$Y_N = k_0 + k_1 \log(CT^{k_2/k_1}) = k_0 + k_1 \log(TL_2) \tag{8.7}$$

where TL_1 and TL_2 are TL parameters 1 and 2. Equations (8.4), (8.6), and (8.7) will only produce identical probability estimates for a given exposure scenario when there are no concentration fluctuations involved or if the toxic load is calculated in such a manner as to ignore the presence or toxic impact of any such fluctuations (see Section 8.5 for additional discussion on this issue) (Yee, 1999, 2001). At present, which of these three equations should be used for fluctuating concentrations profiles cannot be answered via probit analysis because of the absence of dose–population response curve data taken under such conditions. Virtually all the existing acute IH toxicity testing has involved constant vapor concentration exposures over fixed periods (Silver, 1946; MacFarland, 1987; Yee and Ye, 1996). The same situation also exists for PC vapor toxicity testing as well.[2]

For actual exposures, the concentration–time profiles are almost always variable over time. The profiles will involve fluctuations and may include both concentration peaks much higher than the average concentration and periods of zero concentration. The TL may depend on the profile, in addition to the total exposure (defined as the average exposure concentration multiplied by the exposure time). This is illustrated in Figure 8.1a and b. The total area under the curve in both figures is equal, but the differing concentration–time histories will give two different TL values (assuming a non-Haber Law chemical; see Section 8.3 for further discussion).

Saltzman and Fox (1986) did examine the influence of fluctuating concentration–time profiles on acute IH toxicity (conducted on a dose–response severity curve basis). However, only a small number of animals were used (not enough necessary for fitting Equation [8.4]), because the goal was to investigate the pharmacokinetics of toxicant absorption rather than to fit a probit-type equation. Yet, kinetic-type studies may still be useful in providing guidance on the proper probit form (Equations [8.4], [8.6], or [8.7]) for correlating the TL.

For the IH toxicokinetics of chemical warfare (CW) organophosphorus (OP) nerve agents (Benschop and DeJong, 2001; Jakubowski et al., 2003), the evidence suggests that for sarin and soman the rate of respiratory agent absorption depends on the agent vapor concentration in the air rather than on the exposure duration. Thus, at least for OP agents, the appropriate approach appears to be the use of Equation (8.6), although this should be confirmed via collection of dose–population response curve data.

8.3 TOXIC LOAD MODELS

A general expression for the TL received during exposure to an airborne chemical agent, as a function of time, is:

$$TL = \int_0^T \left[C(t)\right]^N dt \tag{8.8}$$

where:

$C(t)$ = the instantaneous agent concentration as a function of time (mg/m^3).
T = the exposure duration (minutes).
N = the TL exponent (dimensionless).

8.3.1 Haber Model

If C is assumed constant with respect to exposure time and N is set equal to 1.0, Equation (8.8) reduces to:

$$\text{TL} = CT \tag{8.9}$$

[2] Inhalation studies often involve whole-body exposures. Simply providing respiratory protection for the test subjects in a whole-body exposure eliminates inhalation as a route of exposure, thereby producing a PC vapor toxicity experiment. Head-only inhalation exposures (described by MacFarland, 1987) are easily converted to body-only or body-part (i.e., arm) PC vapor exposures. Essentially all the PC vapor studies reviewed by Bakshi et al. (1997) were conducted in this manner.

Equation (8.9) indicates that if the concentration is doubled for one-half the exposure time, the toxic load is unchanged. Following this formulation, toxic hazard models (until recently) have assumed that IH hazard toxicity levels were constant with respect to the product of C and T. This may be represented as:

$$\mathrm{ECT}_{50} = K \tag{8.10}$$

where:

ECT_{XX} = the product of C and T that will produce an effect to XX percent of those exposed (mg–min/m^3).
K = a constant (mg–min/m^3).

Equation (8.10) is also known as Haber's Law (Haber, 1924). When the total exposure is numerically integrated by using an incremental time interval, Haber's Law becomes:

$$TL = \sum_{j=1}^{p} C_{\tau}\tau \tag{8.11}$$

where:

TL = the toxic load (mg–min/m^3).
C_{τ} = the mean concentration over interval τ (mg/m^3).
τ = the integration interval (minutes).
p = the number of integration intervals (dimensionless).

By using the Haber model, the probability of a given percent response, such as 50% lethality, is independent of time and depends solely on the person's total dosage (or TL). For chemicals like phosgene, Haber's Law has been found to be an appropriate approach (Sellers, 1993). However, other toxicity testing of some animal species has clearly shown that the probability of a given response depends on the exposure time and the total dosage (see also Section 8.4) (Cresthull et al., 1957; ten Berge and van Heemst, 1983; ten Berge et al., 1986; Yee, 1996a, 1996b; Mioduszewski et al., 2002a, 2002b; Anthony et al., 2004).

From a computational viewpoint, using Haber's Law to model the time dependence of toxicity is far easier to implement in atmospheric transport and dispersion models than a TL model (Yee, 2001). However, overdependence on Haber's Law for the sake of ease of use can lead to problems involving both over- and underestimating of toxicity, as shown in Figure 8.2 (adapted from Sommerville, 2003) for a chemical with $N > 1$. For short exposure durations, Haber's Law (red line) will underestimate the toxicity compared with the TL model (black line), and for longer exposure durations, the toxicity will be overestimated. The opposite will be true for chemicals with $N < 1$.

8.3.2 ten Berge Model

In recognition of the possible time dependence of toxicity, ten Berge and van Heemst (1983) proposed an alternate as given in Equation (8.8). In the numerical integration format used above for Haber's Law, the ten Berge equation becomes:

$$TL = \sum_{j=1}^{p} C_{\tau}^{N}\tau \tag{8.12}$$

Here, N = the dimensionless toxic load exponent and all other variables are as defined in Section 8.3.1.

When $N = 1$, the model reduces to the Haber Model. Use of the ten Berge TL model has been shown to give better fits to animal test data that have a time dependence of the probability of response (ten Berge et al., 1986). However, some recent studies (Mioduszewski et al., 2002a, 2002b; Whalley et al., 2004; and Anthony et al.; 2004) have found that the increase in TL for a given probability of

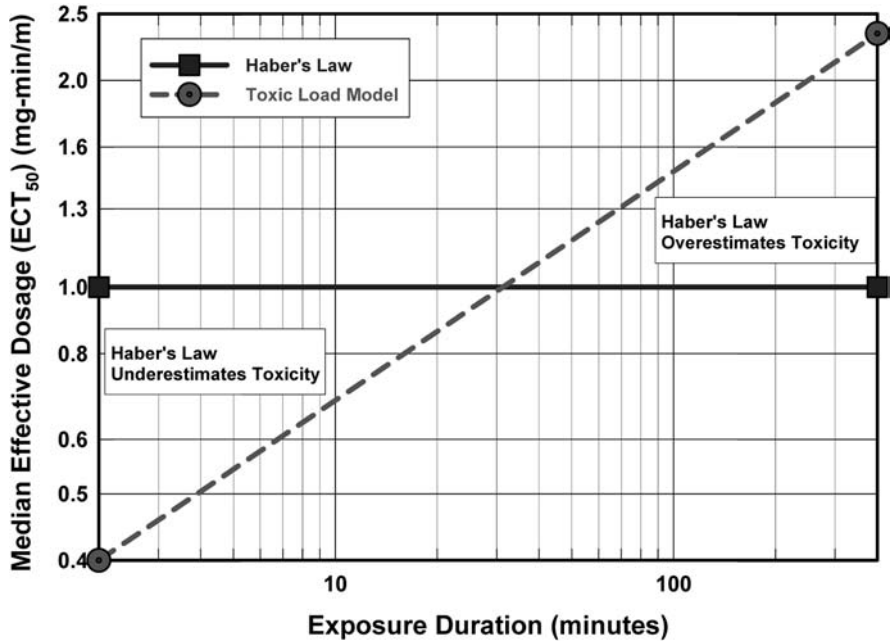


FIGURE 8.2. Sample comparison of Haber's Law ($N = 1$) with TL model (for chemicals with $N > 1$) for extrapolation of time dependence of chemical toxicity (adapted from Sommerville, 2003).

response is nonlinear (on a log–log scale), as opposed to the linear response predicted by the ten Berge model.

8.3.3 Griffiths and Megson Model

Griffiths and Megson (1984) proposed a TL model based on concerns with concentration fluctuations and with periods of zero concentrations (also discussed in Griffiths, 1991; Griffiths and Harper, 1985). They proposed a modification to the ten Berge model as follows:

$$TL = \left[\frac{1}{1-\left(\frac{T-t}{T}\right)}\right]^{N-1} C_T^N T \tag{8.13}$$

where:

TL = the toxic load (mg–min/m^3).
C_T = the mean concentration over interval T (mg/m^3).
T = the total exposure time (minutes).
N = the TL exponent (dimensionless).
t = the total exposure time with nonzero concentrations (minutes).

Because no known toxicity tests using nonconstant concentration challenges have been performed, no validation of this method is possible at the present time.

8.3.4 Ride Model

Ride (1984, 1995), also concerned with the impact of concentration fluctuations, proposed a different approach for addressing fluctuations, using a factor he called "fluctuation intensity" (I). I is based on the statistical standard deviation of the concentrations within a numerical integration time step. Ride first finds the overall mean concentration of the fluctuating concentration time history and presents a general equation for toxic load:

$$C_T = \frac{\sum_{i=1}^{n} C_i(t)}{n} \tag{8.14}$$

$$TL = \int_{t=o}^{t=T} \left[C(t)\right]^N dt \tag{8.15}$$

where:

C_T = the average concentration for the entire exposure (mg/m^3).
$C_i(t)$ = the individual concentration measurements (mg/m^3).
n = the number of measurements (dimensionless).
TL = the toxic load (mg/m^3)^N-min.
T = the total exposure time (minutes).
t = time (minutes).
$C(t)$ = the instantaneous concentration as a function of time (mg/m^3).
N = the TL exponent (dimensionless).

Ride next suggested a normalization factor to eliminate dimensional problems that occur when $N \neq 1$. He proposed that TL be divided by $C_T^N T$, to arrive at the toxic load ratio (TLR). Utilizing Equation (8.16) as a general expression for TLR and defining the fluctuation intensity as per Equation (8.17), Ride develops the alternate TLR expression for one time interval (τ), as given in Equation (8.18):

$$TLR = \frac{\int_{t=o}^{t=T} \left[C(t)\right]^N dt}{C_T^N T} \tag{8.16}$$

$$I_\tau = \frac{\sigma_\tau}{C_T} \tag{8.17}$$

$$TLR = \left[kI_\tau^\lambda + 1\right]^{N-1} \tag{8.18}$$

where:

TLR = toxic load ratio (dimensionless).
I_τ = the fluctuation intensity over the time step τ (dimensionless).
σ_τ = the standard deviation of the concentration over the time step τ (mg/m^3).
τ = an integration interval (minutes).
k = constant (dimensionless).
λ = constant (dimensionless).

Equation (8.18) presents several practical problems in implementation, and Ride (1995) shows that the expressions in Equation (8.10) are simpler to manipulate and produce equally good results. All variables used in Equation (8.19) are as defined above.

$$TLR = \left(N-1\right)^2 I^N + 1$$
$$TL = \left[\left(N-1\right)^2 I^N + 1\right] C_T^N T \tag{8.19}$$

Ride has performed calculations to show the potential impact of his model by comparing results of his model with the results of the Haber model for several agents (in Ride, 1984). He concluded that if his model is correct, current estimates using the Haber Model seriously underestimate toxic effects and the size of the hazard area. Validation of this model again suffers from the lack of available nonconstant challenge toxicity testing. A final note is that Ride mentions $C_{\max,}$ the maximum average interval concentration from among all the integration intervals, as a possible replacement for σ_τ in the fluctuation intensity factor.

8.3.5 Yee Model

Yee and his associates had extensively investigated the statistical properties of dispersing clouds/plumes by using data from numerous full-scale field experiments (taken as part of a cooperative Concentration Fluctuation Experiments [CONFLUX] project) (Yee et al., 1993a, 1993b, 1994a, 1994b, 1995a, 1996; Yee and Chan, 1995). In particular, one goal was to determine how to best model the probability density function (PDF) of the plume (random) concentration profile. Knowledge of the plume concentration PDF gained from the CONFLUX trials was used subsequently (Yee, 1996a, 1996b, 1999; Yee and Ye, 1996) to explicitly model the statistical characteristics of the TL and the resulting probability of effect (as represented by Y_N; see Equations [8.4] and [8.5]) on the exposed individual. PDFs were calculated from 1107 individual CONFLUX concentration-time series involving the continuous and controlled release of a tracer gas into the atmosphere (Yee, 1996a, 1999). These time-series were selected from a larger data set to reflect a wide range of atmospheric conditions and release conditions. The CONFLUX plume concentration PDFs are far more realistic than those used by earlier investigators (Griffiths and Megson, 1984; Ride, 1984) in their work on the effects of concentration fluctuations on the TL (Yee, 1996a, 2001).

Thus, the Yee Model explicitly recognizes that for realistic exposure scenarios Y_N will depend on the combination of two separate random variables: (1) the probability of experiencing a particular toxic response to the received TL and (2) the probability of an individual receiving some TL amount from a toxicant exposure. Some form of the probit relation can be used for describing the former (see Section 8.2), and the latter can be characterized with the appropriate PDF. A detailed discussion of concentration fluctuation PDFs and related statistical properties is beyond the scope of this chapter. However, the reader is referred to Wilson (1995), who has written an introductory book on the subject.

In the initial development of the Yee Model (Yee, 1996a), the TL was defined per Equation (8.15), and a TLR similar to Ride's (see Equation [8.16]) was also defined:

$$TLR = \frac{\int_{t=o}^{t=T} \left[C\left(t\right)\right]^N dt}{C_T^N T} = \left(\frac{1}{T}\right) \int_{t=o}^{t=T} \left(\frac{C\left(t\right)}{C_T}\right)^N dt \tag{8.20}$$

All variables used in Equation (8.20) are as defined previously. The Yee Model views the TLR as a measurement of the impact on toxicity due to the presence of concentration fluctuations. TLR values greater than one suggest that the toxic effect is being enhanced by the presence of concentration fluctuations. The toxic impact may be seriously underestimated as a result of not properly accounting for this possible enhancement effect.

Furthermore, Yee (1996a) and Yee and Ye (1996) took the additional step of defining the TL and TLR in Equation (8.20) in terms of the ensemble-mean concentration, a commonly used parameter in transport and dispersion modeling (Wilson, 1995;DoD, 2002; NRC, 2003):

$$\langle TLR \rangle \equiv \left(\frac{1}{T}\right) \int_{t=0}^{t=T} \left\langle \left(\frac{C(t)}{C_T}\right)^N \right\rangle dt \tag{8.21}$$

where < > denote ensemble-mean properties.

Yee (1996a) proposed that for hazard assessment purposes that <TL> or <TLR> be used for determination of the degree of injury. Validation of this model again suffers from the lack of available nonconstant challenge toxicity testing.

8.3.6 Other Toxic Load Models

A model using a totally different approach was proposed by Rozman and Doull (2000). An attempt is made to formulate a toxic load model based on pseudobiophysical relationships.

$$\text{Toxicity} = f(\text{exposure, toxic-kinetics, toxic-dynamics}) \tag{8.22}$$

where:

Toxic-kinetics = *f* (absorption, elimination)
Elimination = *f* (excretion, distribution, biotransformation)
Absorption = *f* (site, mechanism)
Toxic-dynamics = *f* (injury, recovery)
Recovery = *f* (repair, reversibility, adaptation)
Injury = *f* (site, mechanism)

This discussion is interesting but it does not propose any specific forms or parameters for the functional relationships.

Hilderman et al. (1999) propose a three-parameter numerical integration function for calculating the toxic load where the three parameters have some similarity to the functional relationships proposed by Rozman and Doull (2000). The three parameters are:

- Uptake time constant — The parameter for a first order relationship between the exposure concentration and effective concentration.
- Recovery time constant — The parameter for a first order relationship for the reduction in effective concentration due to recovery mechanisms.
- Saturation concentration — Maximum concentration that can be absorbed based on Michaelis–Menten enzyme reaction kinetics relationship.

A fairly good mapping can be made of the TL relationship between the Hilderman et al. (1999) functions and the Rozman and Doull (2000) functional relationships. Although no values or any basis for values is proposed in the report, first estimates for such a model could probably be made based on toxicity-testing results.

8.4 TYPICAL TOXIC LOAD PARAMETER VALUES

The parameter values used for any toxic load model will depend on the route of entry (e.g., IH, PC, ingestion), species (e.g., rat, mouse, human), and possibly other factors. Other factors that have been

investigated or discussed are sex, age, and health. All or most of these factors are controlled, or at least recorded, in toxicity tests. Toxicity data have most often been collected in non-human species, even though the species of interest is usually human.

As part of the sensitivity study of TL predictions to parameter values (see Section 8,5), it was decided that the ten Berge methodology (Section 8.3.2), normalized as per Ride (Section 8.3.4), would be pursued for the initial sensitivity-modeling efforts. This effort required information into the practical ranges of values for the TL exponent, N, and the integration time step, τ. Also, practical measures for fluctuation intensity, I, proposed by Ride (1984, 1995), were investigated.

8.4.1 ten Berge Toxic Load Exponent, *N*

In addition to ten Berge, others have experimentally derived values for N for various combinations of chemicals, species, and toxicological end points (Cresthull et al., 1957; Eisenberg et al., 1975; Perry and Articola, 1980; ten Berge and van Heemst, 1983 Yee, 1996b; Arts et al., 2000; Mioduszewski et al., 2002a, 2002b; Anthony et al., 2004; Whalley et al., 2004 Benton et al., 2005; Hulet et al., 2005a, 2005b, 2005c). Values for N are documented in several sources (Harris and Moses, 1983; Ride, 1984; Schubach, 1997; Duijm et al., 2000; Ferguson and Hendershot, 2000; Mannan, 2005), with most of the reported values falling in the range of 0.7 to 3.7, though a value of 8.3 was reported for hydrogen sulfide (H_2S) (Arts et al., 2000, Duijm et al., 2000). Mannan (2005) reported that, for acute inhalation of irritant gases, N tends to be greater than unity and is often of the order of two in value.

8.4.2 Integration Time Step, τ

Concentration time history data have been collected in many past field trial tests and in some recent tests with very fine time steps (fractions of a second) (Biltoft, 1997). In making TL calculations from such histories, it has been noted that the choice of an integration time step can greatly impact the calculated TL value (Saltzman and Fox, 1986; Ride, 1995; Saltzman, 1996; Yee and Ye, 1996; Yee, 1996a, 1996b, 1999; Hilderman et al., 1999; Duijm et al., 2000). If a very short time step (e.g., 0.02 sec) is used as the integration time step, very high transient peaks will produce a high toxic load value (for chemicals with $N > 1$). If a longer time step is chosen (e.g., on the order of tens of seconds), these transient high concentration values will be averaged out over the time step and the resulting TL value will be much lower than would result from use of a shorter time step. For chemicals with $N < 1$, the reverse of the above is true. In general, the calculated TL value very much depends on the choice of the integration time step.

For IH exposures, it has been pointed out that there are at least two natural physiologically based concentration-averaging processes to be considered in choosing τ. First, Ride (1995) discusses the respiratory process as a natural averaging process. A finite time of respiration averages out the inhaled vapor concentrations. This time step depends on the respiration rate. If one is at rest, breaths are longer and less frequent than when one is working hard. Complicating the process even further, an exhalation follows each inhalation in a breathing cycle. Ride suggests that τ should be, at best, a few seconds. Yee (1996a), using the same logic as Ride, reached about the same conclusion. Duijm et al. (2000) conclude that the relevant timescale for respiratory absorption is from 5 to 10 sec.

Second, Saltzman (1996) suggests another physiological factor, the biological half-life of a toxicant, which is the time required for half the quantity of the toxicant to be metabolized or eliminated by normal biological processes. He concludes that any time step less than one quarter of the biological half-life is adequate. He cites biological half-lives over a number of pollutants that range from about 10 min to 4 months. The longer times are associated with poisoning from metals, such as lead, and the shortest times are associated with systemic poisons and with highly toxic commercial chemicals. The highly potent, fast acting CW nerve agents have biological half-lives via the IH route on the order of 10 to 60 min (Benschop and DeJong, 2001). Thus, the Saltzman approach would set a maximum τ of less than 2.5 to 15 min for CW nerve agents.

A drawback to the Saltzman approach is the limited amount of experimental data (only one study, Saltzman and Fox, 1986) associated with its development. Saltzman and Fox (1986) investigated just one chemical–species combination, carbon monoxide exposure in rabbits. Their measured values for both the biological and absorption half-lives of carbon monoxide in rabbits are roughly equal, which does not address the issue of how differing reaction rates between the absorption and elimination processes will possibly affect the Saltzman model. For the CW nerve agents, the respiratory absorption process is so rapid that their absorption half-lives are practically zero in value (Benshop and DeJong, 2001). As a result, the absorption process will be very sensitive to concentration fluctuations. Hilderman et al. (1999) argue that the relative contributions of three processes (uptake, recovery, and saturation) instead of just one process (recovery) need to be considered.

The main research emphasis with respect to TL modeling has been on IH exposures. Other routes of vapor exposures (PC and ocular) have been neglected in comparison. This is probably, in part, because toxicants will elicit a greater effect and more rapid response when introduced via inhalation in contrast to the slower and less effective PC (dermal) route of exposure (Andrews and Snyder, 1991; Klaassen and Eaton, 1991). For instance, Riihimaki and Pfaffli (1978) have reported that for the simultaneous IH and PC vapor absorption of several individual volatile organic chemicals (VOCs) only 1 to 2% of the total amount absorbed came via the PC route (or, in other words, the inhalation rate is about 50 to 100 times more rapid than the PC rate). This suggests that the integration time step (τ) should be larger for PC exposures (perhaps on the order of minutes, instead of seconds, as previously recommended for inhalation). The exact time-step value will probably be toxicant and exposure scenario specific, with the more rapidly absorbed toxicants (via the PC route) requiring a smaller value.

8.4.3 Fluctuation Intensity

Concentrations will fluctuate over the total exposure time in actual chemical exposures. The modeling of the complex atmospheric physics of concentration fluctuations have been extensively reviewed and discussed. Yee (1999) summarizes the state-of-the art succinctly as "... the most advanced atmospheric pollutant dispersion models have difficulty in predicting even the two lowest order concentration moments with sufficient accuracy. In fact, in air-pollution studies, it is seldom that characteristics other than the mean concentration are considered."

The model calculations of Griffiths and Megson (1984) and Ride (1995) include factors to compensate for concentration fluctuation. Transport and diffusion models can generate a history of mean concentrations, calculated over fixed integration intervals, at some point. The Griffith model uses the total time of nonzero concentrations as the basic measure in its fluctuations correction factor. This is easily calculated from the concentration history prediction. The Ride model uses σ_τ in its correction factor development. However, Ride also suggests C_{max} as an alternate measure that can be used. σ_τ is not generally available from transport and diffusion models, and it cannot be calculated from the concentration history predictions of the models. On the other hand, C_{max} can be calculated from concentration histories. Therefore, C_{max}/C_T was chosen as the measure for concentration fluctuation in the sensitivity study conducted for this chapter (see Section 8.5). Hanna et al. (1982) states that C_{max}/C_T values can range from 1 to 50 with real data and that C_{max}/C_T decreases with distance.

Yee and Ye (1996) and Yee (1999) have investigated the use of simple mathematical forms of the plume concentration PDF for use in Equation (8.21) in response to the current limitations of the advanced transport and dispersion models presently available. Only the two lowest-order concentration moments (first moment, mean concentration; second moment, mean-square concentration [or the concentration variance]) can be predicted with sufficient accuracy by these models (Yee and Ye, 1996). Note that because <TL> is a random variable, it cannot be adequately described by the first two moments unless the form of the PDF is specified (Yee and Ye, 1996). Using the data from CONFLUX trials, a clipped normal distribution was found to work best for an integration interval (τ) greater than 5 sec, and for the range (1 sec $\leqslant \tau \leqslant$ 5 sec), an exponential distribution provides the best match (Yee, 1999).

8.5 SENSITIVITY ANALYSIS

The following is an overview of a sensitivity analysis recently conducted by the authors. The purpose of the study was to evaluate the sensitivity of TL predictions to variations in the parameters included in the TL equations using actual concentration–time profiles taken from field experiments. Both Yee (1996a) and Duijm et al. (2000) (on a more limited scale) had previously conducted a similar type of study. Some of the details of Yee (1996a) and related papers were discussed previously in this chapter.

8.5.1 Test Data

The nonuniform concentration versus time data set used in the sensitivity analysis was collected during an extensive set of outdoor field trials at the U.S. Army Dugway Proving Ground (DPG), Dugway, UT, in September 1996 (Biltoft, 1997). Yee et al. (1998) had previously reported on the statistical characteristics of the concentration fluctuations from the data of Biltoft (1997). In Biltoft (1997), the dispersal of instantaneous clouds was being studied, in contrast to the mainly continuous emitting sources of the CONFLUX trials with which Yee and his associates were previously involved. Also, the source of the one concentration–time profile investigated by Duijm et al. (2000) is not stated explicitly but appears to be a continuous source.

During the 1996 trials, a line of 48 photoionization detectors was positioned downwind of puff release points. The detectors collected data at a 50-Hz rate. Downwind distances from the release point to the midpoint of the detector line varied from approximately 200 to 1200 m. The lateral spacing of the detectors varied from 6 to 10 m, with it increasing as a function of downwind distance.

The puffs generated during the trials consisted of gaseous propylene. Puff releases were created by using air cannons, which were capable of firing up to 0.36 kg of material in a single shot. A total of nine air cannons could be fired simultaneously, creating puff masses up to 3.2 kg.

In addition to the photoionization detector data, a full range of meteorological data was collected during the trials, including wind speed and direction, temperature, humidity, and incoming and outgoing radiation. Tethersonde and Pibal balloons were used to record data at various elevations, in addition to ground and near-ground level sensors. Wind speed data were collected at different locations and elevations, using both two-axis and three-axis devices. All collected meteorological data were recorded in self-extracting binary files.

In total, 25 individual trials were conducted during the course of the field test, with 22 of the trials yielding useable data. The field trials are documented in Biltoft (1997), which includes a complete description of the trials, test site layout, and instrumentation, as well as a summary of the initial data-reduction efforts conducted on the data. All the summary facts presented above are extracted from this report. In total, data for 4606 puff/sensor interactions (an interaction is defined as one puff passing over one sensor during the field trial) were generated during the field trials.

8.5.2 Sensitivity Analysis Measures

Two measures were calculated for each of the 4606 concentration histories. The first was the ten Berge toxic load, modified to a TLR:

$$TLR = \frac{\sum_{j=1}^{p} \left(C_\tau\right)_j^N \tau}{C_T^N T} \tag{8.23}$$

The values of N and τ used to calculate TLR are presented in Table 8.1. By using a full (3×5) factorial experimental design, 15 different values of TLR were calculated for each concentration history, one for each combination of N and τ. Note that when $N = 1.0$, the results are for the Haber model. Neither Yee (1996a) nor Duijm et al. (2000) used a factorial design approach in varying N, instead they used N values for actual chemicals. Of the two, only Duijm et al. investigated an N value less than one.

I_t is the time increment at which the concentration data used in the sensitivity study was collected, namely, 0.02 sec (50 Hz). T is the duration of an exposure at each given sample point, the time a single trial puff was over an individual sensor. Different T values were extracted from the trial data for each sensor as each puff passed over it.

The second measure calculated was C_{max}/C_T. Note that C_{max} is the maximum concentration measurement during a single integration interval. With nonuniform concentrations time histories, C_{max} varies for each integration interval, τ. The notation used in the study to distinguish these different values was $C_{\tau\text{-max}}/C_{T.}$

8.5.3 Analysis Results

8.5.3.1 ten Berge Toxic Load Exponent, *N*

Tables 8.2 and 8.3 present the percentile distribution (along with minimum, maximum, and mean) of TLR values calculated for $N = 2$ and $N = 0.5$, respectively, subdivided by the different values of τ. Note that $\tau = T$ values are not presented, because for these cases the numerator and denominator of Equation (8.15) are equivalent and the TLR is always equal to 1. The $\tau = 0.02$ values represent the most precise possible use of the data, because they are calculated at the rate of data acquisition. The data show that significant differences exist in TL results for different values of N.

The values of TLR for $N = 2$ are almost always greater than 1.0 and range from 0.36 to 45.49. For $N = 0.5$, the values are almost always less than 1.0 and range from 0.17 to 1.67. Values of TLR less than 1.0 for $N = 2$ and greater than 1.0 for $N = 0.5$ should really be 1.0. The discrepancies are due to an artifact of the calculation procedure.[3]

TABLE 8.1 Values of *N* and τ Used in Sensitivity Study

N	τ
0.5	Δt
1.0	1.0
2.0	2.0
	4.0
	T

TABLE 8.2 Summary Distributions of TLR Values for $N = 2$, Subdivided by τ

τ	Minimum	0.05 P	0.25 P	Median	Mean	0.75 P	0.95 P	Maximum
0.02	0.17	0.59	0.78	0.85	0.82	0.89	0.94	1.00
1	0.28	0.64	0.81	0.86	0.84	0.90	0.95	1.10
2	0.38	0.66	0.82	0.87	0.85	0.91	0.96	1.18
4	0.47	0.69	0.83	0.89	0.87	0.93	0.98	1.67

P = percentile

[3] It was rare to have the exact number of individual $C_{0.02}$ values (C_τ at 50-Hz sampling rate) to perfectly fill out all the averaging periods in a concentration history. As a result, the last averaging period (when $\tau = 1.0$, 2.0, and 4.0 sec) will not normally be perfectly filled with $C_{0.02}$ values (e.g., 50 $C_{0.02}$ values are needed to calculate C_τ for $\tau = 1.0$ sec). Zero $C_{0.02}$ values were added as padding (as needed) onto the end of the data stream in the last averaging period of a concentration history (e.g., if only 15 $C_{0.02}$ values are available for use in the last $\tau = 1.0$ sec, averaging period, then 35 zero $C_{0.02}$ values would be added to the end of this set before C_τ is calculated).

8.5.3.2 Integration Time Step, τ

Tables 8.2 and 8.3 may also be used to analyze the distribution results for τ values of 1.0, 2.0, and 4.0 sec. As the value of τ increases, the range of TLR values decreases. This results from the averaging of concentrations over increasingly longer intervals. As discussed in Section 8.3.2, this numerical smoothing process can be related to the actual smoothing process of breathing. So, the variation in results over the different values of τ is probably representative of the variation that is experienced for different breathing rates.

8.5.3.3 Fluctuation Intensity

Table 8.4 shows the percentile distribution (along with minimum, maximum, and mean) of $C_{\tau\text{-max}}/C_T$ values for τ values of 0.02, 1, 2, and 4. As would be expected, an increase in τ causes a decrease in the average model value. The sensitivity of the results to τ shows that the choice of a value for this parameter is very important in the calculation of TL. Given also that physiological factors such as breathing rate dictate to a great degree appropriate values for τ, care must be taken in the selection of this parameter value for risk assessment-type applications of TL models.

8.5.3.4 Other Parameters

The wide distributions of TLR and $C_{\tau\text{-max}}/C_T$ values discussed in the preceding sections obviously result from differences in the concentration profiles collected by Biltoft (1997). Concentration data were recorded for several test conditions. The following test parameters directly relate to quantification of the differences in the puff profiles:

- The duration of the puff over the sampler, $T = T_p$.
- The two-dimensional distance from the puff source to each of samplers. This can be represented as a combination of:
  - The perpendicular distance from the source point to the line of sensors, which did not intersect the line of sensors at its midpoint.
  - The sensor number, which indicated the lateral distance along the line of sensors from some reference point to the sensor.
- The wind speed, which was measured at both 2- and 8-m elevations.

TABLE 8.3 Summary Distributions of TLR Values for $N = 0.5$, Subdivided by τ

τ	Minimum	0.05 P	0.25 P	Median	Mean	0.75 P	0.95 P	Maximum
0.02	1	1.43	1.78	2.20	2.92	2.96	7.12	45.99
1	0.88	1.37	1.69	2.03	2.41	2.57	4.92	24.97
2	0.72	1.32	1.64	1.95	2.23	2.42	4.27	12.49
4	0.36	1.25	1.54	1.82	2.01	2.23	3.51	8.25

P = percentile

TABLE 8.4 Distribution of $C_{\tau\text{-max}}/C_T$ Values

τ	Minimum	0.05 P	0.25 P	Median	Mean	0.75 P	0.95 P	Maximum
0.02	1.00	2.73	3.81	5.25	7.72	8.25	20.11	634
1	0.92	2.19	2.88	3.61	4.20	4.72	8.49	28.19
2	0.72	1.96	2.60	3.19	3.56	4.03	6.49	14.69
4	0.36	1.66	2.25	2.75	2.95	3.41	4.94	9.25

P = percentile

- The mass of agent in the puffs.
- The trial number, which is a gross indicator of meteorological conditions.
- The time after the start of a trial at which a puff was released, which can be used to correlate puff data with measured meteorological data.

Analysis of variance (ANOVA) was used to investigate the relative contributions of the random factors above (as well as the two fixed parameters, N and τ) on the TLR. The two most important factors on the TLR were T_p and τ, with the puff duration being the more important. This was true for N values of both 0.5 and 2. In the case of an ANOVA on the fluctuation intensity ($C_{\tau\text{-max}}/C_{\text{T}}$) (with τ [4 sec), the same results were obtained, except that τ was more important than T_{p}.

8.6 DISCUSSION

The TLR is proportional to N and inversely proportional to τ. The effect with τ is more pronounced when $N > 1$. If Equation (8.6) is assumed to be the proper form for the probit relation, then the toxic effect of an exposure will increase in severity as τ is decreased in value if $N > 1$. If $N < 1$, then the severity of the toxic effect will increase as τ increases. Duijm et al. (2000) also identified these same trends. Further, for H_2S (with $N = 8.3$), Duijm et al. found that the effect of the integration interval is so great that just minor adjustments of the interval will drastically change the predicted percent effected. Yee (1996a) found that for small (10^{-4} to 10^{-1} sec) and large (greater than 100 sec) values of τ that the TLR is relatively insensitive to changes in τ (for N values of 2.7 and 3.5).

Three major differences exist between the present analysis and those of Yee (1996a) and Duijm et al. (2000). First, these two studies used N values previously reported for specific chemicals. Duijm et al. used N values estimated from rat IH data on several industrial chemicals (Arts et al., 2000), whereas Yee used the N values that had been reported for chlorine (3.5), hydrogen cyanide (2.7), and perfluoroisobutylene (1.2). The N values in the present analysis were varied in a systematic fashion by using design of experiment principles (Box et al., 1978). The latter approach permits a more thorough analysis of how toxicity varies as a function of N, as well as a function of any interactions between N and other parameters. Second, Yee and Duijm et al. only examined a single concentration–time profile versus the 4606 profiles reviewed by the present study. Also, Duijm et al. did not use a TLR-type parameter (see Equation [8.16]) in their analysis of their results. Last, Yee and Duijm et al. examined the concentration time history from a continuous release, whereas an instantaneous cloud release was examined in the present analysis.

The sensitivity of the TLR-to-puff duration, T_p, is intuitively sensible. In general, Puff concentrations may be represented by a trivariate Gaussian distribution, with the highest concentration at the center of the distribution and concentrations falling as the distance from the center increases. Higher TLs will result for sensor points passed over by the high-concentration center of the puff than for points passed over by the low-concentration edge of the puff. The trial puffs were larger than the distance between sensors, and any given puff created differing concentration profiles over a number of sensors. Those sensors near the path of the puff center saw both longer times for the puff to pass the sensor (T_p) and the higher concentration readings inherent to the puff center. Those sensors passed by the edge of the puff saw short durations, sometimes less than the integration intervals used in this analysis, and very low concentration readings. Thus, the significance of T_p indicated by the ANOVA is not unexpected, and T_p may well be a useful parameter in defining fluctuation intensity for the dispersal of instantaneous clouds.

In any event, no final conclusions can be reached on the toxicological significance of these findings until toxicology data become available to identify the proper form of the probit relation to use for chemical exposures involving fluctuating concentrations (either Equations [8.4], [8.6], or [8.7]). The need is greatest for chemicals having TL exponent values greater than about 1.4 to 1.5. Duijm et al. (2000) found that chemicals with N in this range could have a typical response ΔZ shift (see Equation [8.1]) of 4.5 (which is roughly the distance between 1% and 99% individuals responding) for a change of τ from 1000 sec down to 1 sec.

8.7 CONCLUSIONS

The large set of fluctuating concentration–time histories included in the Biltoft (1997) data set has proven suitable for evaluating the sensitivity of TL calculations to parameters included in the TL equation. Conclusions include:

1. The TL results for the TL model measures investigated vary significantly for the different concentration–time histories.
2. The size of the time step, τ, has a significant effect on the model results, with the magnitude of calculated TLR and $C_{\tau\text{-max}}/C_T$ converging toward a value of 1 as τ increases toward T_p in value. Changes in the TLR as a function of τ are more pronounced when $N > 1$.
3. Of all the random factors and fixed parameters of the Biltoft data, the two more important factors on the TLR (found via ANOVA) were T_p and τ, with the puff duration being the more important. This was true for N values of both 0.5 and 2. For the fluctuation intensity ($C_{\tau\text{-max}}/C_T$) (with $\tau = 4$ sec), the same results were obtained, except that τ was more important than T_p.

8.8 SUMMARY

Hazard assessment modeling is a multidiscipline field, requiring the cooperative efforts from many different fields of study: statistics, toxicology, transport/dispersion modeling, etc. An example of this is the modeling of the time dependence of chemical vapor toxicity via the TL model.

First, toxicological research has shown experimentally that many chemical vapor exposures are best described (empirically) by using the TL model (Equation [8.8]). Statistics (probit analysis) originally adapted for Haber's Law assumptions were adapted for use with the new model. However, IH and PC vapor toxicity research has been limited to constant concentration exposure chambers.

In turn, the implications of the TL relationship on hazard assessment predictions have been investigated by the atmospheric transport/dispersion modeling community. The impact of the TL equation on the mathematics and statistics of atmospheric concentration–time histories has been studied by several researchers. In particular, the TL has been expressed and calculated in terms of ensemble mean concentrations, a commonly used parameter in transport and dispersion modeling. Estimates of the appropriate integration timescale (τ) have been made for IH exposures, with human respiratory tract characteristics suggesting that τ (at a minimum) should be in the range of 1 to 4 sec.

However, with the transport/dispersion modeling foundation now laid, further investigation of these models using toxicological data taken under more realistic hazardous event conditions should be pursued. The current paucity of appropriate toxicological data for this purpose needs to be remedied.

ACKNOWLEDGMENT

The authors thank Dr. Eugene Yee of the Defense Research Establishment Suffield, Medicine Hat, Alberta, Canada, for his assistance during the course of this work.

REFERENCES

Andrews, L.S. and Snyder, R., Toxic effects of solvents and vapors, in *Casarett and Doull's Toxicology—The Basic Science of Poisons*, 4th ed., Amdur, M.O., Doull, J., and Klaassen, C.D., Eds., Pergamon Press, New York, 1991.

Anthony, J.S., Haley, M., Manthei, J., Way, R., Burnett, D., Gaviola, B., Sommerville, D., Crosier, R., Mioduszewski, R., and Thomson, S., Inhalation toxicity of cyclosarin (GF) vapor in rats as a function of exposure concentration and duration: potency comparison to sarin (GB), *Inhalation Toxicol.*, 16, 1–9, 2004.

Arts, J.H.E., Mommers, C., and Muijser, H., Toxic Effects from Accidential Releases of Hazardous Substances (TEARHS)—Lethal and Non-Lethal Effects in Rats upon Exposure during Short Periods of Time, TNO Report V99-1136, TNO Nutrition and Food Research, Zeist, The Netherlands., 2000.

Bakshi, K.S., Fusco, L.V., and Crossgrove, R.E., Eds., Review of Acute Human-Toxicity Estimates for Selected Chemical Warfare Agents, National Research Council, Committee on Toxicology, Subcommittee on Toxicology Values for Selected Nerve and Vesicant Agents, National Academy Press, Washington, DC, 1997.

Benschop, H.P. and DeJong, L.P.A., Toxicokinetics of nerve agents, in *Chemical Warfare Agents: Toxicity at Low Levels,* Somani, S.M. and Romano, J.A., Eds., CRC Press, Boca Raton, FL, 2001.

Benton, B.J., Sommerville, D.R., Scotto, J., Burnett, D.C., Gaviola, B.I., Crosier, R.B., Jakubowski, E.M., Jr., Whalley, C.E., Anthony, J.S., Jr., Hulet, S.W., Dabisch, P.A., Reutter, S.A., Forster, J.S., Mioduszewski, R.J., Thompson, S.A., Matson, K.L., Crouse, C.L., Miller, D., Evans, R.A., McGuire, J.M., and Jarvis, J., Low-level effects of VX vapor exposure on pupil size and cholinesterase levels in rats, in *Inhalation Toxicology,* 2nd ed., Salem, H. and Katz, S.A., Eds., CRC Press, New York, 2005.

Biltoft, C.A., Phase 1 of Defense Special Weapons Agency Transport and Dispersion Model Validation, DPG-FR-97–058, Meteorology and Obscurants Division, West Desert Test Center, U.S. Army Dugway Proving Ground, Dugway, UT, 1997.

Bliss, C.I., The method of probits, *Science,* 79, 38–39, 1934.

Box, G.E.P., Hunter, W.G., and Hunter, J.S., *Statistics for Experimenters,* John Wiley & Sons, New York, 1978.

Cresthull, P., Koon, W.S., McGrath, F.P. and Oberst, F.W., Inhalation Effects (Incapacitation and Mortality) for Monkeys Exposed to GA, GB, and GF Vapors, CWLR 2179, U.S. Army Chemical Warfare Laboratories, Army Chemical Center, Edgewood Arsenal, MD, AD 145581 1957.

Crosier, R.B. and Sommerville, D.R., Relationship Between Toxicity Values for the Military Population and Toxicity Values for the General Population, ECBC-TR-224, U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, AD A400214, 2002.

Department of Defense (DoD), Modeling and Risk Characterization of U.S. Demolition Operations at the Khamisiyah Pit, Technical Report 2001137–0000055 Ver. 1.0, Office of the Special Assistant for Gulf War Illnesses, Washington, DC, www.gulflink.osd.mil/khamisiyah_tech/ (last accessed Jan. 2003), 2002.

Duijm, N.J., Markert, F., and Ott, S., TEARHS—Modeling Toxic Impacts on the Airway System from Exposure to Fluctuating Concentrations, Riso-R-1208(EN), Riso National Laboratory, Roskilde, Denmark, 2000.

Eisenberg, N.A., Lynch, C.J., and Breeding, R.J., Vulnerability Model: A Simulation System for Assessing Damage Resulting from Marine Spills, Report CG-D-136–75, Enviro Control, Inc., Rockville, MD, for the U.S. Coast Guard, Office of Research and Development, Washington, DC, NTIS AD A015245, 1975.

Fairhust, S. and Turner, R.M., Toxicological assessments in relation to major hazards, *J. Hazardous Mater.,* 33, 215–227, 1993.

Ferguson, J.S. and Hendershot, D.C., The impact of toxicity dose-response relationships on quantitative risk analysis results, *Process Saf. Prog.,* 19, 91–97, 2000.

Finney, D.J., *Probit Analysis,* 3rd ed., Cambridge University Press, London, 1971.

Finney, D.J., *Statistical Method in Biological Assay,* 3rd ed., Charles Griffen and Co., London, 1978.

Fox, J., *Applied Regression Analysis, Linear Models, and Related Methods,* Sage Publications, Thousand Oaks, CA, 1997.

Franks, A.P., Harper, P.J., and Bilo, M., The relationship between risk of death and risk of dangerous dose for toxic substances, *J. Hazardous Mater.*, 51, 11–34, 1996.

Griffiths, R.F., The use of probit expressions in the assessment of acute population impact of toxic releases, *J. Loss Prev. Process Ind.,* 4, 49–57, 1991.

Griffiths, R.F. and Harper, A.S., A speculation on the importance of concentration fluctuations in the estimation of toxic response to irritant gases, *J. Hazardous Mater.,* 11, 369–372, 1985.

Griffiths, R.F. and Megson, L.C., The effect of uncertainties in human toxic response on hazard range estimation for ammonia and chlorine, *Atmos. Environ*., 18, 1195–1206, 1984.

Haber, F.R., Zur Geschichie des gaskrieges, in *Funf Vortrage aus Jahren 1920–1923*, Springer, Berlin, 1924.

Hanna, S.R., Briggs, G.A., and Hosker, R.P., Handbook on Atmospheric Diffusion, DOE/TIC-11223, U.S. Department of Energy, Oak Ridge, TN, 1982.

Harris, N.C. and Moses, A.M., The use of acute toxicity data in the risk assessment of the effects of accidental releases of toxic gases, in *Fourth International Symposium on Loss Prevention and Safety Promotion in the Process Industries*, Vol. 1, Institute of Chemical Engineers, Rugby, United Kingdom, 1983, p. 136.

Hattis, D., Banati, P., Goble, R., and Burmaster, D., Human interindividual variability in parameters related to health risks, *Risk Anal.,* 19, 705–720, 1999.

Hilderman, T.L., Hrudey, S.E., and Wilson, D.J., A Model for Effective Toxic Load from Fluctuating Gas Concentrations, *J. Hazardous Mater.*, 64, 115–134, 1999.

Hines, W.W. and Montgomery, D.C., *Probability and Statistics in Engineering and Management Science,* 3rd ed., John Wiley & Sons, New York, 1990.

Hulet, S.W., Sommerville, D.R., Benton, B.J., Forster, J.S., Scotto, J.A., Muse, W.T., Crosier, R.B., Reuter, S.A., Mioduszewski, R.J., and Thomson, S.A., Low-Level Sarin Vapor Exposure in the Gottingen Minipig: Effect of Exposure Concentration and Duration on Pupil Size, ECBC-TR-450, U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, in press, 2005a.

Hulet, S.W., Sommerville, D.R., Jakubowski, E.M., Benton, B.J., Forster, J.S., Dabison, P., Miller, D.B., Scotto, J.A., Jarvis, J.R., Crosier, R.B., Muse, W.T., Gaiola, B., Burnett, D., Reutter, S.A., Mioduszewski, R.J., and Thomson, S.A., Estimating Lethal and Severe Effects in Minipigs Following 10, 60 and 180 Minutes of Whole-Body GB Vapor Exposure, ECBC-TR-451, U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, in press, 2005b.

Hulet, S.W., Sommerville, D.R., Benton, B.J., Forster, J.S., Krauthauser, C., Scotto, J.A., Muse, W.T., Crosier, R.B., Reutter, S.A., Mioduszewski, R.J., and Thomson, S.A., Low-Level Cyclosarin Vapor Exposure in the Gottingen Minipig: Effect of Exposure Concentration and Duration on Pupil Size, ECBC-TR-452, U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, in press, 2005c.

Jakubowski, E.M., Mioduszewski, R.J., Hulet, S.W., Manthei, J.H., Benton, B.J., Forster, J.S., Burnett, D.C., Way, R.A., Gaviola, B.I., Edwards, J.L., Muse, W.T., Anthony, J.S., Matson, K.L., Miller, D.B., Crouse, C.L., and Thomson, S.A., Fluoride ion regeneration of sarin (GB) from minipig tissue and fluids after GB inhalation exposure, *The Toxicologist,* 72 (Abstr.), 159, 2003.

Klaassen, C.D. and Eaton, D.L., Principles of toxicology, in *Casarett and Doull's Toxicology—The Basic Science of Poisons,* 4th ed., Amdur, M.O., Doull, J., and Klaassen, C.D., Eds., Pergamon Press, New York, 1991.

MacFarland, H.H., Designs and operational characteristics of inhalation exposure equipment, in *Inhalation Toxicology,* Salem, H.S., Ed., Marcel Dekker, Inc., New York, 1987.

Mannan, S., Ed., Toxic releases, in *Lee's Loss Prevention in the Process Industries,* 3rd ed., Elsevier Butterworth-Heinemann Ltd, Boston, 2005.

Marshall, V.C., The predictions of human mortality from chemical accidents with special reference to the lethal toxicity of chlorine, *J. Hazardous Mater.*, 22, 13–56, 1989.

MINITAB™ Statistical Software, Release 13.31, MINITAB Inc., State College, PA, www.minitab.com/, 2001.

Mioduszewski, R.J., Manthei, J.H., Way, R.A., Burnett, D.C., Gaviola, B.P., Muse, W.T., Anthony, J.S., Durst, H.D., Sommerville, D.R., Crosier, R.B., Thomson, S.A., and Crouse, C.L., ECBC Low-Level Operational Toxicology Program: Phase 1—Inhalation Toxicology of Sarin Vapor in Rats as a Function of Exposure Concentration and Duration, ECBC-TR-183, U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, AD A394372, 2001.

Mioduszewski, R.J., Manthei, J.A., Way, R.A., Burnett, D.C., Gaviola, B.P., Muse, W.T., Thomson, S.A., Sommerville, D.R., and Crosier, R.B., Interaction of exposure concentration and duration in determining acute toxic effects of sarin vapor in rats, *Toxicol. Sci.*, 66, 176–184, 2002a.

Mioduszewski, R.J., Manthei, J., Way, R., Burnett, D., Gaviola, B., Muse, W., Thomson, S., Sommerville, D., and Crosier, R., Low-level Sarin Vapor Exposure in Rats: Effect of Exposure Concentration and Duration on Pupil Size, ECBC-TR-235, U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, AD A402869, 2002b.

National Research Council (NRC), *Acute Exposure Guideline Levels for Selected Airborne Chemicals*, Vol. 1, Subcommittee on Acute Exposure Guideline Levels (Krewski, D., chair), Committee on Toxicology, The National Academics Press, Washington, DC, 2000.

National Research Council (NRC), *Acute Exposure Guideline Levels for Selected Airborne Chemicals*, Vol. 2, Subcommittee on Acute Exposure Guideline Levels (Krewski, D., chair), Committee on Toxicology, The National Academics Press, Washington, DC, 2002.

National Research Council (NRC), *Tracking and Predicting the Atmospheric Dispersion of Hazardous Material Releases: Implications for Homeland Security*, Committee on the Atmospheric Dispersion of Hazardous Material Releases (Serafin, R.J., chair), The National Academies Press, Washington, DC, 2003.

Neumann, D.A. and Kimmel, C.A., Eds., *Human Variability in Response to Chemical Exposures,* International Life Sciences Institute, CRC Press, Boca Raton, FL, 1998.

Perry, W.W. and Articola, W.P., Study to Modify the Vulnerability Model of the Risk Management System, Report CG-D-22–80, U.S. Coast Guard, NTIS AD A084214, 1980.

Poblete, B.R. and Lees, F.P., The assessment of major hazards: estimation of injury and damage around a hazard source using an impact model based on inverse square law and probit relations, *J. Hazardous Mater*., 9, 355–371, 1984.

Ride, D.J., An assessment of the effects of fluctuations on the severity of poisoning by toxic vapours, *J. Hazardous Mater*., 9, 235–240, 1984.

Ride, D.J., A practical method of estimating toxic loads in the presence of concentration fluctuations, *Environmetrics*, 6, 643–650, 1995.

Riihimaki, V. and Pfaffli, P., Percutaneous absorption of solvent vapors in man, *Scand. J. Work Environ. Health*, 4, 73–85, 1978; as cited by Heinrich-Ramm, R., Jakubowski, M., Heinzow, B., Christensen, J.M., Olsen, E., and Hertel, O., Biological monitoring for exposure to volatile organic compounds (VOCs), *Pure Appl. Chem.*, 72, 385–436, 2000.

Rozman, K.K. and Doull, J., Dose and time as variables of toxicity, *Toxicology*, 144, 169–178, 2000.

Salem, H., Principles of inhalation toxicology, in *Inhalation Toxicology: Research Methods, Applications, and Evaluation,* Salem, H, Ed., Marcel Dekker, Inc., New York, 1987, pp. 1–33.

Saltzman, B.E. and Fox, S.H., Biological significance of fluctuating concentrations of carbon monoxide*, Environ. Sci. Technol*., 20, 916–923, 1986.

Saltzman, B.E., Assessment of health effects of fluctuating concentrations using simplified pharmacokinetic algorithms, *J. Air Waste Manage. Assoc*., 46, 1022–1034, 1996.

Schubach, S., Comparison of probit expressions for the prediction of lethality due to toxic exposure, *J. Loss Prev. Process Ind.*, 8, 197–204, 1995.

Schubach, S., A measure of human sensitivity in acute inhalation toxicity, *J. Loss Prev. Process Ind.*, 10, 309–315, 1997.

Sellers, G., Ed., *Phosgene Toxicity—A Report of the Major Hazards Assessment Panel Toxicity Working Party*, Major Hazards Monograph, Institution of Chemical Engineers, Rugby, United Kingdom (1993).

Silver, S.D., Constant flow gassing chambers: Principles influencing design and operation, *J. Lab. Clin. Med*., 31, 1153–1161, 1946.

Sommerville, D.R., Translation of toxicity data into CW agent toxicity estimates, in *Proceedings of the 2002 Joint Service Scientific Conference on Chemical & Biological Defense Research, 19–21 November 2002*, Berg, DA, Ed., Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, AD M001523, 2003.

ten Berge, W.F. and van Heemst, M.V., Validity and accuracy of a commonly used toxicity-assessment model in risk analysis, in *Fourth International Symposium on Loss Prevention and Safety Promotion in the Process Industries*, Vol 1., Institute of Chemical Engineers, Rugby, United Kingdom, 1983, p. 11.

ten Berge, W.F., Zwart, A., and Appelman, L.M., Concentration-time mortality response relationship of irritant and systemically acting vapours and gases, *J. Hazardous Mater*., 13, 301–309, 1986.

Whalley, C.E., Benton, B.B., Manthei, J.H., Way, R.A., Jakubowski, E.M., Burnett, D.C., Gaviola, B.I., Crosier, R.B., Sommerville, D.R., Muse, W.T., Forster, J.S., Mioduszweski, R.J., Thomson, S.A., Scotto, J.A., Miller, D.B., Crouse, C.L., Matson, K.L., Edwards, J.L., Low-Level Cyclo-Sarin (GF) Vapor Exposure in Rats: Effect of Exposure Concentration and Duration on Pupil Size, ECBC-TR-407, U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, AD A429234, 2004.

Wilson, D.J., *Concentration Fluctuations and Averaging Time in Vapor Clouds*, Center for Chemical Process Safety, American Institute of Chemical Engineers, New York City, 1995.

Withers, R.M.J. and Lees, F.P., The assessment of major hazards: the lethal toxicity of chlorine, part I. Review of information on toxicity, *J. Hazardous Mater.*, 12, 231–282, 1985.

Yee, E., A Rational Basis for Accounting for the Impact of Concentration on Toxicological Assessment and Estimation of Injury Resulting from the Release of Chemical and Biological Warfare Agents, SR-634, Defence Research Establishment Suffield, Alberta, Canada, AD A304587, 1996a.

Yee, E., A Non-Linear Dose-Response Model with an Application to the Reconstruction of the Human Mortality Response Surface from Acute Inhalation Toxicity with Sarin, SM-1476, Defence Research Establishment Suffield, Alberta, Canada, AD# A309486, 1996b.

Yee, E., An Impact-Effect Mathematical Model Incorporating the Influence of Exposures to Fluctuating Concentrations in a Dispersing Plume of Pollutant in the Atmosphere, *Journal of Exposure Analysis and Environmental Epidemiology*, 9, 300–311, 1999.

Yee, E., personal correspondence with Douglas R. Sommerville 2001 to 2003.

Yee, E. and Chan, R., Fractal characteristics of isoconcentration surfaces in plumes dispersing in the atmospheric surface layer, *Physics Fluids*, 7, 2715–2724, 1995.

Yee, E., Kosteniuk, P.R., Chandler, G.M., Biltoft, C.A., and Bowers, J.F., Statistical characteristics of concentration fluctuations in dispersing plumes in the atmospheric surface layer, *Boundary-Layer Meteorol.*, 65, 69–109, 1993a.

Yee, E., Kosteniuk, P.R., Chandler, G.M., Biltoft, C.A., and Bowers, J.F., Recurrence statistics of concentration fluctuations in plumes within a near-neutral atmospheric surface layer, *Boundary-Layer Meteorol.*, 66, 127–153, 1993b.

Yee, E., Chan, R., Kosteniuk, P.R., Chandler, G.M., Biltoft, C.A., and Bowers, J.F., Experimental measurements of concentration fluctuations and scales in a dispersing plume in the atmospheric surface layer obtained using a very fast response concentration detector, *J. Appl Meteorol.*, 33, 996–1016, 1994a.

Yee, E., Chan, R., Kosteniuk, P.R., Chandler, G.M., Biltoft, C.A., and Bowers, J.F., Concentration fluctuation measurements in clouds released from a quasi-instantaneous point source in the atmospheric surface layer, *Boundary-Layer Meteorol.*, 71, 341–373, 1994b.

Yee, E., Chan, R., Kosteniuk, P.R., Chandler, G.M., Biltoft, C.A., and Bowers, J.F., The vertical structure of concentration fluctuation statistics in plumes dispersing in the atmospheric surface layer, *Boundary-Layer Meteorol.*, 76, 41–67, 1995a.

Yee, E., Chan, R., Kosteniuk, P.R., Chandler, G.M., Biltoft, C.A., and Bowers, J.F., Measurements of level-crossing statistics, *Boundary-Layer Meteoral.*, 73, 53–90, 1995b.

Yee, E., Chan, R., Kosteniuk, P.R., Biltoft, C.A., and Bowers, J.F., Multiscaling properties of concentration fluctuations in dispersing plumes revealed using an orthonormal wavelet decomposition, *Boundary-Layer Meteorol.*, 77, 173–207, 1996.

Yee, E., Kosteniuk, P.R., and Bowers, J.F., A study of concentration fluctuations in instantaneous clouds dispersing in the atmospheric surface layer for relative turbulent diffusion: basic descriptive statistics, *Boundary-Layer Meteorol.*, 87, 409–457, 1998.

Yee, E. and Ye, H., Military Casualty Estimation for Realistic Chemical and Biological Warfare Agent Cloud Concentration Challenges, SR-661**,** Defence Research Establishment Suffield, Alberta, Canada, AD 322185, 1996.

Additional Articles Concerning Use of Toxic Load and Related Topics

Anthony, J.S., Haley, M.V., Manthei, J.H., Way, R.A., Burnett, D.C., Gaviola, B.P., Sommerville, D.R., Crosier, R.B., Mioduszewski, R.J., Thomson, S.A., Crouse, C.L., and Matson, K.L., Inhalation Toxicity of GF Vapor in Rats as a Function of Exposure Concentration and Duration and its Potency Comparison to GB, ECBC-TR-335, U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, AD A417763, 2003.

Anthony, J.S., Haley, M.V., Manthei, J.H., Way, R.A., Burnett, D.C., Gaviola, B.P., Sommerville, D.R., Crosier, R.B., Mioduszewski, R.J., Thomson, S.A., Crouse, C.L., and Matson, K.L., The Inhalation Toxicity of Sarin (GB) Vapor in Rats as a Function of Equilibration Time for Ten Minute Exposures, in *Proceedings of the 2003 Joint Service Scientific Conference on Chemical & Biological Defense Research, 17–20 November 2003, ECBC-SP-018,* Berg, DA, Ed., Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, 2004.

Bliss, C.I., The relationship between exposure, time, concentration and toxicity in experiments on insecticides, *Ann. Entomol. Soc. Am.*, 33, 721–766, 1940.

Chatwin, P.C., Lewis, D.M., and Mole, N., Atmospheric diffusion: some new mathematical models, *Adv. Computational Math.*, 6, 227–242, 1996.

Chatwin, P.C., Lewis, D.M., and Mole, N., Comments on the properties and uses of atmospheric dispersion datasets, *Soc. Ital. Fisico*, 20, 475–489, 1997.

Grotte, J.H. and Yang, L.I., Eds. Report of the Workshop on Chemical Agent Toxicity for Acute Effects, Institute for Defense Analyses, May 11–12, 1998, Institute of Defense Analyses Document D2176, Institute for Defense Analyses, Alexandria, VA, AD A392849, 2001.

Lewis, D.M. and Chatwin, P.C., A three-parameter PDF for the concentration of an atmospheric pollutant, J. Appl. Meteorol., 36, 1064–1075, 1997.

Mioduszewski, R.J., et al., ECBC Low Level Operational Toxicology Program: Phase 1 – Inhalation Toxicity of Sarin Vapor in Rats as a Function of Exposure Concentration and Duration, ECBC-TR-183, U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, AD A394372, 2001.

National Research Council (NRC), *Acute Exposure Guideline Levels for Selected Airborne Chemicals,* Vol. 3, Subcommittee on Acute Exposure Guideline Levels (Krewski, D., chair), Committee on Toxicology, The National Academies Press, Washington, DC, 2003b.

National Research Council (NRC), *Acute Exposure Guideline Levels for Selected Airborne Chemicals*, Vol. 4, Subcommittee on Acute Exposure Guideline Levels (Krewski, D., chair), Committee on Toxicology, The National Academies Press, Washington, DC, 2004.

Nielsen, M., Chatwin, P.C., Jorgen sen, H.E., Mole, N., Munro, R.J., and Ott, S., Concentration Fluctuations in Gas Releases by Industrial Accidents—Final Report, Riso-R-1329(EN), Riso National Laboratory, Roskilde, Denmark, 2002.

Rappaport, S.M. and Spear, R.C., Physiological damping of exposure variability during brief periods, *Ann. Occup. Hyg*., 32, 21–33, 1988.

Reutter, S., Hazards of chemical weapons release during war: new perspectives, *Environ. Health Perspect.*, 107, 985–990, 1999.

Rozman, K.K. and Doull, J., The role of time as a quantifiable variable of toxicity and the experimental conditions when Haber's c × t product can be observed: implications for therapeutics, *J. Pharmacol. Exp. Ther.*, 296, 663–668, 2001.

Schopflocher, T.P. and Sullivan, P.J., Spectral analysis of heavy contamination clouds, *Environmetrics*, 8, 603–612, 1997.

Sommerville, D.R., Relationship between the dose response curves for lethality and severe effects for chemical warfare nerve agents, in *Proceedings of the 2003 Joint Service Scientific Conference on Chemical & Biological Defense Research, 17–20 November 2003, ECBC-SP-018,* Berg, DA, Ed., Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, 2004.

Sweatman, W.L. and Chatwin, P.C., Dosages from instantaneous releases of dense gases in wind tunnels and into a neutrally stable atmosphere, *Boundary-Layer Meteorol*., 77, 211–231, 1996.

Withers, R.M.J. and Lees, F.P., The assessment of major hazards: the lethal toxicity of vhlorine. Part II. Model of toxicity to man, *J. Hazardous Mater*., 12, 283–302, 1985.

Yee, E., et al., Measurements of level-crossing statistics, *Boundary-Layer Meteorol*., 73, 53–90, 1995b.

Zwart, A., Arts, J.H.E., Klokman-Houweling, J.M., and Schoen, E.D., Determination of concentration-time-mortality relationships to replace LC_{50} values, *Inhalation Toxicol*., 2. 105–117, 1990.

Zwart, A., Arts, J.H.E., ten Berge, W.F., and Appelman, L.M., Alternate acute inhalation toxicity testing by determination of the concentration-time-mortality relationship: experimental comparison with standard LC_{50} testing, *Regul. Toxicol. Pharmacol.*, 15, 278–290, 1992.

9 Inhalation Toxicology and Carcinogenicity Studies of the National Toxicology Program

Elizabeth K. Weisburger and Vera W. Hudson

CONTENTS

9.1 INTRODUCTION

In 1971 the "War on Cancer" drive provided funds for the National Cancer Institute (NCI) to initiate long-term testing of a series of halogenated aliphatics. Although these compounds were widely used as solvents, fumigants, gasoline additives, and intermediates, most had had little or no testing for possible carcinogenicity. Within three years, there was a publication describing the potential for 1,2-dibromoethane (DBE) and 1,2-dibromo-3-chloropropane (DBCP) to induce stomach and other cancers in rodents after gavage in corn oil (Olson et al., 1974).

The NCI staff considered that although traces of DBE remained in grain that had been fumigated, the greater exposure to DBE and DBCP was through inhalation. DBCP was employed as a soil fumigant whereas DBE was a gasoline additive and a fumigant for fruits and vegetables, especially those from tropical areas. Therefore, an inhalation toxicity study with these two compounds was initiated at Hazelton Laboratories (now Covance). The NCI testing program was transferred to the National Toxicology Program (NTP) in 1978, and the study was completed under NTP direction. Final results were quite definite; both DBE and DBCP were carcinogens by the inhalation route and induced tumors of the nasal passages. DBCP was more dangerous for the tumors it induced tended to invade the brain (Reznik et al., 1980). This example reinforced the need for inhalation studies with certain compounds.

There are several advantages to testing by inhalation. Often, inhalation is the route by which humans are exposed to workplace or environmental chemicals; thus inhalation is a more relevant method of testing. Furthermore, the vapor/aerosol affects the respiratory tract directly, without the intervention or complication of prior metabolism by the liver. Another advantage is that there is no need for a solvent, as used in gavage studies; thus complications from food oils or other solvents are eliminated. An NTP trial of corn and safflower oils demonstrated the confounding effects of these oils in carcinogenesis experiments (National Toxicology Program, l994a).

Since 1978 the NTP has tested 40 or more compounds by inhalation. Most are volatile liquids but some are gases and a good proportion are solids. Results of these tests are presented here. However, for more information on these compounds with respect to human health effects, toxicity, environmental fate, exposure, and regulations, refer to the National Library of Medicine Hazardous Substances Data Bank (HSDB) (http://toxnet.nlm.nih.gov). Some of the compounds have also been evaluated by the American Conference of Governmental Industrial Hygienists (ACGIH) and by the International Agency for Research on Cancer (IARC). However, HSDB probably has the most comprehensive treatment of each compound. This is especially true for compounds that fall outside the special interests of ACGIH or IARC, and thus they are not considered by these groups.

9.2 GENERAL PROCEDURE

The general procedures used by the NTP are based on the NCI "Guidelines for Carcinogen Bioassay in Small Rodents" (National Cancer Institute, 1976), the first number of the NCI/NTP Technical Reports. Slight modifications have evolved with the program, and details are given in various review chapters (Hamm, 1994; Jameson and Goehl, 1994; Prejean, 1994).

The animal species and strains originally used by the NCI are the mainstay of the NTP operation, namely the Fischer or F344/N rat and the B6C3F1 mouse (Goodman et al., 1994). As for animal diets, during the first few inhalation bioassays, the animals were fed Wayne Lab-Blox ad libitum when not in the exposure chambers, but in later experiments, the NIH-07 diet was fed. However, since 1995, the NTP-2000 diet has been used (Table 9.1) This diet was designed to increase longevity and decrease the incidence and severity of spontaneous neoplastic and nonneoplastic lesions. It contains more fiber and fat than the NIH-07 diet and less protein. Drinking water was available to animals at all times.

Animals were caged singly in stainless steel wire bottom cages to avoid problems. The exposure chambers varied somewhat, but most were stainless steel, made by Harford Systems of Lab Products, Aberdeen, MD.

Each chemical to be tested was obtained from a reputable supplier and tested for identity, purity, and stability, against a reference standard (Jameson and Goehl, 1994).

TABLE 9.1 Constituents of NIH-07 and NTP-2000 Rat and Mouse Ration

	NIH-07,%	NTP-2000,%
Ground hard winter wheat	23.0	22.26
Ground #2 yellow shelled corn	24.5	22.18
Wheat middlings	10.0	15.0
Oat hulls	—	8.5
Alfalfa meal	4.0	7.5
Corn gluten meal (60% protein)	3.0	—
Dried skim milk	5.0	—
Soy oil	2.5	3.0
Dried brewer's yeast	2.0	1.0
Soybean meal	12.0	5.0
Purified cellulose	—	5.5
Corn oil	—	3.0
Fish meal	10.0	4.0
Dry molasses	1.5	—
Calcium carbonate	—	0.9
Ground limestone	0.5	—
Dicalcium phosphate	1.25	0.4
Sodium chloride	0.5	0.3
Choline chloride	—	0.26
Methionine	—	0.2
Premixes	0.25	—
Vitamin premix	—	0.5
Mineral premix	—	0.5

The ration was checked for nutrient composition, levels of vitamins and minerals, and the possible presence of contaminants (heavy metals, bacteria, mycotoxins, and pesticides).

If no data on the toxicity of the test chemical were available, five animals of each species and sex were exposed at each of several levels for single-dose studies. The data thus obtained served to set tentative levels for 14- to 16-day repeated-dose studies, and in sequence, levels for 14-week or 90-day tests, with 10 animals in each exposure group. These tests were conducted for 6 h/d, 5 d/week. An allowance of approximately 12–25 min for the exposure chamber concentration to reach the desired level was factored into the exposure times.

TABLE 9.2 Tissues Sampled for NTP Studies

1972–1978	1978–present
Adrenal	Adrenal
Brain	Bone with marrow
Diaphragm	Brain
Duodenum	Clitoral gland
Femur	Gall bladder (mice)
Heart	Esophagus
Jejunum/ileum	Heart
Kidney	Kidney
Large intestine	Large intestine (cecum, colon, rectum)
Liver	Larynx
Lung	Liver
Lymph nodes	Lung
Ovary/testis	Lymph nodes (mandibular, mediastinal, mesenteric, bronchial)
Pancreas	Mammary gland
Pituitary	Nose
Prostate/uterus	Ovary
Salivary gland	Pancreas
Skin	Parathyroid
Spleen	Pituitary
Stomach	Preputial gland
Thyroid and parathyroid	Prostate
Trachea	Salivary gland
Urinary bladder	Skin
Any gross lesions/tissue masses	Spleen
	Stomach
	Testis with epididymis and seminal vesicle
	Thymus
	Thyroid gland
	Trachea
	Urinary bladder
	Uterus
	Any other gross lesions/tissue masses

Sperm mobility and vaginal cytology were investigated in animals from the 14-week studies. Results from the 13/14 week tests enabled the NTP to select two or three dose levels for the definitive 2-year study, the aim being to have levels that would allow survival over the 2-year period without causing more than a 10% weight difference from the controls. In the 2-year study, 50 male and 50 female rats and mice were started for each group: controls plus two or three exposure levels. In a few cases more animals were placed on test in order that a sample could be necropsied and examined at interim periods. Animals from each phase of a study were subjected to a careful necropsy and subsequent histopathological examination to explore specific indications of toxicity. Tissues from animals in the 2-year study were examined microscopically by the study pathologist (Table 9.2), followed by a review by the NTP Pathology Working Group. Thus the final diagnosis represented a consensus opinion. Quality assurance measures were followed throughout each study (Boorman et al., 1994).

Various statistical analyses were employed (Cox, 1972; Tarone, 1975; Williams, 1972), with the Fisher exact test serving as the indicator of statistical significance, although other tests such as the trend test were also used. More recently, the Poly-3 test served to assess prevalence of neoplastic and nonneoplastic lesions (Portier and Bailer, 1989).

Results of the animal tests were classified according to the following criteria:

- Clear evidence of carcinogenicity is demonstrated by studies that are interpreted as showing a dose-related increase of malignant neoplasms, or an increase of a combination of malignant and benign neoplasms, or marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.
- Some evidence of carcinogenic activity is demonstrated by studies that are interpreted as showing a chemically related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.
- Equivocal evidence of carcinogenic activity is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemically related.
- No evidence of carcinogenic activity is demonstrated by studies that are interpreted as showing no chemically related increases in malignant or benign neoplasms.
- Inadequate study of carcinogenic activity is demonstrated by studies that because of major qualitative or quantitative limitations cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

The written report included the results of the animal tests, with their classifications, the histopathological summaries and the statistical evaluations. The report was reviewed by a panel of experts before release to the public.

9.3 INDIVIDUAL COMPOUNDS

Because of the diverse nature of the compounds tested and the results, each compound is presented separately. Gases tested by the NTP are presented first, in order of their report numbers, then liquids, and finally solids.

9.3.1 Gases

9.3.1.1 Propylene NTP #272

Propylene (CAS No. 115–07–1 formula C_3H_6; molecular weight, 42.08; boiling point, −48°C; melting point, −185°C) is a colorless flammable gas with an olefin-like odor. It is used as a feedstock for octane improvement in gasoline, as an intermediate in making plastics and compounds such as

propanol, cumene, and acrylonitrile. Propylene was tested at Battelle Pacific Northwest Laboratories (Battelle) under a contract with the NTP (National Toxicology Program, 1985a).

9.3.1.1.1 Vapor Generation

Propylene was purchased as such in a gas cylinder; the pressure was reduced by piping the propylene through a regulator into metering valves and thence to the exposure chambers. Concentrations in the chambers were monitored automatically 10 times each exposure day with a Hewlett-Packard 5840 gas chromatograph equipped with a flame ionization detector.

9.3.1.1.2 Procedure

In the preliminary studies, five male and five female rats and mice were exposed to concentrations of 0, 625, 1250, 5000, or 10,000 ppm for 6 h/d, 5 d/week for 14 days. Since no toxic effects were observed, 9 or 10 animals of each species and sex were exposed to the same levels, under the same conditions, for 14 weeks. In the final 2-year test, groups of 49–50 rats and mice of each sex were exposed to propylene at concentrations of 0, 5000, or 10,000 ppm for 6 h/d, 5 d/week for a total of 103 weeks.

9.3.1.1.3 Results

No toxic effects were noted in the preliminary tests, explaining why the 5000 and 10,000 ppm levels were chosen for the 2-year study. In that part of the study, survival of the test animals and the controls was similar, but body weights of exposed rats were slightly lower (within 5%) than those of controls. Nonneoplastic lesions noted were squamous metaplasia, hyperplasia, and inflammation of the nasal cavity in rats, especially in females. In male mice, there was a decrease in the spontaneous type alveolar/bronchiolar tumors. The NTP concluded that there was no evidence for carcinogenicity in either rats or mice. The American Conference of Governmental Industrial Hygienists (ACGIH) has not recommended a threshold limit value (TLV) for propylene but considers it a simple asphyxiant if oxygen levels fall below 18%. The major danger from propylene is the possibility of fire and explosion (ACGIH, 2001).

9.3.1.2 Butadiene NTP #288 and #434

1,3-Butadiene (CAS No. 106–99–0; formula C_4H_6; molecular weight, 54.09; boiling point, −4.44°C; melting point, −108.9°C; vapor pressure, 1900 mmHg at 20°C) is a colorless, highly flammable gas that is the most important monomer in the synthetic rubber industry. Because of its widespread production, it was tested by Battelle under contracts with the NTP (National Toxicology Program, 1984, 1993a).

9.3.1.2.1 Vapor Generation

The liquified gas was piped through stainless steel tubing to a distribution manifold and flow control system, with metering valves and flow meters. Concentrations in the exposure chambers were measured by a gas chromatograph and photoionization detector.

9.3.1.2.2 Procedure

Because there were data on inhalation exposures with rats, the NTP experiments were done only in mice. In the first series of experiments, the 15-day and 14-week trials were done by Industrial Biotest, and the long-term tests were done by Battelle. In the 15-day trial, five male and five female mice were exposed by inhalation for 6 h/d, 5 d/week to levels of 0, 625, 1250, 2500, 5000, and 8000 ppm. The 14-week study used 10 mice of each sex at 0, 625, 1250, 2500, 5000, and 8000 ppm at the usual schedule for 14 weeks. In the long-term study, 50 mice of each sex were exposed at 0, 625, or 1250 ppm at the usual schedule. Originally this was to be for 105 weeks, but the study was terminated at 60 or 61 weeks. In the second study, groups of 70 male and female mice were exposed at 0, 6.25, 20, 62.5, 200, or 625 ppm for 6 h/d, 5 d/week for up to 103 weeks. In addition a group of

50 male mice was exposed at 200 ppm for 40 weeks; another group at 312 ppm for 52 weeks; a third group at 625 ppm for 13 or 26 weeks. After the exposures all were kept under control conditions until 103 weeks.

9.3.1.2.2 Results

In the initial 15-day trial, all the mice survived, but body weights were lower in all exposed groups. The 14-week test showed that levels over 1250 ppm were toxic; body weights were also lower. The 60- to 61-week study showed that body weights were not affected, but survival was poor. Many tumors occurred in both sexes: malignant lymphomas, hemangiosarcomas of the heart, alveolar/bronchiolar neoplasms, squamous cell neoplasms of the forestomach, and, in females, mammary carcinomas, granulosa cell neoplasms of the ovary, and hepatocellular neoplasms.

In the second long-term trial, animals at 625 ppm died early of malignant lymphomas. There were significant increases in many types of tumors: neoplasms of the heart, lung, forestomach, Harderian gland, mammary gland, ovary, and liver. Even the lowest level, 6.25 ppm, led to increased lung tumors in female mice. Thus, there clearly was evidence for the carcinogenicity of 1,3-butadiene in both male and female mice.

In contrast, the 2-year study with rats (Owen and Glaister, 1990) was performed with exposures of 1000 and 8000 ppm. There were increases in tumors but mainly of the endocrine system.

The enhanced susceptibility of mice to 1,3-butadiene has been linked to their efficient conversion of butadiene to activated metabolites rather than to detoxification products (ACGIH, 2001).

The ACGIH has set a TLV of 2 ppm for butadiene and has given it the A2 designation, suspected human carcinogen (ACGIH, 2001).

9.3.1.3 Ethylene Oxide NTP #326

Ethylene oxide (CAS No. 75–21–8: formula C_2H_4O; molecular weight, 44.05; boiling point, 10.73°C; melting point, −111°C; vapor pressure, 146 mmHg at 20°C) is a colorless gas used primarily as an intermediate in the production of antifreeze, surface-active agents; glycol ethers; ethanol amine, and choline. Lesser uses are as a sterilant and fumigant. Because of its widespread use it was tested at Battelle under a contract with the NTP (National Toxicology Program, 1987a).

9.3.1.3.1 Vapor Generation

Ethylene oxide was obtained as a liquid in a cylinder; from the cylinder the liquid was dispensed through an eductor tube to a boiler located in a 55°C water bath by pressurizing with nitrogen. From the boiler the vapor was routed through a manifold to gas-metering valves that controlled gas flow to each exposure chamber. Monitoring the levels of ethylene oxide was accomplished with a photoionization detector and then a Hewlett-Packard Model 5840 gas chromatograph equipped with a flame ionization detector.

9.3.1.3.2 Procedure

Because there already were tests of the long-term effects of ethylene oxide in rats, the NTP study was performed using only mice. In the single-dose exposure, groups of five male and five female mice were exposed to 100, 300, 400, 800, or 1600 ppm of ethylene oxide for 4 h. In the 14-day study, similar groups of mice were exposed by inhalation at 0, 50, 100, 200, 400, or 800 ppm for 6 h/d, 5 d/week for 10 exposures over a 14 day period. The 14-week step involved 10 mice of each sex at 0, 50, 100, 200, 400, or 600 ppm of ethylene oxide for 6 h/d, 5 d/week for 14 weeks. In the 2-year study, groups of 50 male and 50 female mice were exposed by inhalation for 6 h/d, 5 d/week for 102 weeks at 0, 50, or 100 ppm.

9.3.1.3.3 Results

In the single-dose study, levels of 800 ppm and above were toxic; the same held for the 14-day section of the test. Doses of 400 ppm or more led to deaths in the 14-week study. However, no clinical signs of toxicity were apparent.

In the 2-year part of the test, mean body weights and survival of exposed mice were similar to that of controls. Histopathological examination at the end showed that both male and female mice had significant increases in alveolar and bronchiolar carcinomas and adenomas and papillary cystadenomas of the Harderian gland. Females also had significant increases in malignant lymphomas and uterine adenocarcinomas. The NTP decided the results showed clear evidence of carcinogenicity in both male and female mice.

Confirming the result with the mice was an inhalation test of ethylene oxide in female A/J mice. A significant increase in pulmonary adenomas in this susceptible strain was observed (Adkins et al., 1986).

The comparable studies with rats showed that ethylene oxide caused significant increases in various types of tumors, namely subcutaneous fibromas, peritoneal mesotheliomas, pancreatic and pituitary adenomas, brain tumors, and mononuclear cell leukemia (Garman et al., 1986; Lynch et al., 1984; Snellings et al., 1984). It thus appeared that rats were affected more by ethylene oxide than were mice.

The epidemiological studies of humans exposed to ethylene oxide afford limited evidence for the carcinogenicity of the substance for the studies are conflicting and often confounded by exposure to other chemicals. These studies have been summarized by IARC (IARC, 1994). IARC concluded that although there is limited evidence in humans for the carcinogenicity of ethylene oxide, the compound is a human carcinogen, based on the mechanism of action and the similarity of other effects in both humans and animals.

On the other hand, the same data led ACGIH to designate ethylene oxide as a suspected human carcinogen, with the A2 designation. ACGIH set a TLV of 1 ppm for ethylene oxide to avoid potential oncogenic risks and nonneoplastic effects on many organs and the central nervous system (ACGIH, 2001).

9.3.1.4 Chloroethane (Ethyl Chloride) NTP #346

Chloroethane (CAS No. 75–00–3; formula C_2H_5Cl; molecular weight, 64.52; boiling point, 12.3°C; melting point, −138.7°C; vapor pressure 1000 mmHg at 20°C) is a colorless gas which has been used as an intermediate, as a solvent, propellant, an anesthetic, in refrigeration, and in formulation of pesticides. It was tested at Battelle under a contract with the NTP (National Toxicology Program, 1989a).

9.3.1.4.1 Vapor Generation

The chloroethane was led from the shipping container into a stainless steel boiler maintained at about 60°C by a water bath. The vapor was routed through a gas-metering valve and a purge/expose valve into a pipe at the chamber inlets and was mixed with air entering the exposure chamber. Monitoring of chamber concentrations was done with a Hewlett-Packard Model 5840 gas chromatograph equipped with a flame ionization detector.

9.3.1.4.2 Procedure

In the preliminary phase, five male and five female rats and mice were exposed to 19,000 ppm for 4 h. All survived; therefore, in the 14-day study five male rats and five female rats and mice were at 19,000 ppm for 6 h/d, 5 d/week for 10 exposure days. All animals survived without effect on body weights. Thus, in the 13-week phase, 10 male and 10 female animals of each species were exposed to 0, 2500, 5000, 10,000, or 19,000 ppm for 6 h/d, 5 d/week for 13 weeks. In the 2-year study, 50 rats and 50 mice of each sex were exposed at 0 or 15,000 ppm for 6 h/d, 5 d/week for 100 weeks (mice) or 102 weeks (rats).

9.3.1.4.3 Results

In the three preliminary parts of the test, there seemed to be no effect of chloroethane on body weights and survival. However, in the 2-year study body weights were lower than controls in the exposed animals and survival was low in the male rats on test. Histopathological examinations

revealed that male rats had an increase in benign and malignant epithelial neoplasms, whereas in female rats there was an increase in malignant astrocytomas of the brain. For male mice, the study was considered inadequate, but there was an increase in lung neoplasms. Female mice had very poor survival, but there was a highly significant incidence of endometrial uterine carcinomas, along with some increases in liver tumors.

These results caused the NTP to decide that there was equivocal evidence for carcinogenicity in male and female rats; that the male mouse study was inadequate, but that there was clear evidence of carcinogenicity in female mice.

In humans exposure to chloroethane at levels over 13,000 ppm leads to anesthesia, but lower levels can cause incoordination, cyanosis, and nausea. Human exposure has been without untoward results, even though chloroethane has been widely used to relieve the pain of acute injury and in sports medicine. The ACGIH has recommended a TLV of 100 ppm for this compound, with the A3 designation, a confirmed animal carcinogen with unknown relevance to humans (ACGIH, 2001).

9.3.1.5 Methyl Bromide NTP #385

Methyl bromide (CAS No. 74–83–9; formula CH_3Br; molecular weight, 94.95; boiling point, 3.56°C; melting point, −93.66°C; vapor pressure, 1420 mmHg at 20°C) is a colorless nonflammable gas used as a fumigant in pest control and as a methylating agent in the chemical industry. Methyl bromide was tested at Brookhaven National Laboratory under a contract with the NTP (National Toxicology Program, 1992a). Because of previous negative inhalation and feeding studies with rats, methyl bromide was tested in mice only.

9.3.1.5.1 Vapor Generation

The methyl bromide, obtained as a compressed gas, was delivered from the cylinder through a shrouded delivery tube to a distribution plenum; rotameters controlled the gas flow to each exposure chamber. The concentration in each chamber was determined by a MIRAN 80 infrared spectrometer; additional monitoring by gas chromatography was done biweekly.

9.3.1.5.2 Procedure

In the preliminary studies, five male and five female mice in each group were exposed to 0, 12, 25, 50, 100, or 200 ppm methyl bromide for 6 h/d, 5 d/week for 10 exposures over a 14-day period. In the 13-week phase, groups of 10 male and 10 female mice were exposed by inhalation at 0, 10, 20, 40, 80, or 120 ppm for 6 h/d, 5 d/week. In the 2-year study 86 male and female mice were exposed at 0, 10, 33, or 100 ppm for 6 h/d, 5 d/week for up to 2 years. At four or six and 15 months 10 mice from each group were taken for interim examination, while 16 mice were used for neurobehavorial studies.

9.3.1.5.3 Results

In the 14-day phase, most of the mice exposed to 200 ppm died and hematuria was noted. Little organ toxicity was observed in the 13-week test, but there were decreases in body weight, relative to the controls. In the 2-year study, animals at the high-dose level were discontinued after 20 weeks because of high mortality, especially in the males. After two years no carcinogenic effects were noted, but there was irritation of the respiratory tract.

Rats exposed to methyl bromide for up to 29 months also showed degenerative and hyperplastic changes of the olfactory epithelium but no neoplastic changes (Reuzel et al., 1991).

In humans exposure to methyl bromide has led to pulmonary edema, loss of coordination, convulsions, lung irritation, paralysis, and coma and death at high levels of exposure. The ACGIH has recommended a TLV of 1 ppm to minimize the potential for skin and respiratory tract irritation. There is no evidence of carcinogenicity in humans, despite the acute toxic effects. In view of the negative carcinogenicity studies, the ACGIH has given methyl bromide the A4 designation, not classifiable as a human carcinogen (ACGIH, 2001).

9.3.1.6 Ozone NTP #440

Ozone (CAS No. 10028–15–6; formula O_3; molecular weight, 47.99; boiling point, −111.9°C; melting point, −193°C) is a bluish liquid or gas; at high concentrations the odor is pungent and irritating. Ozone is used as a disinfectant for air and water, for bleaching oils and waxes and textiles, and in organic syntheses. Because of its harmful effects on pulmonary function, it was tested by inhalation at Battelle under a contract with the NTP (National Toxicology Program, 1994b).

9.3.1.6.1 Vapor Generation

The ozone was generated by a silent corona discharge ozonator and delivered to the exposure chambers. Chamber concentrations were monitored with an ultraviolet spectrophotometric analyzer; a Hewlett-Packard computer controlled the stream and the operation of each monitor.

9.3.1.6.2 Procedure

The bioassay began with a 4-week test in which groups of five male and five female rats and mice were exposed to ozone at levels of 0, 0.5, or 1 ppm 6 h/d, 5 d/week for 20 exposures over the period. In the 2-year study, groups of 50 male and 50 female rats and mice were exposed at 0, 0.12, 0.5, or 1 ppm for 6 h/d, 5 d/week for 105 weeks. In the lifetime phase, groups of 50 male and 50 female rats and mice received exposures of 0, 0.5, or 1 ppm for 125 weeks at the 6 h/d, 5 d/week schedule.

To determine whether ozone had a promoting action on a known lung carcinogen, groups of 48 male rats received injections of NNK (4-(methylnitrosamino)-l-(3-pyridyl)-l-butanone) and the same ozone exposure as in the 2-year trial. Thus, groups of male rats received ozone at 0 or 0.5 ppm for 6 h/d, 5 d/week for 105 weeks. During the first 20 weeks, they were injected subcutaneously three times weekly with 0, 0.1, or 1 mg NNK in trioctanoin.

9.3.1.6.3 Results

In the 4-week phase, all the rats and mice survived, although body weights were lower at the 1 ppm level. The 2-year study demonstrated that male rats were more susceptible to the effects of ozone for they had the lowest survival rate. The lifetime trial of 125 weeks led to very poor survival in both controls and test animals, since it was at the limit of their life span. The male rats on the NNK-ozone trial had survival and body weights similar to those of controls, except for the 1 mg NNK group. These rats had lower body weights.

As for carcinogenicity and promoting effects, there was no evidence for this in any of the rat studies. Animals developed hyperplasia and metaplasia of the nose, larynx, and lung, but there was no increase in neoplastic effects. Furthermore, the rats treated with NNK plus ozone showed no promoting action by ozone on the lung tumors induced by NNK.

Mice were apparently more susceptible to any effects of ozone, for their body weights were somewhat lower than those of controls, although survival rates were similar. Mice also had hyperplasia and metaplasia similar to the case with the rats. However, both male and female mice had uncertain or nonstatistically significant increases in lung tumors, slightly more in the females. The NTP interpreted these results as equivocal evidence in male mice and some evidence in female mice.

Humans exposed to ozone have shown effects ranging from discomfort to pulmonary congestion. The ACGIH has recommended different TLVs for ozone, depending on whether the exposed individuals are doing light to heavy work. Thus, for heavy work a TLV of 0.05 ppm has been set; for moderate work 0.08 ppm, and for light work 0.1 ppm. ACGIH has also given ozone the A4 designation, not classifiable as a human carcinogen (ACGIH, 2001).

9.3.1.7 Tetrafluoroethylene NTP #450

Tetrafluoroethylene (CAS No. 116–14–3; formula C_2F_4; molecular weight, 100.02; boiling point, 76.3°C; melting point, −142.5°C; vapor pressure, 438.4 mmHg) is a colorless, odorless, highly flammable gas used to make Teflon in closed systems. Because of lack of information on its long-term effects, it was tested at Battelle under a contract with the NTP (National Toxicology Program, 1997a).

9.3.1.7.1 Vapor Generation

Tetrafluoroethylene was metered through regulators directly from the shipping cylinder to the exposure chambers. Concentrations were monitored by an on-line gas chromatograph.

9.3.1.7.2 Procedure

Because acute toxicity data were available, 16-day exposure studies were the initial tests. Groups of five male and five female rats and mice were exposed for 6 h/d, 5 d/week for 12 exposure days in a 16-day period to levels of 0, 312, 625, 1250, 2500, and 5000 ppm. For the 13-week study, 10 male and 10 female rats and mice were exposed for 6 h/d, 5 d/week for 13 weeks to levels of 0, 312, 625, 1250, 2500, or 5,000 ppm. In the 2-year test, groups of 60 male rats were exposed to 0, 156, 312, and 625 ppm tetrafluoroethylene for 6 h/d, 5 d/week for 104 weeks. Groups of 60 female rats and 58 male and female mice were exposed at 0, 312, 625 and 1250 ppm for 95–96 weeks (mice) or 104 weeks (female rats). At 15 months, 10 animals from each group were evaluated for hematology, clinical chemistry, organ weight, and urinalysis parameters (rats).

9.3.1.7.3 Results

In the 16-day study, all rats survived but body weights were appreciably lower at the top exposure levels in both sexes. Likewise, all mice survived without an effect on body weight. In the 13-week study all rats survived but body weights were lower at the top level. All mice survived the 13-week test, but with somewhat lower body weights and karyomegaly of the renal tubule epithelial cells in mice exposed at higher than 1250 ppm.

For the 2-year study, it was noted that survival in male rats at the top level and in females at all exposure levels was less than in controls. Body weights of both male and female rats at the highest level were lower than those of controls. Histopathological examination showed increases in renal tubule hyperplasia in both males and females and significant increases in renal adenomas or carcinomas in both sexes. In addition, the incidences of liver tumors, namely hepatocellular adenoma or carcinoma, were higher in both males and females, and mononuclear cell leukemia was higher. On the other hand, the incidence of mammary gland fibroadenomas decreased in female rats.

As for the mice, all survived the 16-day study, and body weights were similar in controls and exposed mice. In the 13-week test, all mice survived without effect on body weights, but karyomegaly of the kidney occurred in both sexes at the three highest dose groups. In the 2-year study survival rates of both males and females at all dose levels were lower than for controls; thus the study was ended at 96 weeks. The liver was a target organ in the mice with hemangiomas and/or hemangiosarcomas, hepatocellular adenomas or carcinomas, and histiocytic sarcomas of many organs being observed.

The results of the study led NTP to decide there was clear evidence of carcinogenicity in all four test groups—male rats, female rats, male mice, and female mice.

The ACGIH has set a TLV of 2 ppm for tetrafluoroethylene to avoid liver and kidney damage (ACGIH, 2001), with the A3 designation, confirmed animal carcinogen with unknown relevance to humans.

9.3.2 Liquids

9.3.2.1 1,2-Dibromo-3-Chloropropane NTP #206

1,2-Dibromo-3-chloropropane (DBCP) (CAS No. 96–12–8; formula $C_3H_5Br_2Cl$: molecular weight, 236.4; boiling point, 196°C; vapor pressure, 8 mmHg at 21°C) is a brown liquid formerly used as a nematocide. It was tested under a contract from the NTP (National Toxicology Program, 1982a) with Hazelton Laboratories (now Covance).

9.3.2.1.1 Vapor Generation

Filtered dry air was bubbled through liquid DBCP and the vapor was pumped into the inhalation chambers through Teflon tubing. The level of DBCP was determined with a Varian 600-D

gas chromatograph. The inhalation chambers were monitored continuously by an HNU Systems analyzer.

9.3.2.1.2 Procedure

Groups of five male and five female rats or groups of 8–10 male mice and 10–12 female mice were exposed by inhalation to 0, 1, 5, or 25 ppm DBCP for 6 h/d, 5 d/week for 13 weeks, as the preliminary study. In the 2-year study, groups of 50 male and 50 female rats and mice were exposed to 0, 0.6, or 3 ppm DBCP for 6 h/d, 5 d/week for 76 to 103 weeks.

9.3.2.1.3 Results

In the 13-week portion of the test, there were some deaths at the higher dose level. Inflammatory necrotizing lesions of the nasal cavity were observed in both rats and mice. In the 2-year study, survival decreased sharply at 76 weeks for animals on the higher dose. Histopathology showed that DBCP induced significant increases in squamous cell papillomas and carcinomas of the nasal cavity in both sexes of rats and mice. In rats, these tumors tended to invade the brain (Reznik et al., 1980). In rats there were significant incidences of squamous cell papillomas of the tongue and pharynx, whereas in mice lung tumors were increased significantly. Mammary gland fibroadenomas in female rats and mesotheliomas of the tunica vaginalis in male rats also appeared related to DBCP exposure. Thus, inhalation exposure to DBCP was clearly related to carcinogenicity in both male and female rats and male and female mice.

The ACGIH has not set any TLV for DBCP. However, IARC has concluded that DBCP belongs in their Group 2B, namely that it is possibly carcinogenic to humans (IARC, 1999).

9.3.2.2 1,2-Dibromoethane (Ethylene Dibromide) NTP #210

1,2-Dibromoethane (CAS No. 106–93–4; formula $C_2H_4Br_2$; molecular weight, 187.66; boiling point, 131–2°C; melting point, −9°C; vapor pressure, 11 mmHg at 25°C) is a clear liquid formerly used as a gasoline additive to sequester lead and as a fumigant for grains, fruits, and vegetables. It was tested by inhalation at Hazelton Laboratories (Covance) under a contract with the NTP (National Toxicology Program, 1982b).

9.3.2.2.1 Vapor Generation

The dibromoethane was held in a large flask and vaporized by a stream of dry air. The concentration in the exposure chambers was monitored by a Varian 600-D gas chromatograph.

9.3.2.2.2 Procedure

A 13-week test was performed with groups of five male and five female rats and 8–10 male mice and 10–12 female mice exposed at 0, 3, 15 or 75 ppm for 6 hrs/day, 5 days/week. The final 2-year study began with 50 male and 50 female rats and mice exposed at 0, 10, or 40 ppm for 6 hrs/day, 5 days/week for 78–90 and 103 weeks.

9.3.2.2.3 Results

Although the toxicity of dibromoethane was somewhat lower than that of DBCP, the types of tumors induced were similar. In male and female rats, there were significant incidences of adenomas, carcinomas, and adenocarcinomas of the nasal cavity and hemangiosarcomas of the circulatory system. Males also had a significant incidence of mesotheliomas of the tunica vaginalis while females showed an increase in fibroadenomas of the mammary gland. In the mice, males appeared somewhat less susceptible, but they had significant incidences of adenomas and carcinomas of the lung and total respiratory tumors. Female mice reacted more like the rats with notable increases in tumors of the nasal cavity and hemangiosarcomas or hemangiomas of the circulatory system plus increases in respiratory tumors and adenocarcinoma of the mammary gland.

Thus, there was definite evidence for the carcinogenicity of 1,2-dibromoethane in the four animal models tested, male and female rats and male and female mice.

When the U.S. EPA banned the use of lead compounds as antiknock agents in gasoline, the major use and means of exposure of 1,2-dibromoethane ended. Shortly thereafter EPA also cancelled the application as a fumigant for grains and tropical fruits and vegetables.

The ACGIH has not set any TLV for this compound, but has given it the A3 designation, confirmed animal carcinogen with unknown relevance to humans (ACGIH, 2001).

9.3.2.3 Propylene Oxide NTP #267

Propylene oxide (CAS No. 75–56–9; formula C_3H_6O; molecular, 58.08; boiling point, 34.2°C; melting point, −112°C; vapor pressure, 445 mmHg at 20°C) is a colorless, highly volatile and flammable liquid which is an important material for production of polyols and propylene glycols for polyester resins. The long-term study of propylene oxide was performed by Battelle under a contract with the NTP (National Toxicology Program, 1985b).

9.3.2.3.1 Vapor Generation

Propylene oxide, contained in a stainless steel reservoir housed in a vapor hood, was pumped to a vaporizer and delivered to the exposure chambers using micrometering pumps and a manifold distribution system. Concentrations in the Chambers were monitored with a gas chromatograph.

9.3.2.3.2 Procedure

Single and repeated exposure trials were done at Industrial Biotest. In the single exposure, groups of five males and five females of each species were exposed to propylene oxide by inhalation for 4 h; rats at 1277, 2970, 3794, or 3900 ppm; mice at 387, 859, 1102, 1277, or 2970 ppm. In the repeat-exposure group of five males and five females of each species were exposed for 6 h/d, 5 d/week for 10 exposures; rats at 0, 47.2, 98.5, 196, 487, or 1433 ppm; mice at 0, 20.1, 47.2, 98.5, 196, or 487 ppm under the same schedule. In the 13-week trial, groups of 10 males and 10 females of each species were exposed at 0, 31, 63, 125, 250, or 500 ppm for 6 h/d, 5 d/week for 13 weeks. In the 2-year study, groups of 50 males and 50 females of each species were exposed to propylene oxide by inhalation for 6 h/d, 5 d/week for 103 weeks, at levels of 0, 200, or 400 ppm.

9.3.2.3.3 Results

In the single exposure, rats died at levels of 1277 and above; 1102 and higher led to deaths in the mice. In the repeated exposure trial, rats died or showed signs of toxicity at the top level; although no mice died, they had dyspnea and hypoactivity at the highest level. The 13-week study led to depression in weight gain or deaths at 500 ppm. The 2-year study showed survival in rats was similar to that of controls, but body weights were depressed slightly at the top dose. Both survival and body weights of exposed mice were lower than in the controls. In both species, there were exposure-related increases in inflammation, hyperplasia, and squamous metaplasia of the nasal turbinates. Furthermore, exposed male and female rats had a low incidence of papillary adenomas of the nasal turbinates, whereas exposed mice had adenocarcinomas, hemangiomas, and hemangiosarcomas of the nasal mucosa. Based on these results, the NTP concluded there was some evidence for the carcinogenicity of propylene oxide in male and female rats and clear evidence in male and female mice.

Other inhalation studies of propylene oxide in male F344 rats (Lynch et al., 1984) and male and female Wistar rats (Kuper et al., 1988) have confirmed the carcinogenic action of propylene oxide.

However, epidemiological surveys of workers exposed to a variety of chemicals, including propylene oxide, have not shown increases in cancer mortality. Nevertheless, to protect against deleterious effects, the ACGIH has set a TLV of 2 ppm, with the A3 designation, confirmed animal carcinogen with unknown relevance to humans (ACGIH, 2001).

9.3.2.4 Dichloromethane (Methylene Chloride) NTP #306

Dichloromethane (CAS No. 75–09–2; formula CH_2Cl_2; molecular weight, 84.93; boiling point, 40°C at 76 mmHg; melting point, −95.1°C; vapor pressure, 349 mmHg at 20°C) is a colorless,

volatile nonflammable liquid which has been widely used as a solvent in paint removers, in plastics, as a degreasing agent, a blowing agent in foams, and as a propellant in aerosol containers. It was tested by Battelle under a contract with the NTP (National Toxicology Program, 1986a).

9.3.2.4.1 Vapor Generation

Dichloromethane was vaporized at 38–42°C, diluted with air and introduced into the exposure chambers. Concentrations were monitored with an on-line gas chromatograph.

9.3.2.4.2 Procedure

The preliminary studies, namely single dose and 19-day, were done by Industrial Biotest. For the single dose, five rats and five mice of each sex were exposed by inhalation to 15,500, 16,500, 16,800, 17,250, 18,500, or 19,000 ppm for 4 h. In the 19-day part of the test, five rats and five mice of each sex were exposed to 0, 1625, 3250, 6500, 13,000, or 16,000 ppm for 6 h/d, 5 d/week for 11 exposures over 19 days. In the next step, 10 rats and 10 mice of each sex were exposed at 0, 524, 1050, 2100, 4200, or 8400 ppm 6 h/d, 5 week for 13 weeks. In the 2-year study, 50 rats of each sex were exposed to levels of 0, 1000, 2000, or 4000 ppm for 6 h/d, 5 d/week for 102 weeks. Similar groups of mice were exposed at levels of 0, 2000, or 4000 ppm for 102 weeks.

9.3.2.4.3 Results

In the single-exposure study, there were deaths in the various groups; likewise in the 19-day test, there were deaths at the higher levels. Dyspnea, ataxia, and hyperactivity were noted. The higher levels in the 13-week study also led to deaths, and body weights were lower. During the 2-year study, exposures of rats to 1000 ppm or higher led to liver disorders. In male rats, there were increases in neoplastic nodules and carcinomas of the liver, and the same types of tumors were increased in female rats. However, both sexes showed an increase in benign, but not malignant, mammary tumors. Mice of both sexes had lower survival and body weights at the higher exposure levels. There were significant increases in lung adenomas and carcinomas and liver tumors. Thus, rats were more sensitive to the hepatotoxicity of dichloromethane; mice to the carcinogenicity.

The NTP decided there was some evidence for carcinogenicity in male rats, clear evidence in female rats, and clear evidence in both male and female mice.

The carcinogenicity of dichloromethane apparently is dependent on strain in rats. Comparable inhalation studies, with even higher exposures, led to no tumors in Sprague–Dawley rats, although there was evidence of hepatotoxicity (Burek et al.,1984; Nitschke et al., 1988).

Epidemiological studies on various groups of workers exposed to dichloromethane were insufficient to confirm or refute an increased risk of cancer in humans (ACGIH, 2001). The ACGIH has decided on a TLV of 50 ppm for dichloromethane to prevent central nervous system effects and anoxia. In view of the NTP carcinogenicity results, the compound has been given the A3 designation, confirmed animal carcinogen of unknown relevance to humans (ACGIH, 2001).

9.3.2.5 Tetrachloroethylene NTP #311

Tetrachloroethylene (perchloroethylene) (CAS No. 127–18–4; formula C_2Cl_4; molecular weight, 165.8; boiling point, 121°C; melting point, −22.4°C; vapor pressure, 20 mmHg at 26.3°C) is a clear colorless liquid which has been extensively used in dry cleaning, metal degreasing, and in manufacture of fluorocarbons. It was tested by Battelle under a contract with the NTP (National Toxicology Program, 1986b).

9.3.2.5.1 Vapor Generation

Liquid tetrachloroethylene was pumped to a cylinder with a glass fiber wick, maintained at 110°C, from which the material vaporized and then was led into the exposure chambers. A Hewlett–Packard gas chromatograph and an automatic sampling valve were on line to monitor levels in the exposure chambers.

9.3.2.5.2 Procedure

The single and 14-day studies were performed at Industrial Biotest. Five rats of each sex were exposed to 2445, 3786, 4092, 4513, or 5163 ppm for 4 h. Likewise five mice of each sex were exposed to 2328, 2445, 2613, 2971, or 3786 ppm for 4 h. In the 14-day test, five male and five female rats and mice were exposed at 0, 100, 200, 425, 875, or 1750 ppm for 6 h/d, 5 d/week for 10 exposures over the 14-day period. In the 13-week part of the test, 10 rats and mice of each sex were exposed by inhalation at 0, 100, 200, 400, 800, or 1600 ppm at the usual schedule for 13 weeks. In the 2-year study, groups of 50 male and female rats were exposed to 0, 200, or 400 ppm at the usual schedule for 103 weeks, whereas similar groups of mice were exposed to 0, 100, or 200 ppm for the same period.

9.3.2.5.3 Results

The preliminary test led to deaths in most animals, indicating that the doses were too high. In similar fashion, the highest doses in the 14-day and 13-week tests led to deaths in the animals. The 2-year exposure led to increases in mononuclear cell leukemia in both male and female rats, plus nonsignificant increases in renal tubule adenomas and carcinomas in the males. However, interpretation of the finding on leukemia has been questioned (ACGIH, 2001). Mice from the 2-year study showed hepatic degeneration and necrosis, with significant increases in hepatocellular adenomas or carcinomas in males and females.

The results led the NTP to state there was clear evidence of carcinogenicity in male rats, some evidence in female rats, and clear evidence in both male and female mice.

Epidemiological studies of laundry and dry-cleaning workers have been compromised by exposure to other solvents besides tetrachloroethylene (ACGIH, 2001). The ACGIH has set a TLV of 25 ppm for tetrachloroethylene to minimize headaches, dizziness, incoordination, and sleepiness from exposure. Because of the carcinogenic effects in rats and mice, the A3 designation, confirmed animal carcinogen with unknown relevance to humans, has been assigned to this solvent (ACGIH, 2001).

9.3.2.6 Methyl Methacrylate NTP #314

Methyl methacrylate (CAS No. 80–62–6; formula $C_5H_8O_2$; molecular weight, 100.13; boiling point, 101°C; melting point, −48°C; vapor pressure 40 mmHg at 25.5°C) is a colorless liquid with an acrid fruity odor. The compound is an intermediate for various polymers, binders, paints, adhesives, and in dental or orthopedic surgery. It was tested by inhalation by Battelle under a contract with the NTP (National Toxicology Program, 1986c).

9.3.2.6.1 Vapor Generation

Liquid methyl methacrylate was pumped from a cylinder to a glass fiber wick maintained at 50°C; the vaporizers were located in the ducts leading directly to the exposure chambers. A gas chromatograph and a photoionization device were used to monitor concentrations in the exposure chambers.

9.3.2.6.2 Procedure

Several of the preliminary tests were done at Industrial Biotest. For the single exposure, five male and five female rats and mice were exposed by inhalation to 1191, 2159, 2220, 4055, 4446, 4632, or 16,000 ppm for 4 h. In 10- or 11-day protocols, five male and five female rats and mice were exposed at 0, 75, 125, 250, 500, or 1000 ppm for 6 h/d, 5 d/week for 9 exposures, or at 0, 500, 1000, 2000, 3000, or 5000 ppm at the same schedule for 10 exposures over 11 days. In the 14-week study, 10 animals of each sex and species were exposed to 0, 500, 1000, 2000, 3000, and 5000 ppm at the usual schedule over the 14-week period. The 2-year study entailed groups of 50 male rats exposed to 0, 500, and 1000 ppm; groups of 50 female rats exposed at 0, 250, and 500 ppm; groups of 50 male and female mice at 0, 500, or 1000 ppm at the 6 h/d, 5 d/week schedule for 102 weeks.

9.3.2.6.3 Results

The preliminary tests showed that a single exposure to 16,000 ppm was toxic; the 10- or 11-day tests showed 5000 ppm as toxic in both species. Likewise, in the 14-week study, 1000 ppm appeared to be an upper limit for exposure to methyl methacrylate. In the 2-year study, there was no evidence of a carcinogenic effect in either species or sex; there were negative trends for the commonly seen tumors. Inflammation of the nasal cavity and atrophy of the olfactory epithelium were observed in exposed animals.

In humans, exposure to methyl methacrylate has led to allergic dermatitis, sensitization, and irritation. The ACGIH has set a TLV of 50 ppm to avoid these undesirable effects, with the A4 designation, not classifiable as a human carcinogen (ACGIH, 2001). IARC has concluded there is evidence suggesting lack of carcinogenicity in experimental animals and that methyl methacrylate is in Group 3, not classifiable as to its carcinogenicity in humans (IARC, 1994).

9.3.2.7 1,2-Epoxybutane NTP #329

1,2-Epoxybutane (CAS No. 106–88–7; C_4H_8O; molecular weight, 72.1; boiling point, 63°C) is a clear colorless liquid used as a stabilizer in chlorinated hydrocarbon solvents. It was tested by inhalation by Battelle under a contract with the NTP (National Toxicology Program, 1988a).

9.3.2.7.1 Vapor Generation

Liquid epoxybutane, held in a reservoir in a vapor hood, was pumped to a cylinder covered with a glass fiber wick, from which the liquid was vaporized and sent to the exposure chambers. To monitor the vapor concentration, a photoionization device and a gas chromatograph were used.

9.3.2.7.2 Procedure

Groups of five rats and five mice of each sex were exposed to levels of 398, 721, 1420, and 2050 ppm and an additional group of rats at 6550 ppm for 4 h. In the 14-day trial, groups of five male and five female rats and mice were exposed at 0, 400, 800, 1600, 3200, or 6400 ppm for 6 h/d, 5 d/week for 10 exposures over 14 days. In the 13-week test, group size was 10 animals and exposure levels were 0, 50, 100, 200, 400, and 800 ppm at the usual schedule. In the 2-year portion of the test, there were 50 animals in each group exposed at 0, 200, and 400 ppm for 102 weeks (rats) and 0, 100, and 200 ppm (mice), also for 102 weeks.

9.3.2.7.3 Results

The single-exposure trial showed that 6550 ppm was toxic to all rats; there was eye irritation at 142 ppm. With mice there were deaths at 398 ppm and above, as well as eye irritation at 1420 ppm. In the 14-day trial, 1600 ppm and above was toxic; mice showed toxicity at 800 ppm and above. The 13-week study led to survival, but with lower body weights at 800 ppm for rats. At 800 ppm all mice died; there was respiratory tract irritation. Survival of all animals in the 2-year study was fairly good, and body weights were only slightly less-than those of controls. However, papillary adenomas of the nasal cavity, as well as lung neoplasms, were significantly higher in the high-dose male rats. The increase in nasal cavity tumors was not statistically significant in female rats. Although both male and female mice had nonneoplastic lesions of the nasal cavity, there was no increase in tumors.

The NTP decided that there was clear evidence for the carcinogenicity of 1,2-epoxybutane in male rats, equivocal evidence in female rats, and no evidence in both male and female mice.

The ACGIH has not set any TLV for 1,2-epoxybutane.

9.3.2.8 Bromoethane (Ethyl Bromide) NTP #363

Bromoethane or ethyl bromide (CAS No. 74–96–4; formula C_2H_5Br; molecular weight, 108.98; boiling point, 38.4°C; melting point, −119°C; vapor pressure, 400 mmHg at 21°C) is a colorless volatile liquid used as an alkylating agent and solvent. It was tested by inhalation at Battelle under a contract with the NTP (National Toxicology Program, 1989b).

9.3.2.8.1 Vapor Generation

Liquid bromoethane was pumped from a reservoir to a vaporizer without need for warming; the vapor was diluted with air before entering the exposure chambers. The concentration in the chambers was determined with a gas chromatograph.

9.3.2.8.2 Procedure

In the preliminary trial, five rats and five mice of each sex were exposed to the vapor at 625, 1250, 2500, 5000, or 10,000 ppm for 4 h. The 14-day study began with groups of five rats and five mice of each sex exposed to 0, 250, 500, 1000, 2000, or 4000 ppm for 6 h/d, 5 d/week for 10 exposures over 14 days. In the 14-week trial, 10 male and 10 female rats and mice received exposures of 0, 100, 200, 400, 800, or 1600 ppm 6 h/d, 5 d/week for 65 exposures over 14 weeks. The 2-year study began with 49 or 50 rats and mice of each sex at 0, 100, 200, or 400 ppm at the usual schedule for 103 or 104 weeks.

9.3.2.8.3 Results

In the single exposure, levels of 5000 or more were toxic to rats, whereas at 1250 ppm or higher, the mice died. The 14-day trial showed that both rats and mice died at levels of 2000 ppm and above. In the 14-week test, 1600 ppm was toxic for rats; for mice 800 ppm and above led to deaths.

Histopathological examination of the animals from the 2-year study showed that male rats had an increased incidence of pheochromocytomas of the adrenal medulla, gliomas of the brain, and pulmonary tumors. Female rats also had brain and lung tumors. Male mice had an increase in lung tumors, whereas female mice showed a significant incidence of adenomas, carcinomas, adenocarcinomas, and squamous cell carcinomas of the uterus. The results led NTP to conclude there was some evidence of carcinogenicity in male rats, equivocal evidence in female rats and male mice, and clear evidence in female mice.

On the basis of the animal tests and reports on exposed humans, ACGIH has recommended a TLV of 5 ppm for bromoethane to avoid liver, kidney, and central nervous system effects. In view of the NTP study, an A3 designation, confirmed animal carcinogen with unknown relevance to humans, was assigned (ACGIH, 2001).

9.3.2.9 Toluene NTP #371

Toluene (CAS No. 108–88–3; formula C_7H_8; molecular weight, 92.13; boiling point, 110.63°C; melting point, −94.97°C; vapor pressure, 59.3 mmHg at 40°C) is a colorless flammable liquid with a characteristic aromatic odor. It is used to increase fuel and gasoline octane ratings, as a solvent, and in the synthesis of explosives, polymer intermediates, and other products. It was tested by inhalation by International Research and Development Corporation under a contract with the NTP (National Toxicology Program, 1990a).

9.3.2.9.1 Vapor Generation

Liquid toluene was pumped to an atomizer, heated to 80°C; the toluene vapor was diluted with air to the desired concentration. Levels of toluene in the chambers were measured by a MIRAN gas-phase spectrometer coupled to a computer.

9.3.2.9.2 Procedure

Because of the extensive database on the toxicity of toluene, the studies began with 14- and 15-week trials. Groups of 10 male and 10 female rats and mice were exposed at 0, 100, 625, 1250, 2500, or 3000 ppm for 6.5 h/d, 5 d/week. Sperm morphology and vaginal cytology were examined in the survivors at the 0, 100, 625, or 1250 ppm levels. The next phase was a combination 15-month and 2-year trial. Thus, groups of 60 male and female rats were exposed at levels of 0, 600, or 1200 ppm for 6.5 h/d, 5 d/week for 15 months or 103 weeks. Similar groups of male and female mice were exposed at 0, 120, 600, or 1200 ppm under the same conditions as the rats. At 15 months 10 animals from each group were examined for blood parameters.

9.3.2.9.3 Results

The 14- to 15-week study showed that 3000 ppm was toxic to rats; whereas 1250 ppm was a top limit for mice. In the long-term study, various nonneoplastic lesions were noted in both rats and mice such as nephropathy in rats, inflammation and degeneration of the olfactory and respiratory epithelium. However, there was no increase in neoplastic lesions in either the rats or the mice.

The ACGIH has set a TLV of 50 ppm for toluene to avoid central nervous system effects such as headache, dizziness, memory lapse, and other effects. In view of the negative carcinogenicity results in both male and female rats and mice, an A4 designation has been given, namely, not classifiable as a human carcinogen (ACGIH, 2001).

9.3.2.10 Vinyl Toluene NTP #375

Vinyl toluene (CAS No. 25013–15–4; formula C_9H_{10}; molecular weight, 118.18; boiling point, 172°C; freezing point, −77°C; vapor pressure, 1.1 mmHg at 20°C) is a colorless combustible liquid with a disagreeable odor. Commercial material is a mixture of meta and para isomers and is used as a monomer in the plastics and surface coating industries. Vinyl toluene was tested for the NTP under a contract with Midwest Research Institute (National Toxicology Program, 1990b).

9.3.2.10.1 Vapor Generation

Liquid vinyl toluene, contained in a reservoir, was metered into a tube through which warmed air passed and carried the vapor to the ducts leading to the exposure chambers. The concentration in the chambers was monitored by an automatic sampling system coupled to a gas chromatograph.

9.3.2.10.2 Procedure

The test began with 15-day studies in which groups of five male and five female rats and mice were exposed to 0, 200, 400, 800, or 1300 ppm for 6 h/d, 5 d/week for 10 exposures. In the 13-week trial, groups of 10 male and female rats were exposed to 0, 25, 60, 160, 400, or 1000 ppm for the 6 h/d, 5 d/week schedule. Similar groups of mice received 0, 10, 25, 60, or 160 ppm exposures. The 2-year study involved groups of 49 or 50 rats of each sex exposed at the usual schedule to 0, 100, or 300 ppm for 103 weeks, while similar groups of mice had exposures to 0, 10, or 25 ppm.

9.3.2.10.3 Results

In the 15-day study, mice were more susceptible to the toxicity of vinyl toluene for deaths occurred at 200 ppm and above. However, rats survived but body weights were affected at 400 ppm. In the 13-week trial, all rats survived but body weights were lower than in controls at 160 ppm. Increased liver weights and nephropathy occurred in male rats at the higher dose levels. Body weights of mice at 25 ppm and higher were affected, and pulmonary inflammation occurred at the highest dose level.

In the 2-year study, survival was not reduced by vinyl toluene exposure; rats showed relatively little effect on body weights. Mice at the 25 ppm level had weights 10 to 23% lower than did the controls. In both rats and mice, there were degenerative lesions of the nasal mucosa and other nonneoplastic lesions. However, no increase in neoplastic lesions was noted in either sex of the rats or the mice. The incidence of the spontaneous lung and liver tumors of the mice was lower.

The NTP concluded that there was no evidence for the carcinogenicity of vinyl toluene in either male and female rats and male and female mice.

The ACGIH has set a TLV of 50 ppm for vinyl toluene to minimize ocular and mucous membrane irritation. In view of the negative carcinogenicity results, the compound was given the A4 designation, not classifiable as a human carcinogen (ACGIH, 2001).

9.3.2.11 Allyl Glycidyl Ether NTP #376

Allyl glycidyl ether (CAS No. 106–92–3; formula $C_6H_{10}O_2$; molecular weight, 114.14; boiling point, 153.9°C; freezing point, −100°C; vapor pressure, 4.7 mmHg at 25°C) is a colorless flammable liquid

used as a reactive diluent, as a resin intermediate, and as a stabilizer of chlorinated compounds, vinyl resins and rubber. It was tested at Battelle under a contract with the NTP (National Toxicology Program, 1990c).

9.3.2.11.1 Vapor Generation

For generation of allyl glycidyl ether vapor, the liquid was pumped from a reservoir to a vaporizer maintained at approximately 105°C; the vapor was directed into the ducts leading to the exposure chambers. A gas chromatograph with a flame ionization detector monitored the concentrations of the test material.

9.3.2.11.2 Procedure

In this study, Osborne–Mendel rats and B6C3F$_1$ mice, were used. For the 14-day study, five male and five female rats were exposed to 0, 25, 50, 100, 200, or 500 ppm of the allyl compound for 6 h/d, 5 d/week for a total of 10 exposures. Groups of five mice of each sex were exposed under the same schedule to 0, 25, 40, or 100 ppm. In the 13-week test, 10 male and 10 female rats were exposed by inhalation to 0, 4, 10, 30, 100, or 200 ppm 6 h/d, 5 d/week for the 13-week period. The mice, in groups of 10 of each sex, were exposed to 0, 1, 4, 10, or 30 ppm for the same period. For the 2-year test, groups of 50 male and female rats and mice were exposed to 0, 5 or 10 ppm at the 6 h/d, 5 d/week schedule for 102 or 103 weeks.

9.3.2.11.3 Results

In the preliminary studies, survival and body weights of rats were adversely affected at levels above 25 ppm; for mice the situation was similar. In the 13-week study, all the animals survived, but body weights were lower than in controls. In the final study, there was a high incidence of inflammation, hyperplasia, and degeneration of the respiratory and olfactory epithelium. In male rats three separate tumors occurred in the respiratory epithelium, leading the NTP to decide there was equivocal evidence of carcinogenicity in male rats. For female rats, there was no evidence of carcinogenicity, despite the inflammation of the respiratory tract. Male mice were considered to show some evidence for carcinogenicity, based on respiratory tract adenomas; for female mice there was equivocal evidence.

Workers exposed to allyl glycidyl ether have shown dermatitis, irritation, sensitization, and allergic reactions. Therefore, the ACGIH has set a TLV of 1 ppm to prevent irritation. In view of the equivocal results from the carcinogenicity test, ACGIH has decided on the A4 designation, not classifiable as a human carcinogen (ACGIH, 2001).

9.3.2.12 Tetranitromethane NTP #386

Tetranitromethane (CAS No. 509–14–8; formula CN_4O_8; molecular weight, 196.04; boiling point, 126°C; melting point, 13.8°C; vapor pressure, 8.4 mmHg at 20°C) is a colorless to yellow oily liquid which has been used as an explosive, as an additive in diesel fuel to increase octane rating, and in rocket propellants. It was tested under a contract from the NTP with Midwest Research Institute (National Toxicology Program, 1990d).

9.3.2.12.1 Vapor Generation

The vapor was generated by bubbling nitrogen through the liquid; the vapor then entered the air-stream at the top of the exposure chambers and was diluted with air in the chamber plenum. The concentration in the chambers was monitored with a Wilks Miran Infrared Process Analyzer or a Miran Infrared Gas Analyzer.

9.3.2.12.2 Procedure

The tests began with the 14-day experiments. Groups of five male and five female rats and mice were exposed to the vapor at levels of 0, 2, 5, 10, or 25 ppm for 6 h/d, 5 d/week for 10 days over a 14-day period for the rats; mice had the same treatment except that a 50 ppm group was also added.

In the 13-week trial, groups of 10 rats and 10 mice of each sex received 0, 0.2, 0.7, 2, 5, or 10 ppm exposures over the 13-week period. In the 2-year study, groups of 50 male and female rats received 0, 2, or 5 ppm for 6 h/d, 5 d/week. Similar groups of mice had 0, 0.5, or 2 ppm exposures on the same schedule.

9.3.2.12.3 Results

In the 14-day trial, all rats on the highest level died, and body weights were lower than controls in the 10 ppm group. For mice, the same situation held. Animals at the top level had metaplasia or inflammation of the respiratory epithelium.

Rats at the top level in the 2-year study did not survive well and had reduced body weights after about 70 weeks. Alveolar/bronchiolar adenomas and carcinomas increased significantly in both male and female rats. Similar results were noted in mice where the lung tumors tended to metastasize. Hyperplasia and inflammation occurred in exposed rats and mice.

These results caused the NTP to conclude that there was clear evidence for the carcinogenicity of tetranitromethane in male and female rats and male and female mice.

The ACGIH has set a TLV of 0.005 ppm for tetranitromethane to avoid irritation, with the A3 designation, confirmed animal carcinogen with unknown relevance for humans (ACGIH, 2001).

9.3.2.13 Hexachlorocyclopentadiene NTP #437

Hexachlorocyclopentadiene (CAS No. 77–47–4; formula C_5C_{16}; molecular weight, 272.77; boiling point, 239°C at 753 mmHg; melting point, −9°C; vapor pressure, 0.08 mmHg at 25°C) is a pale yellow to amber nonflammable liquid with a pungent odor. It is primarily used as an intermediate in the production of some pesticides. It was tested by Battelle under a contract with the NTP (National Toxicology Program, 1994c).

9.3.2.13.1 Vapor Generation

Liquid hexachlorocyclopentadiene was pumped under nitrogen to a vaporizer which had a glass fiber wick; the vaporizer was warmed to 81°C for the 2-year studies and 100°C for the 13-week tests. Filtered fresh air was drawn across the vaporizer, thus taking the vapor into the exposure chambers. A condensation nuclei detector was used to ensure that the vapor and not an aerosol was present. Concentrations were monitored by a gas chromatograph.

9.3.2.13.2 Procedure

The bioassay began with the 13-week studies. Groups of 10 male and 10 female rats and mice were exposed to 0, 0.04, 0.15, 0.4, 1, or 2 ppm for 6 h/d, 5 d/week for 13 weeks. In the 2-year trial, groups of 60 male and female rats and mice were exposed to 0, 0.01, 0.05, 5 d/week for 103 or 104 weeks. At 15 months 10 animals per group were taken for interim evaluation.

9.3.2.13.3 Results

In the 13-week trial, levels of 2 ppm were toxic to rats while 1 ppm was toxic to the mice. Body weights were lower in exposed animals. In the 2-year study, survival of exposed animals was good, although body weights were affected somewhat at the highest level. Exposed animals showed some pigmentation of the lungs and irritation of the mucous membranes. However, no neoplastic effects were noted. The NTP decided that there was negative evidence for the carcinogenicity of hexachlorocyclopentadiene in male and female rats and mice.

The ACGIH has set a TLV of 0.01 ppm for this compound, with the A4 designation, not classifiable as a human carcinogen (ACGIH, 2001).

9.3.2.14 Acetonitrile NTP #447

Acetonitrile (CAS No. 75–05–8; formula CH_3CN; molecular weight, 41.05; boiling point, 81.6°C; melting point, −45°C; vapor pressure, 74 mmHg at 20°C) is a volatile, clear, colorless liquid with

an ether-like odor. It is used to extract fatty acids, animal and vegetable oils, for crystallization of various pharmaceutical and agricultural products, as a solvent for spinning fibers, for casting and molding plastics, and as a solvent in numerous laboratory operations. Because of widespread exposure, it was tested by inhalation by Battelle under a contract with the NTP (National Toxicology Program, 1996a).

9.3.2.14.1 Vapor Generation

Liquid acetonitrile, held in a stainless steel reservoir under a nitrogen atmosphere, was pumped to a vaporizer, namely a heated stainless steel cylinder with a glass fiber wick. The vapor was mixed with filtered air, drawn into a distribution manifold, diluted with air to the desired concentration, and delivered to the exposure chambers. Concentrations were monitored with an on-line gas chromatograph.

9.3.2.14.2 Procedure

The bioassay began with 13-week studies since toxicity data on both single and short-term exposures were available. Groups of 10 male and 10 female rats and mice were exposed by inhalation to levels of 0, 100, 200, 400, 800, or 1600 ppm for 6 h/d, 5 d/week for 13 weeks. The animals were used for clinical pathology studies at the end of the exposure.

In the 2-year study, groups of 56 male and 56 female rats were exposed at the usual schedule to 0, 100, 200, or 400 ppm for 103 weeks. At 15 months 8 male and 8 female rats from each group were evaluated for hematology and clinical chemistry. Groups of 60 male and 60 female mice were exposed similarly to 0, 50, 100, or 200 ppm for 103 weeks. At 15 months, 10 animals from each group were taken for evaluation.

9.3.2.14.3 Results

The 13-week trial in rats led to early deaths at exposures of 800 ppm and higher, but body weights were not affected. In the 2-year study, survival and body weights of exposed rats were similar to those of controls. At the 2-year period, there were increases in liver tumors in male rats, as well as increases in pheochromocytomas of the adrenal at the two lower doses. Female rats did not show any increases in tumors. The 13-week trial in mice led to deaths at 400 ppm and higher. Body weights were also affected. However, in the 2-year study survival and body weights were similar to those of controls. Male mice at the top dose showed some increase in lung adenomas, whereas male mice at the middle dose had an increase in liver tumors. Female mice showed no increases in these tumors, but both male and female mice had a marginal increase in squamous hyperplasia of the forestomach.

The NTP concluded there was equivocal evidence for carcinogenicity in male rats, but no evidence in female rats and male and female mice.

The ACGIH has recommended a TLV of 40 ppm for acetonitrile to minimize the potential for headaches, nausea, and respiratory distress in exposed workers. Based on the results of the NTP bioassay, ACGIH has given acetonitrile the A4 designation, not classifiable as a human carcinogen (ACGIH, 2001).

9.3.2.15 Isobutyl Nitrite NTP #448

Isobutyl nitrite (CAS No. 542–56–3; formula $C_4H_9NO_2$; molecular weight, 103.12; boiling point, 67°C) is a colorless volatile liquid that has been used to a limited extent as an intermediate in synthesis of aliphatic nitriles, as a jet propellant, and as an incense or room odorizer. It was tested by the IIT Research Institute (Illinois Institute of Technology) under a contract with the NTP (National Toxicology Program, 1996b).

9.3.2.15.1 Vapor Generation

Isobutyl nitrite vapor was generated by pumping the liquid from reservoir bottles to glass vapor transpiration bubblers. Nitrogen gas passed through and carried the vapor to the exposure chambers. Chamber concentrations were measured by gas chromatography.

9.3.2.15.2 Procedure

Acute toxicity values for isobutyl nitrite were known from the literature. Thus 16-day studies were initiated at levels of 0, 100, 200, 400, 600, or 800 ppm for groups of five male and five female rats and mice. Exposure was by inhalation for 6 h/d, 5 d/week for 12 exposures during a 16-day period.

The 13-week studies were conducted at levels of 0, 10, 25, 75, 150, or 300 ppm isobutyl nitrite in groups of 10 male and 10 female rats and mice, also at the 6 h/d, 5 d/week schedule. In the 2-year test, groups of 56 male and 56 female rats were exposed to 0, 37.5, 75, or 150 ppm for 6 h/d, 5 d/week for 103 weeks. After 15 months 10 animals from each group were evaluated for clinical pathology. Likewise, groups of 60 male and 60 female mice were exposed to the same concentrations at the same schedule as the rats. At 15 months 10 animals from each group were evaluated for both clinical pathology and histopathology.

9.3.2.15.3 Results

In the 16 day study, levels of 600 or 800 ppm were fatal to all rats, whereas lower body weights and lethargy were noted at 400 ppm. Mice died at 800 ppm; lung weights of mice at 600 ppm were higher than in controls; body weights were lower. In the 13-week segment, all rats survived, but body weights at the top level were lowered. A similar situation held for the mice. At levels over 75 ppm, both rats and mice had higher methemoglobin, anemia, and epithelial cell hyperplasia of the respiratory tract. In the 2-year study, exposed rats and mice generally had better survival than did the controls. At the 15-month interim period, minimal methemoglobinemia was noted in rats at 37.5 ppm or higher; this condition appeared in mice at 75 ppm or higher. By two years, there were significant increases in alveolar/bronchiolar adenomas or carcinomas in both male and female rats and alveolar epithelial hyperplasia was higher. Mice also showed increases in the same conditions. The results led the NTP to conclude that isobutyl nitrite was clearly carcinogenic in rats, but that for mice the verdict was that there was some evidence of carcinogenicity.

The ACGIH has not proposed a TLV for isobutyl nitrite, but methemoglobin inducers are covered under the Biological Exposure Indices (BEI).

9.3.2.16 Nitromethane NTP #461

Nitromethane (CAS No. 75–52–5; formula CH_3NO_2; molecular weight, 61.02; boiling point, 101.2°C; melting point, −29°C; vapor pressure, 27.8 mmHg at 20°C) is a colorless oily liquid used as a chemical stabilizer, as a fuel additive for rockets, racing cars, boats, model engines, as an intermediate in synthesis, and in explosive mixtures for mining, oil well drilling, and in seismic exploration. It was tested by inhalation at Battelle under a contract with the NTP (National Toxicology Program, 1997b).

9.3.2.16.1 Vapor Generation

Nitromethane was pumped under a nitrogen blanket from a reservoir to a liquid distribution manifold leading to heated wick vaporizers; the vapor was diluted with air and delivered to the inhalation chambers. Concentrations were monitored with an on-line gas chromatograph.

9.3.2.16.2 Procedure

Since there were data on the toxicity of nitromethane, 16-day studies were initiated directly. Groups of five male and five female rats and mice were exposed to 0, 94, 188, 375, 750, or 1500 ppm by inhalation for 6 h/d, 5 d/week for 16 days. In the 13-week part of the test, groups of 10 male and 10 female rats and mice were exposed to 0, 94, 188, 750, or 1500 ppm 6 h/d, 5 d/week for 13 weeks. Groups of 10 additional male and female rats were exposed under the same conditions for clinical pathology workup at 3 and 23 days. In the 2-year test, groups of 50 male and 50 female rats were exposed at 0, 94, 188, or 375 ppm for 6 h/d, 5 d/week for 103 weeks. Similar groups of 50 male and 50 female mice were exposed at 0, 188, 375, or 750 ppm for 103 weeks.

9.3.2.16.3 Results

All rats survived in the 16-day test, but body weights were lower at the top dose. Degeneration of the olfactory epithelium and sciatic nerve degeneration were observed at the three highest dose levels. All mice in the 16-day study survived with no effect on body weights and minimal effect on the respiratory epithelium. All rats survived the 13-week exposure, but body weights were reduced at the two top levels. Hindlimb paralysis occurred as well as a microcytic anemia. Mice survived the 13-week exposure, but there were lower sperm motility, longer estrus cycle times, increased kidney weights, and degeneration and hyaline droplets of the olfactory and respiratory epithelium.

Body weights and survival rates for both male and female rats in the 2-year study were similar to those of the controls. In females mammary gland tumors were significantly increased at the two higher dose levels. Renal tubule hyperplasia and adenomas in males increased but not significantly; even after step sectioning of the kidneys. Mice in this part of the study had survival and body weights similar to those in controls. However, there were significant increases in Harderian gland tumors for both males and females. Hepatocellular adenomas and carcinomas were significantly increased in females, while both sexes showed increases in lung tumors. Nonneoplastic lesions of the nose were noted in mice in all exposure levels.

The results of these tests led the NTP to declare there was no evidence of carcinogenicity in male rats, but clear evidence for the carcinogenicity of nitromethane in female rats and male and female mice.

The ACGIH has set a TLV of 20 ppm for nitromethane, with the A3 designation, confirmed animal carcinogen with unknown relevance to humans (ACGIH, 2001).

9.3.2.17 Ethylbenzene NTP #466

Ethylbenzene (CAS No. 100–41–4; formula C_8H_{10} molecular weight, 106.16; boiling point, 136.2°C; melting point, −95.0°C; vapor pressure, 10 mmHg at 25.7°C) is a colorless flammable aromatic liquid used mainly in production of styrene. Other uses include manufacture of diethylbenzene, acetophenone, and ethylanthraquinone, as well as a solvent in insecticide sprays, degreasing cleaners, paints, adhesives, rust preventives and as an antiknock agent in fuels. The potential for widespread exposure led NTP to test it under a contract with IIT Research Institute (National Toxicology Program, 1999a).

9.3.2.17.1 Vapor Generation

From a heated reservoir of the liquid ethylbenzene, the vapor was carried by nitrogen gas into heated stainless steel transfer lines that led to exposure chambers. Chamber concentrations were monitored by an on-line gas chromatograph equipped with a flame ionization detector.

9.3.2.17.2 Procedure

Because there were relatively extensive data on ethylbenzene toxicity, no preliminary studies were done. Groups of 50 male and 50 female rats and mice were exposed by inhalation at 0, 75, 250, or 750 ppm for 6 h/d, 5 d/week for 103 weeks (mice) or 104 weeks (rats).

9.3.2.17.3 Results

Male rats at the 750 ppm level survived less well than did controls. Exposed female rats generally had lower body weights than did controls. The exposed rats had relatively high incidences of renal tubular adenomas and carcinomas, especially when step sections of the kidneys were made. Interstitial cell adenomas of the testis were increased in male rats, but mononuclear cell leukemia decreased in males at the top dose.

In the mice mean body weights of 75 ppm females were greater than those of controls; survival of test animals was similar to that of controls. Alveolar/bronchiolar adenomas or carcinomas were increased somewhat in female mice and significantly in male mice. Although male mice had significant increases in hepatocyte hypertrophy, necrosis, and other alterations in the liver after the

2-year exposure, there were no liver tumors. However, female mice had significant increases in hepatocellular adenomas or carcinomas.

The NTP concluded that under these conditions, there was clear evidence of carcinogenicity in male rats, some evidence in female rats, and likewise, some evidence in both male and female mice.

The ACGIH has set a TLV of 100 ppm for ethylbenzene to avoid irritation and central nervous system effects. The compound also has the A3 designation, confirmed animal carcinogen with unknown relevance to humans (ACGIH, 2003).

9.3.2.18 Chloroprene NTP #467

Chloroprene (CAS No. 126–99–8; formula C_4H_5Cl; molecular weight, 88.54; boiling point, 59.4°C; vapor pressure, 188 mmHg at 20°C) is a colorless flammable liquid used as an intermediate in the production of neoprene. It was tested at Battelle under a contract with the NTP (National Toxicology Program, 1998a).

9.3.2.18.1 Vapor Generation

Chloroprene was pumped at a steady rate from a chilled reservoir into a rotating flask kept in a hot-water bath with a temperature-controlled condenser which returned material boiling higher than chloroprene to the rotating flask. The vapor was led to a distribution manifold, diluted with air or nitrogen, and pumped through metering valves to the exposure chambers. Levels of chloroprene were monitored by an on-line gas chromatograph.

9.3.2.18.2 Procedure

In view of data on the acute toxicity of chloroprene, the testing process began with 16-day studies. Groups of 10 male and 10 female rats and mice were exposed at levels of 0, 32, 80, 200, or 500 ppm (rats) or 0, 12, 32, 80, or 200 ppm (mice) for 6 h/d, 5 d/week for 16 days. Additional groups of 10 male and 10 female mice were included for hematology and clinical chemistry analyses. For the 13-week test, groups of 10 male and 10 female rats were exposed to levels of 0, 5, 12, 32, 80, or 200 ppm whereas groups of 10 male and 10 female mice were exposed to 0, 5, 12, 32, or 80 ppm for 6 h/d, 5 d/week for 13 weeks. Additional groups of both rats and mice were exposed and then evaluated for clinical pathology, tissue glutathione, coagulation, and hematology. In all 120 male rats, 120 female rats, 40 male mice, and 40 female mice were used for these auxiliary studies. For the 2-year study, groups of 50 male and 50 female rats and mice were exposed by inhalation at levels of 0, 12.8, 32, or 80 ppm for 6 h/d, 5 d/week for 105 weeks.

9.3.2.18.3 Results

In the 16-day test, rats at the two higher dose levels died, while 200 ppm was toxic to the mice. In the 13-week study, rats had survival and body weights comparable to those of controls, but although all mice survived, their body weights were affected. In the 2-year study, male rats had lower survival than did controls; female rats had body weights and survival equivalent to that of controls. Squamous cell papillomas and carcinomas of the oral cavity were increased significantly in both sexes; males had follicular cell adenomas or carcinomas of the thyroid. Lung tumors increased in both sexes; whereas females had more fibroadenomas of the mammary gland, males showed a tendency to develop kidney tumors.

In the mice, survival and body weights were lower than controls. Both males and females had significant increases in adenomas and carcinomas of the lung. Hemangiomas or hemangiosarcomas of many organs increased in both sexes; carcinomas of the Harderian gland, mammary tumors, and liver tumors increased. These results led the NTP to conclude that there was clear evidence for the carcinogenicity of chloroprene in both male and female rats and mice.

The ACGIH has set a TLV of 10 ppm for chloroprene to prevent irritation and effects on the liver and reproductive systems (ACGIH, 2001). IARC has decided that although there is inadequate

evidence in humans for the carcinogenicity of chloroprene, the compound belongs in Group 2B, namely possibly carcinogenic to humans (IARC, 1994).

9.3.2.19 Isobutyraldehyde NTP #472

Isobutyraldehyde (CAS No, 78–84–2; formula C_4H_8O; molecular weight, 72.1; boiling point, 64°C; melting point, −65.9°C; vapor pressure, 173 mmHg at 25°C) is a colorless liquid with a pungent odor and fruity taste. It is used as an intermediate in synthesis of various products, such as plasticizers, resins, rubber antioxidants, accelerators. It is a natural constituent of many foods and flavoring agents. Isobutyraldehyde was tested by Battelle under a contract with the NTP (National Toxicology Program, 1999b).

9.3.2.19.1 Vapor Generation

The vapor was generated by bubbling nitrogen through a column of liquid isobutyraldehyde at constant temperature in a water bath; the vapor was then conducted to a manifold for delivery to the exposure chambers. Concentrations were monitored by an infrared spectrometer for the 13-week studies and by an on-line gas chromatograph for the 2-year exposure.

9.3.2.19.2 Procedure

Groups of 10 male and 10 female rats and mice were exposed to isobutyraldehyde at levels of 0, 500, 1000, 2000, 4000, or 8000 ppm for 6 h/d, 5 d/week for 13 weeks. In the 2-year study, groups of 50 male and 50 female rats and mice were exposed by inhalation to 0, 500, 1000, or 2000 ppm for 6 h/d, 5 d/week for 105 weeks. Animals were then observed for 3–7 days prior to necropsy.

9.3.2.19.3 Results

In the 13-week study, survival and body weights were adversely affected at levels over 2000 ppm. Inflammation, necrosis, and hyperplasia of the nasal cavity occurred. In the 2-year study, survival and mean body weights of exposed male and female rats were similar to those of controls. Male mice at the top dose level had lower survival rates than controls, but female survival was like that of controls. During the second year, body weights of exposed female mice were lower than those of controls.

In the 2-year study, rats showed squamous metaplasia of the respiratory epithelium, degeneration of the olfactory epithelium, and inflammation. There was no significant incidence of neoplastic lesions. Mice also demonstrated degeneration of the olfactory epithelium. Liver tumor incidence was lower in exposed mice.

The NTP concluded there was no evidence of carcinogenic activity in male and female rats and mice exposed to isobutyraldehyde.

9.3.2.20 Tetrahydrofuran NTP #475

Tetrahydrofuran (CAS No. 109–99–9; formula C_4H_8O; molecular weight, 72.10; boiling point, 66°C; melting point, −108.5°C; vapor pressure, 114 mmHg at 15°C; and 204 mmHg at 30°C) is a colorless volatile liquid with an ethereal odor and a pungent taste. It has many uses as a reaction medium, as an intermediate, in fabrication of articles for packaging, transporting, and storing of foods, and as an intermediate in polymerization solvents for various materials. It was tested by inhalation by Battelle under a contract with the NTP (National Toxicology Program, 1998b).

9.3.2.20.1 Vapor Generation

Tetrahydrofuran vapor was generated with a rotary evaporation system; vapor from the condensing column of the evaporator entered a distribution manifold with individual delivery lines for each exposure chamber. The vapor was injected into the chamber inlet and diluted with filtered air to achieve the desired exposure concentration. A small particle detector was in place to ensure that the vapor and not an aerosol was produced. Chamber concentrations were monitored by an on-line gas chromatograph with a flame ionization detector.

9.3.2.20.2 Procedure

For the 14-week part of the test, groups of 10 male and 10 female rats and mice were exposed to tetrahydrofuran vapor at concentrations of 0, 66, 200, 600, 1800, and 5000 ppm for 6 h/d, 5 d/week for 14 weeks. Hematology and clinical chemistry studies were performed on the rats at the end of the exposure period. For the 2-year study, groups of 50 rats and mice of each sex were exposed at 0, 200, 600, and 1800 ppm by inhalation for 6 h/d, 5 d/week for 105 weeks.

9.3.2.20.3 Results

In the 14-week study, all rats survived and body weights were similar to those of controls. However, all rats at 5000 ppm exhibited ataxia. In the 2-year trial, body weights of exposed rats were similar to those of controls, while survival was comparable until approximately the last two months of the study. Renal tubule adenomas were marginally but not statistically increased in male rats at the 600 and 1800 ppm levels and testicular adenomas were elevated somewhat. In female rats, the slight increase in mammary fibroadenomas was not considered related to exposure. The 14-week study in mice showed that 5000 ppm was too toxic for male mice. Body weights in exposed mice were comparable to those of controls, but 1800 and 5000 ppm led to narcosis in the mice. In the 2-year trial, survival of male mice at 1800 ppm was less than that of controls; all other test mice had survival similar to that of controls. Male mice at 1800 ppm also exhibited narcosis. Although liver neoplasms were significantly greater in exposed female mice, male mice showed a lower incidence of these tumors.

The data led NTP to conclude there was some evidence for carcinogenicity in male rats, none in female rats and male mice and clear evidence in female mice.

The ACGIH has set a TLV of 200 ppm for tetrahydrofuran to avoid respiratory tract irritation and damage (ACGIH, 2001).

9.3.2.21 Furfuryl Alcohol NTP #482

Furfuryl alcohol (CAS No. 98–00–0; formula $C_5H_6O_2$; molecular weight, 98.1; boiling point, 170°C; vapor pressure, 0.4 mmHg at 20°C) is a clear liquid used as starting material for various polymers and resins, especially binders in foundry sands. The inhalation studies were done at Battelle under contact to the NTP (National Toxicology Program, 1999c).

9.3.2.21.1 Vapor Generation

Furfuryl alcohol was pumped into a glass evaporation column from which a heated nitrogen stream vaporized the alcohol which then passed to a mixing chamber. It was then pumped into the distribution line for the exposure chambers. Vapor concentrations were monitored by a gas chromatograph.

9.3.2.21.2 Procedure

Because there were data on the acute effects of furfuryl alcohol, the protocol began with the 16-day studies. Groups of five male and five female rats and mice were exposed to 0, 16, 31, 63, 125, or 250 ppm 6 h/d, 5 d/week for 12 exposures over 16 days. The 14-week portion of the test used groups of 10 male and 10 female rats and mice exposed at 0, 2, 4, 8, 16, or 32 ppm for 6 h/d, 5 d/week for the 14 weeks. In the 2-year study, groups of 50 male and 50 female rats and mice were exposed to 0, 2, 8, or 32 ppm for 6 h/d, 5 d/week for 105 weeks.

9.3.2.21.3 Results

In the 16-day study, levels of 125 and 250 led to obvious toxicity in both rats and mice. All animals survived in the 14-week study, but body weights were lower at the top levels.

In the 2-year study, male rats at the 32 ppm level had poor survival, but there was little effect in females. Mice in this part of the study had body weights and survival similar to that of the controls. In both rats and mice there was inflammation and hyperplasia of the nose, atrophy of the olfactory epithelium, and hyaline degeneration of the respiratory epithelium. Male rats showed a significant increase in tumors of the nose at the top-dose level, while females had a small increase in renal

tubule neoplasms. Although female mice had no evidence of carcinogenicity, male mice at the top dose had an increase in renal tubule neoplasms, after step sections were made. The results led the NTP to conclude there was some evidence for the carcinogenicity of furfuryl alcohol in male rats and male mice, equivocal evidence in female rats, and no evidence in female mice.

The ACGIH has decided on a TLV of 10 ppm for furfuryl alcohol to avoid irritation (ACGIH, 2001).

9.3.2.22 2-Butoxyethanol NTP #484

2-Butoxyethanol (CAS No. 111–76–2; formula $C_6K_{14}O_2$; molecular weight, 118.17; boiling point, 171°C; melting point, −70°C; vapor pressure 0.88 mmHg at 25°C) is a colorless liquid used as a solvent in lacquers, enamels, varnish, latex paint, in various paint thinners or strippers, in inks, degreasers, and in industrial and household cleaners, in addition to being an intermediate for production of acetate esters and plasticizers. Because of its extensive use, it was tested at Battelle under a contract with the NTP (National Toxicology Program, 2000a).

9.3.2.22.1 Vapor Generation

2-Butoxyethanol was pumped into a glass column warmed by electric heat tape and distributed from there to a vapor distribution manifold; from there the vapor went to the exposure chambers. Chamber concentrations were monitored by an on-line gas chromatograph; samples were drawn every 16 min for the 14-week study and every 30 min for the 2-year test.

9.3.2.22.2 Procedure

Because of previous tests with 2-butoxyethanol, the single- and repeated-dose tests were not considered as needed, and a 14-week study was the initial test. Groups of 10 male and 10 female rats and mice were exposed to 0, 31, 62.5, 125, 250, and 500 ppm by inhalation for 6 h/d, 5 d/week for 14 weeks. In the 2-year segment, groups of 50 male and 50 female rats were exposed to 0, 31.2, 62.5, or 125 ppm for 6 h/d, 5 d/week for 104 weeks. Mice, in groups of 50 of each sex, were exposed to 0, 31.2, 62.5, or 125 ppm on the same schedule as the rats for 104 weeks. In addition, for histological analysis and bone marrow studies, groups of 9 to 30 rats or mice were exposed at the various levels for 3 to 12 months.

9.3.2.22.3 Results

In the 14-week study, female rats at the higher exposure levels had decreased body weights and various clinical signs such as anemia and tail lesions. Mice at the top level for 14 weeks showed reduced body weight, anemia, lethargy, and deaths. In the 2-year study with rats, survival and body weights were equivalent to that of controls, but regenerative anemia was observed in males and was more prevalent in females. No neoplastic lesions occurred in the male rats, but females showed an increase in pheochromocytomas of the adrenal medulla. In male mice at the top level in the 2-year test, hemangiosarcomas of the liver were increased. Female mice showed an increase in forestomach squamous cell papillomas or carcinomas (combined) at the top dose only. Splenic hematopoietic cell proliferation and hemosiderin pigmentation were increased in males and females. Hyaline degeneration of the nasal epithelium was higher in exposed female mice.

The NTP concluded that there was no evidence of carcinogenicity in male rats, equivocal evidence in female rats, and some evidence of carcinogenicity in male and female mice.

The ACGIH has set a TLV of 20 ppm for 2-butoxyethanol to avoid irritation and central nervous system effects, with the A3 designation, confirmed animal carcinogen with unknown relevance to humans (ACGIH, 2003).

9.3.2.23 Isoprene NTP #486

Isoprene (CAS No. 78–79–5; formula C_5H_8; molecular weight, 68.1; boiling point, 34°C; melting point, −146°C; vapor pressure, 493 mmHg at 20°C) is a colorless, volatile, flammable liquid obtained

as a by-product when naphtha is cracked for production of ethylene. It is used to prepare elastomers and butyl rubber. It occurs as the monomer unit of natural rubber, is emitted from plants and trees, and has been identified as the major endogenous hydrocarbon in human breath. Isoprene was tested at Battelle under a contract with the NTP (National Toxicology Program, 1999d).

9.3.2.23.1 Vapor Generation

Isoprene was pumped from a bulk reservoir by a liquid micrometering pump into a rotating flask partially immersed in a hot water bath. The vapor passed into a condenser and was carried by a stream of nitrogen to a distribution manifold. Individual delivery lines carried metered amounts of vapor to each exposure chamber. Concentrations were monitored by an on-line gas chromatograph.

9.3.2.23.2 Procedure

Due to extensive previous tests in mice, preliminary studies were not done and the 2-year study was done only in rats. Groups of 50 male and 50 female rats were exposed by inhalation to isoprene at levels of 0, 220, 700, or 7000 ppm for 6 h/d, 5 d/week for 105 weeks. Additional groups of 10 male and 10 female rats were exposed to the same levels for a period of four weeks for a lung fibroblast assay.

9.3.2.23.3 Results

Survival and mean body weights of all exposed groups of males and females were similar to those of the controls. Histopathological examination at the end showed increases in mammary fibroadenomas in male rats, especially at the highest dose level, and in all exposed groups of female rats. Male rats also had increases in renal tubule adenomas or carcinomas, especially when step sections of the kidney were made. Interstitial cell adenomas of the testis were higher in males at the 700 and 7000 ppm levels.

NTP concluded that the results showed clear evidence of carcinogenicity in male rats and some evidence in female rats. A previous test of isoprene in mice also led to carcinogenicity (Melnick et al., 1996).

ACGIH has not evaluated isoprene. However, IARC has examined the isoprene data and concluded that although there is insufficient evidence for the carcinogenicity of isoprene in humans, the compound is possibly carcinogenic to humans (IARC, 1994).

9.3.2.24 Isobutene NTP #487

Isobutene (CAS No. 115–11–7; formula C_4H_8; molecular weight, 56.1; boiling point, −6.9°C; melting point, −139°C; vapor pressure 400 mm Hg at 21.6°C) is a colorless volatile liquid from refinery gases, and it is used for polymers and in production of isooctane and high octane aviation gasoline. It was tested by inhalation by Battelle under a contract with the NTP (National Toxicology Program, 1998c).

9.3.2.24.1 Vapor Generation

Liquid isobutene was vaporized by a warm water system; the gas was passed through a manifold to metering valves with flow meters which allowed the vapor to enter the exposure chambers. Concentrations were monitored with an on-line gas chromatograph. Samples were drawn approximately every 20 min.

9.3.2.24.2 Procedure

The bioassay began with the 14-week study. Groups of 10 male and 10 female rats and mice were exposed to isobutene at 0, 500, 1000, 2000, 4000, or 8000 ppm for 6 h/d, 5 d/week for 14 weeks. In the 2-year study, groups of 50 male and 50 female rats and mice were exposed to 0, 500, 2000, or 8000 ppm for 6 h/d, 5 d/week for 105 weeks.

9.3.2.24.3 Results

In the 14-week study, all the rats and mice survived and body weights of test animals were similar to those of controls. Likewise, in the 2-year part of the test, survival and mean body weights of exposed

animals were similar to those of controls. Hyaline degeneration of the olfactory epithelium occurred in exposed animals, especially mice at the higher dose levels. Male rats at the 8000 ppm dose level had an increase in follicular cell carcinoma of the thyroid, but not to a statistically significant degree. In the female rats and the male and female mice, there was no evidence of carcinogenicity.

These results led the NTP to conclude that there was some evidence for carcinogenicity in male rats but no evidence in female rats and mice and no evidence in male mice. The ACGIH had not set any TLV for isobutene.

9.3.2.25 Glutaraldehyde NTP: #490

Glutaraldehyde (CAS No. 111–30–8; formula $C_5H_8O_2$; molecular weight, 100.13; boiling point, 60–61°C; freezing point, −14°C; vapor pressure, 0.116 mmHg at 25°C) is a colorless liquid with an odor of rotten apples. It is used as a cold disinfectant in the health care industry, as a hardener in x-ray film processing, as a cross-linking agent, preservative, biocide, tissue fixative, and in many other products. It was tested by inhalation by Battelle under a contract with the NTP (National Toxicology Program, 1999e).

9.3.2.25.1 Vapor Generation

Glutaraldehyde vapor was generated from the liquid by a rotary evaporation system with a hot-water bath; nitrogen gas carried the vapor through a generator to the distribution manifold and through a valve system into the exposure chambers. A small-particle detector was used to ensure that the vapor and not aerosol was produced. An on-line gas chromatograph monitored chamber concentrations.

9.3.2.25.2 Procedure

Since previous toxicity studies indicated that 750 ppb was an approximate upper limit, the 2-year study began with groups of 50 male and 50 female rats exposed at levels of 0, 250, 500, or 750 ppb for 6 h/d, 5 d/week for 104 weeks. Similarly, groups of 50 male and 50 female mice were exposed by inhalation at 0, 62.5, 124, or 250 ppb for 6 h/d, 5 d/week for 104 weeks.

9.3.2.25.3 Results

Survival and body weights of female rats at 500 and 750 ppb were lower than those of controls; male rats of all exposed groups had survival rates like that of controls, but body weights were lower in the 500 and 750 ppb groups. Hyperplasia and inflammation of the respiratory epithelium and degeneration of the olfactory epithelium were increased in both exposed males and females. Nevertheless, there was no evidence of any increase in neoplastic lesions, and mammary gland tumors decreased in exposed female rats. Survival of test mice was similar to that of controls, but body weights of females at the top dose were lower than those of the control females. As in the rats, inflammation, metaplasia, and degeneration of the respiratory epithelium occurred, but there was no increase in tumor incidence. Hepatocellular adenomas in both males and females decreased significantly compared with controls.

The results led the NTP to conclude there was no evidence for the carcinogenicity of glutaraldehyde in either male and female rats or in male and female mice. The ACGIH has set a TLV of 0.05 ppm to avoid sensitization and irritation, with the A4 designation, not classifiable as a human carcinogen (ACGIH, 2001).

9.3.3 Solids

Testing of solids by inhalation required attention to many details, including size of the aerosol particles, the need to prevent particles from becoming electrically charged, and other problems.

9.3.3.1 *o*-Chlorobenzalmalononitrile NTP #377

o-Chlorobenzalmalononitrile (CAS No. 2698–41–1; formula $C_{10}H_8ClN_2$; molecular weight, 188.52; boiling point, 310–315°C; melting point, 95–96°C; vapor pressure, 0.000034 mmHg at 20°C) is also

known as CS2 and is used primarily by law enforcement and military personnel for riot control. It was tested by inhalation by Battelle under a contract with the NTP (National Toxicology Program, 1990e).

9.3.3.1.1 Aerosol Generation

The CS2 was prepared as an aerosol of 94% CS2, 5% Cab-o-Sil colloidal silica and 1% hexamethyl disilane. The aerosol was generated within a glove box from the original powder with a brush dust feed mechanism and passed through a deionizer into the distribution box. The aerosol concentration was controlled by adjusting the rotational speed of the feed brush or by altering the total airflow in the duct to the exposure chambers. A light scattering monitor determined aerosol concentrations, while a gas chromatograph measured CS2 levels (vapor plus aerosol).

9.3.3.1.2 Procedure

In the initial 14-day test, five rats and five mice of each sex were exposed to 0, 1, 10, or 100 mg/m^3 for 6 h/d. Since most of the animals died, a second test was begun with 0, 3, or 30 mg/m for 6 hrs/day for 14 days. In the 13-week portion, 10 male and 10 female were exposed to 0.4, 0.75, 1.5, 3 or 6 mg/m^3 for the 6 h/day, 5 d/week schedule. In the 2-year study, groups of 50 male and female rats were exposed to 0, 0.075, 0.25, or 0.75 mg/m^3 for the 6 h/d 5 days/week schedule for 105 weeks. Similar groups of mice were exposed at the 0,0.75 or 1.5 mg levels.

9.3.3.1.3 Results

As mentioned, the animals in the first 14-day test died; in the second trial, levels of 3 mg and above were fatal. The 13-week trial showed that even the 3-mg level was toxic.

In the 2-year study, survival was fairly good compared with controls, but body weights were somewhat lower. Histopathological examination at the end of the test period showed no neoplastic lesions in any of the test groups, although degeneration of the olfactory epithelium, inflammation, and hyperplasia of the respiratory tract were noted. The NTP concluded that there was no evidence for the carcinogenicity of CS2 in any of the test groups.

Human volunteers exposed to CS2 developed headaches, skin, eye and nasal irritation, and lacrimation with dizziness and nausea if exposure continued for more than a few minutes. On the basis of these results, the ACGIH has set a TLV ceiling of 0.05 ppm (0.39 mg/m^3) for CS2 to minimize these effects. In view of the negative animal studies, ACGIH has given CS2 the A4 designation, not classifiable as a human carcinogen (ACGIH, 2001).

9.3.3.2 2-Chloroacetophenone NTP #379

2-Chloroacetophenone (CAS No. 532–27–4; formula C_8H_7ClO; molecular weight, 154.6; boiling point, 244–245°C; melting point, 58–59°C; vapor pressure, 0.0059 mmHg at 20°C) is a colorless to gray crystalline solid with a pungent odor, used in riot control and personal protective devices because of its lacrimating action. It was tested by Battelle under contract with the NTP (National Toxicology Program, 1990f).

9.3.3.2.1 Aerosol Generation

A stream of preheated nitrogen was bubbled through a reservoir of melted 2-chloroacetophenone and carried the material to the exposure chambers. Concentrations were monitored with a gas chromatograph.

9.3.3.2.2 Procedure

For the 14-day trial, groups of five rats and five mice of each sex were exposed by inhalation to 0, 4.8, 10, 19, 43, or 64 mg/m^3 for 6 h/d, 5 d/week for 10 exposure periods over 14 days. In the 13-week study, groups of 10 rats and 10 mice of each sex were exposed to 0, 0.25, 0.5, 1, 2, or 4 mg/m^3 for 6 h/d, 5 d/week. In the 2-year phase, 60 rats of each sex were exposed to 0, 1, or 2 mg/m^3 for 103 weeks. Similar groups of mice were exposed to 0, 2, or 4 mg/m^3 at the same schedule for 103 weeks. At 15 months 10 animals of each group were taken for blood samples and other tests.

9.3.3.2.3 Results

The 14-day test indicated that levels of 10 mg/m^3 and above were harmful for rats; in mice 4.8 mg/m^3 was the highest dose that could be tolerated. In the 13-week study, all the animals lived, but body weights were lowered and some eye irritation was noted in the mice. Animals in the 2-year study survived well although body weights were a little lower than in controls. Inflammation, hyperplasia, and squamous metaplasia of the respiratory epithelium appeared in rats and mice, but mice were affected somewhat less. In male rats there was no increase in tumors; female rats had a marginal increase in mammary fibroadenomas; in both male and female mice there was no increase in tumors. The NTP concluded there was no evidence for the carcinogenicity of 2-chloroacetophenone in male rats and male and female mice and equivocal evidence in female rats. The ACGIH has set a TLV of 0.05 ppm or 0.32 mg/m^3 for 2-chloroacetophenone and has given it the A4 designation, not classifiable as a human carcinogen (ACGIH, 2001).

9.3.3.3 *1*-Epinephrine Hydrochloride NTP #380

1-Epinephrine hydrochloride (CAS No. 55–31–2; formula $C_3H_{13}NO_3$.HCl; molecular weight, 219.7; melting point, 157°C) is a crystalline solid used to treat allergic reactions and bronchospasm associated with asthma. It was tested by inhalation by Battelle under a contract with the NTP (National Toxicology Program, 1990g).

9.3.3.3.1 Aerosol Generation

An aqueous solution of epinephrine hydrochloride was supplied from a reservoir to a nebulizer. Output from the nebulizer went to an aerosol collection manifold and then to the inlet tube for the exposure chamber. On-line measurement of chamber concentrations was accomplished with a forward light-scattering photometer. The aerosol produced was within the respirable size range (1.8–2.8 μm).

9.3.3.3.2 Procedure

In the 14-day trial, five rats and five mice of each sex were exposed at levels of 0, 12.5, 25, 50, 100, or 200 mg/m^3 for 6 h/d, 5 d/week for 10 exposures over a 14-day period. In the 13-week test, 10 rats and 10 mice of each sex were exposed at 0, 2.5, 5, 10, 20, or 40 mg/m^3 for 6 h/d, 5 d/week for a total of 65 exposures. In the 2-year part of the study, groups of 60 male and 60 female rats were exposed at 0, 1.5, and 5 mg/m^3 for 6 h/d, 5 d/week while similar groups of mice were exposed at 0, 1.5, and 3 mg/m^3.

9.3.3.3.3 Results

In the 14-day trial, levels of 50 mg/m^3 were toxic to male rats and the mice; female rats were affected at 100 mg/m^3. The 13-week study showed that many rats died at 40 mg/m^3 and mice at levels as low as 5 mg/m^3 died. In the 2-year study, survival and body weights were comparable to those of controls. However, the 10 animals of each group sampled at 15 months had inflammation of the nasal passages, some hyaline degeneration of the respiratory and olfactory epithelium. But at the end of the 2-year period, there was no increase in neoplastic lesions in any of the exposed animals. Thus, there was negative evidence for the carcinogenicity of epinephrine hydrochloride in male and female rats and in male and female mice. The NTP review panel considered that this study was "inadequate" since a maximum tolerated dose had not been given. In view of the pronounced physiological actions of epinephrine, it is not clear that the animals might survive for two years if exposed to a high level. Although epinephrine does not appear to be an animal carcinogen, epidemiological studies of humans who used the drug are not available.

9.3.3.4 Naphthalene NTP #410 and #500

Naphthalene (CAS No. 91–20–3; formula $C_{10}H_8$; molecular weight, 128.18; boiling point, 218°C; melting point, 80.2°C; vapor pressure, 0.01 mmHg) is a white crystalline solid which is used as an

intermediate in the synthesis of dyestuffs, insecticides, plasticizers, synthetic resins, and as a moth repellant. It is a combustion product of wood, occurs in coal tar, tobacco smoke and in crude oil. Because of widespread exposure it was tested by inhalation under contracts from the NTP with Northrop Services (mice) and Battelle (rats) (National Toxicology Program 1992b, 2000b).

9.3.3.4.1 Vapor Generation

Naphthalene, contained in a heated flask, was vaporized by a stream of heated nitrogen and transported through a heated Teflon line to the distribution manifold. Naphthalene concentrations were monitored by a MIRAN Infrared analyzer or by a gas chromatograph. A small-particle detector was used to ensure that naphthalene vapor and not an aerosol was produced.

9.3.3.4.2 Procedure

In the mouse study, groups of 75 male and 75 female mice were exposed to 0, 10, and 30 ppm of naphthalene for 6 h/d, 5 d/week for 104 weeks. Additional groups of 75 mice of each sex were also exposed to 30 ppm at the same schedule. At 14 days, five mice of each group were taken for hematology studies; likewise similar numbers of mice from the 30 ppm groups were examined at 3- or 6-month intervals, starting at 3 months.

In the rat study, groups of 50 male and female rats were exposed at levels of 0, 10, 30, or 60 ppm for 6 h/d, 5 d/week for 105 weeks. Additional groups of 9 rats of each sex were exposed at levels of 10, 30, or 60 ppm under the same schedule for toxicokinetic studies at 2 weeks, 3, 6, 12, and 18 months.

9.3.3.4.3 Results

In exposed male mice, survival was better than in controls and body weights were only slightly lower. There was inflammation of the nose and metaplasia of the olfactory epithelium, but there was no increase in neoplasms. Exposed female mice showed the same nonneoplastic lesions, but in addition, there was a significant increase in alveolar/bronchiolar adenomas or carcinomas in the lung.

Exposed rats had survival rates comparable to that of controls, and body weights were somewhat lower only in males. In both sexes, there were the usual nonneoplastic lesions of the nose. However, male rats had significant increases in adenomas of the nose and an increase in neuroblastomas of the nose. Female rats also showed significant increases in nasal tumors, especially at the 60 ppm level.

The results led the NTP to conclude there was no evidence for the carcinogenicity of naphthalene in male mice, some evidence in female mice, and clear evidence in both male and female rats.

The ACGIH has set a TLV of 10 ppm for naphthalene to avoid irritation and effects on the eyes and blood (ACGIH, 2001).

9.3.3.5 Talc NTP #421

Talc (CAS No. 14807–96–6; formula $Mg_3Si_4O_{10}(OH)_2$; molecular weight, 379.26; melting point, 900–1000°C) is a mineral product composed largely of a hydrated silicate of magnesium. The relatively pure form is used in cosmetics, but many other products contain talc. It was tested by inhalation at the Lovelace Biomedical and Environmental Research Institute under a contract with the NTP (National Toxicology Program, 1993b).

9.3.3.5.1 Aerosol Generation

The aerosol was generated with a fluid bed generator; talc was mixed with the stainless steel bed material and compressed air released the small talc particles and carried them to the exposure chambers. A Kr-85 discharger was placed above the bed to reduce the charges on the particles. A continuous aerosol monitor was used to check the stability of the aerosol (2.7–3.6 μm diameter).

9.3.3.5.2 Procedure

The bioassay began with a 4-week study. Groups of 10 male and 10 female rats and mice were exposed at levels of 0, 2, 6, and 18 mg/m^3 for 6 h/d, 5 d/week for the 4-week period. In the 2-year

phase, groups of 50 male and 50 female rats and mice were exposed at 0, 6, or 18 mg/m^3 for 6 h/d, 5 d/week for 113 weeks (male rats), 122 weeks (female rats), and 104 weeks (mice). Additional groups of 22 male rats, 22 female rats, 40 male mice, and 40 female mice were exposed for interim evaluations at 6, 11, 12, and 18 months.

9.3.3.5.3 Results

In the 4-week phase, all the rats survived; with the mice some of the males at the lower levels died within a week. However, in the 2-year study, body weights were slightly lower, but survival was comparable to that of the controls. Male rats showed inflammation of the lung, along with metaplasia and hyperplasia. There was some increase in pheochromocytomas of the adrenal. Female rats had statistically significant increases in adenomas and carcinomas of the lung, as well as pheochromocytomas of the adrenal. In both male and female mice, there was inflammation of the lungs, but there were no neoplastic effects. On the basis of these results, the NTP concluded there was some evidence for the carcinogenicity of talc in male rats, clear evidence in female rats, and no evidence in male or female mice.

The ACGIH has set a TLV of 2 mg/m^3 for the respirable particulate fraction of talc that contains no asbestos fibers. The A4 designation, not classifiable as a human carcinogen, was assigned to the asbestos-free talc (ACGIH, 2001).

9.3.3.6 Nickel Oxide NTP #451

Nickel oxide (CAS No. 1313–99–1; formula NiO; molecular weight, 74.71; melting point, 2,090°C) is an olive gray powder used in production of stainless steel, various chemicals, and in petroleum refining. It was tested by inhalation by the Lovelace Inhalation Toxicology Research Institute under contract with the NTP (National Toxicology Program, 1996c).

9.3.3.6.1 Aerosol Generation

Nickel oxide aerosol was generated from fluid bed generators; a Kr-85 discharger was placed in the generator to reduce the electrical charge on the aerosol. The aerosol was mixed with air to achieve the desired concentration and delivered to the exposure chambers. An aerosol monitor was used to check the aerosol concentrations. Particle sizes ranged from 1.896 to 3.29 μm.

9.3.3.6.2 Procedure

In the 16-day test, groups of five male and five female rats and mice were exposed at levels of 0, 1.2, 2.5, 5, 10, or 30 mg/m^3 for 6 h/d, 5 d/week for 12 exposure periods within 16 days. Extra groups of five animals were exposed at the 1.2, 5 or 10 mg/m^3 levels for tissue burden studies. In the 13-week study, 10 male and 10 female rats and mice per group were exposed to 0, 0.6, 1.2, 2.5, 5 or 10 mg/m^3 at the 6 h/d, 5 d/week schedule for 13 weeks. Additional groups of 18 male and 18 female rats and six male and six female mice were exposed at the 0, 0.6, 2.5, or 10 mg levels for tissue burden studies.

For the 2-year study, groups of 65 male and female rats were exposed at levels of 0, 0.62, 1.25, or 2.5 mg/m^3 for 6 h/d, 5 d/week for 104 weeks. Likewise, groups of 79 male and 76 female mice were exposed to 0, 1.25, or 5 mg/m^3 under the same schedule. After seven months as many as seven male rats and five female rats as well as five mice from each group were evaluated for histopathology and tissue burden. At 15 months five animals of each group were studied for histopathology, hematology, and tissue burden.

9.3.3.6.3 Results

In the 16-day study all rats and mice survived and body weights were similar to those of controls. At the higher levels inflammation and hyperplasia of the lungs were noted. In the 13-week test, there were no consistent findings related to nickel oxide exposure, apart from inflammation and hyperplasia. There was a considerable burden of nickel in the lungs.

For the 2-year study, survival of exposed rats and mice was similar to that of controls; body weights were only slightly affected. Exposed rats showed inflammation and hyperplasia but both

males and females had increases in alveolar/bronchiolar tumors and pheochromocytomas of the adrenal. However, in the male mice there was no increase in tumors, whereas in female mice there was a marginal increase in lung tumors.

These results led the NTP to conclude there was some evidence of carcinogenicity for male and female rats; no evidence in male mice; and equivocal evidence in female mice.

The ACGIH has set a TLV of 0.2 mg/m^3 for insoluble inorganic nickel compounds to protect against cancer, irritation and dermatitis, with the A1 designation, confirmed human carcinogen (ACGIH, 2001).

9.3.3.7 Nickel Subsulfide NTP #453

Nickel subsulfide (CAS No. 12035–72–2; formula Ni_3S_2; molecular weight, 240.25; melting point, 790°C) is a black powder used in manufacture of lithium batteries. It is an intermediate in refining of certain nickel ores. It was tested by inhalation at the Lovelace Inhalation Toxicology Institute under a contract with the NTP (National Toxicology Program, 1996d).

9.3.3.7.1 Aerosol Generation

Nickel subsulfide aerosol was generated from 2-inch fluid bed generators, with a Kr-85 discharger in the generator to reduce the electrical charge on the aerosol. The aerosol was mixed with air to obtain the proper concentration and was delivered to the exposure chambers. A continuous aerosol monitor was used to check the stability of the aerosols. Particle size ranged from 1.65 to 2.99 μm.

9.3.3.7.2 Procedure

The initial part of the process was the 16-day study. Groups of five male and five female rats and mice were exposed to nickel subsulfide at concentrations of 0, 0.6, 1.2, 2.5, 5, or 10 mg/m^3 for 6 h/d, 5 d/week for 12 exposure days during a 16-day period. Tissue burdens in kidney and lung were done on three male and three female rats and mice exposed at the 0, 0.6, 2.5, and 10 mg levels. In the 13-week study, groups of 10 male and female rats and mice were exposed by inhalation at concentrations of 0, 0.15, 0.3, 0.6, 1.2, or 2.5 mg/m^3 for 6 h/d, 5 d/week for 13 weeks. Lungs of six male rats in each group were examined for nickel after 4, 9, or 13 weeks of exposure; kidneys and testes of six male rats from each group were checked for nickel burden after 13 weeks. Female rats and male and female mice were tested for nickel burden in the lungs after 13 weeks.

For the 2-year study, groups of 63 male and female rats and 80 male and female mice were exposed by inhalation at levels of 0, 0.6, or 1 mg/m^3 for 6 h/d, 5 d/week for 104 week (rats), or at 0, 0.6, or 1.2 mg/m^3 for 105 weeks (mice). After seven months, five male and five female rats and mice from each group were taken for histopathology, while seven male and seven female rats and five male and five female mice from each group were evaluated for nickel tissue burden. After 15 months five animals from each group were examined for hematology, histopathology, and tissue burden.

9.3.3.7.3 Results

In the 16-day test, levels above 2.5 or 5 mg/m^3 led to deaths or weight loss. In the 13-week test, the animals survived with inflammation of the respiratory tract and nonneoplastic lesions, as atrophy of the olfactory epithelium. In the 2-year study, survival of all animals was similar to that of controls, but body weights were lower. Both male and female rats, especially at the top dose, had significant increases in alveolar/bronchiolar adenomas or carcinomas of the lung and pheochromocytomas of the adrenal medulla. Nonneoplastic lesions were also observed. Although mice in the 2-year test bore the usual nonneoplastic lesions, there was no increase in tumors.

These results led the NTP to conclude that there was clear evidence for the carcinogenicity of nickel subsulfide in male and female rats, based on the increases in lung and adrenal tumors. It was concluded that there was no evidence for the carcinogenicity of nickel subsulfide in male and female mice.

The ACGIH has set a TLV of 0.1 mg/m^3 for nickel subsulfide and has given it the A1 designation, confirmed human carcinogen.

9.3.3.8 Nickel Sulfate Hexahydrate NTP #454

Nickel sulfate hexahydrate (CAS No. 10101–97–0; formula $NiSO_4 \cdot 6H_2O$; molecular weight, 262.86; melting point, 53.3°C) is a blue–green water-soluble nickel compound used in nickel plating, as a mordant in textile processing, and in the manufacture of other nickel compounds. It was tested by inhalation at Lovelace Inhalation Toxicology Research Institute under a contract with the NTP (National Toxicology Program, 1996e).

9.3.3.8.1 Aerosol Generation

Nickel sulfate hexahydrate aerosol was generated from an aqueous solution (62.1 g/l) which was atomized with a Retec nebulizer; the aerosol was then mixed with air to obtain the proper concentration and airflow rate. Water then evaporated from the aerosol droplets, leaving the nickel sulfate hexahydrate aerosol. Particle size of the aerosol ranged from 1.8 to 3.1 μm but was generally 2.2–2.5 μm. A continuous aerosol monitor was used to follow stability of the aerosol concentration and the need to adjust the generation system during exposures.

9.3.3.8.2 Procedure

Since there was information on the acute toxicity of nickel compounds, the test began with the 16-day studies. Groups of five male and five female rats and mice were exposed at levels of 0, 3.5, 7, 15, 30, or 60 mg/m^3 for 6 h/d for 12 exposure periods during 16 days. An additional four or five male and female rats and mice were exposed at 0, 3.5, 15, or 30 mg/m^3 to determine the tissue burden of nickel in lung (rats and mice) and kidney (rats). For the 13-week test, groups of 10 male and female rats and mice were exposed by inhalation at levels of 0, 0.12, 0.25, 0.5, 1, or 2 mg/m^3 for 6 h/d, 5 d/week for 13 weeks. Additional groups of five or six male and female rats and mice were exposed at the 0, 0.12, 0.5, or 2 mg/m^3 levels for tissue burden studies. Samples were also collected from rats and mice at the 0, 0.5, 1, and 2 mg/m^3 levels for sperm morphology and vaginal cytology. For the 2-year portion of the test, groups of 63 to 65 male and 63 or 64 female rats were exposed at levels of 0, 0.12, 0.25, or 0.5 mg/m^3 for 6 h/d, 5 d/week for 104 weeks. At seven months, five animals from each group were evaluated for histopathology and an additional seven were examined for nickel tissue burden. At 15 months, five animals from each group were examined for hematology, nickel tissue burden in the lungs and histopathology. Similarly groups of 50 male and female mice were exposed at concentrations of 0, 0.25, or 1 mg/m^3 for 6 h/d, 5 d/week for 104 weeks. At seven months, five males and five females from each group were evaluated for histopathology, and an equal number were evaluated for nickel tissue burden in lung and kidney. At 15 months five males and five females from each group were also examined for nickel burden in lung and kidney.

9.3.3.8.3 Results

In the 16-day study, the higher exposure levels led to deaths in both rats and mice, with inflammation, atrophy and hyperplasia noted in the respiratory tract. For the 13-week test, body weights and survival in both rats and mice were hardly affected. In the 2-year study, survival of all groups of rats and mice was similar to that of controls. Lung weights of animals on the higher concentrations were increased, due to the nickel burden. Inflammatory lesions of the lung, and hyperplasia, as well as atrophy of the olfactory epithelium were noted. However, no neoplasms attributable to exposure to nickel sulfate were noted in either rats or mice.

The results led the NTP to conclude that there was no evidence of carcinogenic activity of nickel sulfate hexahydrate in either male or female rats and mice.

The ACGIH has set a TLV of 0.1 mg/m^3 for soluble nickel compounds with the A4 designation, not classifiable as a human carcinogen (ACGIH, 2001).

9.3.3.9 Molybdenum Trioxide NTP #462

Molybdenum trioxide (CAS No. 1313–27–5; formula MoO_3; molecular weight, 143.95; boiling point, 1155°C; melting point, 795°C) is a white to blue powder used as an additive in steel, as a

chemical intermediate, catalyst, pigment, flame retardant, and component of ceramics, glass and enamels. Molybdenum is an essential element for both plants and animals because it is a cofactor for several enzyme systems. Molybdenum trioxide was tested by inhalation at Battelle, under a contract with the NTP (National Toxicology Program, 1997c).

9.3.3.9.1 Aerosol Generation

In the 14-day and 13-week studies, the molybdenum trioxide dust was generated by Wright dust-feed mechanisms on top of elutriators which opened into the top of the exposure chambers. The dust was swept into the chambers by compressed air. For the 2-year studies, the aerosol generation system was composed of a flexible dust feed mechanism, a Trost air impact mill, an aerosol charge neutralizer, and an aerosol distribution system. A pump drew material from the distribution system into the chamber inlet. A real-time aerosol monitor was used to follow chamber concentrations. Particle size varied from 1.3 to 1.8 μm.

9.3.3.9.2 Procedure

Groups of five male and five female rats and mice were exposed to molybdenum trioxide at levels of 0, 3, 10, 30, 100 or 300 mg/m^3 for 6 h/d, 5 d/week over a period of 14 days. In the next phase, groups of 10 male and 10 female rats and mice received exposure at the 0, 1, 3, 10, 30, or 100 mg/m^3 levels according to the usual schedule for a period of 13 weeks. For the 2-year study, groups of 50 male and 50 female rats and mice were exposed at 0, 10, 30, or 100 mg/m^3 for 6 h/d, 5 d/week for 105 (mice) or 106 weeks (rats).

9.3.3.9.3 Results

In the 14-day and 13-week tests, there were no special effects noted. Survival of test animals was comparable to that of controls. The same held for the 2-year study, except that female mice were affected more. Control female mice had lower weight gains over the 2-year span than did the test females. Rats exposed for the 2–year period had inflammation of the respiratory tract; the severity was greater at the higher levels. There was a nonsignificant increase in lung tumors in male rats, but in females there was no evidence of carcinogenicity. Male and female mice also had increases in lung tumors. Nonneoplastic lesions in both species were inflammation, hyaline degeneration, and squamous metaplasia of the epiglottis.

The NTP concluded there was equivocal evidence for the carcinogenicity of molybdenum trioxide in male rats, no evidence in female rats, and some evidence in male and female mice.

The ACGIH has recommended a TLV of 0.5 mg/m^3 for soluble molybdenum compounds, with the A3 designation, animal carcinogen of unknown relevance to humans (ACGIH, 2003).

9.3.3.10 Cobalt Sulfate Heptahydrate NTP #471

Cobalt sulfate heptahydrate (CAS No. 10026–24–1; formula $CoSO_4 \cdot 7H_2O$; molecular weight 281.13) is a reddish, crystalline, water-soluble powder used in the electroplating and electrochemical industries, as a coloring agent for ceramics, as a drying agent in inks, paints, and varnishes, and as a mineral supplement in animal feeds. Cobalt is an essential trace element since it is part of vitamin B_{12}. This salt was tested by inhalation at Battelle under a contract with the NTP (National Toxicology Program, 1998d).

9.3.3.10.1 Aerosol Generation

A solution of the heptahydrate in deionized water was passed into a nebulizer reservoir, then aspirated into the nebulizer chamber and expelled as a stream. Shear forces broke the stream into droplets that were evaporated to leave dry particles of the heptahydrate. Aerosols thus generated were passed through the aerosol charge neutralizer to reduce static charges. A distribution line carried the aerosol to the exposure chambers. Chamber concentrations of the aerosol were monitored by computer-controlled real line aerosol monitors. Particle sizes ranged from 1.4 to 1.6 μm.

9.3.3.10.2 Procedure

Groups of 50 male and 50 female rats and mice were exposed to aerosols containing 0, 0.3, 1.0, or 3.0 mg/m^3 of cobalt sulfate heptahydrate for 6 h/d, 5 d/week for 105 weeks.

9.3.3.10.3 Results

Body weights and survival of exposed male and female rats were similar to those of unexposed controls. Females at the 3 mg/m^3 level showed episodes of irregular breathing. Inflammation, hyperplasia, and metaplasia of the respiratory tract were noted in both sexes. Likewise, in both sexes the incidences of alveolar/bronchiolar neoplasms were greater than in unexposed controls, especially in females at the two higher exposure levels. Furthermore, pheochromocytomas of the adrenal medulla were significantly higher in females at the top dose.

In mice, although survival was similar to that of controls, body weights of exposed animals were lower. The incidence of alveolar/bronchiolar neoplasms in both males and females was significantly greater than that of controls. Hemangiosarcomas of the liver were increased in test males and in females at the 1 mg/m^3 level. Atrophy, hyperplasia, and inflammation of the nose also occurred in the test animals.

Under these conditions, the NTP concluded that there was some evidence for carcinogenicity in male rats, clear evidence in female rats, and clear evidence in both male and female mice.

The ACGIH has set a TLV of 0.02 mg/m^3 as cobalt for elemental and inorganic forms. The A3 designation, confirmed animal carcinogen with unknown relevance to humans, has been given for cobalt compounds (ACGIH, 2001).

9.3.3.11 Gallium Arsenide NTP #492

Gallium arsenide (CAS No. 1303–00–0; formula GaAs; molecular weight, 144.64; melting point, 1238°C) is a dark-gray crystal with a metallic sheen and a garlic odor when moistened. It is used extensively in the microelectronics industries to make light-emitting diodes, lasers, laser windows, photodetectors, infrared emitters and detectors, solar cells, semiconductors, and microwave devices. It was tested by inhalation by Battelle under a contract with the NTP (National Toxicology Program, 2000c).

9.3.3.11.1 Aerosol Generation

Gallium arsenide aerosol was generated through a flexible brush dust feed mechanism, an air impact mill, cyclone separator, an aerosol charge neutralizer, and an aerosol distribution system. Before the aerosol entered the exposure chambers, it was diluted with filtered air to the appropriate concentration. The chamber aerosol concentrations were followed with real-time aerosol monitors that used a pulsed-light-emitting diode in combination with a silicon detector to sense light scattered by particles traversing the sensing volume. Particle sizes ranged from 0.8 to 1.9 μm.

9.3.3.11.2 Procedure

The test began with 16-day studies in which groups of five male and five female rats and mice were exposed by inhalation to gallium arsenide at levels of 0, 1, 10, 37, 75, or 150 mg/m for 6 h/d, 5 d/week for 16 days. In the 14-week portion of the test, groups of 10 male and 10 female rats and mice were exposed at 0, 0.1, 1, 10, 37, or 75 mg/m^3 levels for 6 h/d, 5 d/week for 14 weeks. Groups of 10 male and 10 female rats were exposed to the same levels for up to 23 days for clinical pathology evaluation, while four additional male rats were exposed for 14 weeks to do lung burden analyses.

In the 2-year study, groups of 50 male and 50 female rats were exposed at 0, 0.01, 0.1, or 1 mg/m^3 at the usual schedule for 105 weeks. Similar groups of 50 male and 50 female mice were exposed at 0, 0.1, 0.5, or 1 mg/m^3 for 105 (males) or 106 weeks (females). Groups of up to 30 male rats were exposed at the same levels as the main group, but they were evaluated at 1,2, 4, 6, 12, and 18 months for gallium arsenide distribution (five rats from the 0, 0.1, and 1 mg/m^3 groups at each time period).

Likewise, four or five male rats from the 0.01 mg/m^3 group were evaluated at 2, 12, and 18 month time periods.

9.3.3.11.3 Results

In the 16-day test, all the rats and mice survived with body weights similar to those of controls. However, mice at the two top levels showed abnormal posture and hypoactivity. The 14-week study demonstrated that body weights were adversely affected at the 37 and 75 mg/m^3 levels, especially in male rats and mice. At the end of the 2-year study, survival rates of rats were comparable to those of controls although rats at the 1mg/m^3 level had lower body weights.

Although exposure to gallium arsenide led to increases in inflammation and proliferative lesions of the lung, larynx, and nose in both sexes, there was no increase in neoplasms in the male rats. However, females had a significantly greater incidence of lung neoplasms, as well as increases in tumors of the adrenal and mononuclear cell leukemia.

The 2-year trial in mice showed that survival and body weights were comparable to those of controls. Nonneoplastic lesions of the lung and nose, including inflammation, hyperplasia, and hyaline degeneration occurred in the mice, especially at the two higher dose levels. However, there was no increase in neoplasms in either male or female mice.

The NTP concluded the results showed no evidence of carcinogenicity in male rats and male and female mice, but clear evidence in female rats.

The ACGIH has not set any TLV for gallium arsenide, but there is a TLV of 0.01 mg/m^3 for arsenic and its inorganic compounds, as arsenic, with the A1 designation, or confirmed human carcinogen (ACGIH, 2001).

9.3.3.12 Indium Phosphide NTP #499

Indium phosphide (CAS No. 22398–80–7; formula InP; molecular weight, 145.80; melting point, 1,070°C) is a dark-gray powder or brittle metallic solid. It is used extensively in the microelectronics industry to make semiconductors, injection lasers, solar cells, photodiodes, and similar devices. It was tested by inhalation by Battelle under a contract with the NTP (National Toxicology Program, 2002).

9.3.3.12.1 Aerosol Generation

In the preliminary study, the aerosol generation system consisted of a flexible brush dust feed mechanism, an air-impact mill, an aerosol charge neutralizer, and a stainless steel aerosol distribution system. In the 2-year trial, indium phosphide, released from a rotating drum, filled metering ports and was dispersed when a nitrogen solenoid opened. The aerosol passed through a corona discharge air-ionizing neutralizer and into the distribution lines to the exposure chambers. Concentrations were monitored with real-time aerosol monitors. Particle sizes ranged from 1.2 to 1.5 μm.

9.3.3.12.2 Procedure

Sufficient data on toxicity were available to allow starting with the 14-week trial. Groups of 10 male and 10 female rats and mice were exposed to the aerosol at levels of 0, 1, 3, 10, 30, or 100 mg/m^3 for 6 h/d, 5 d/week for 9 of the 14 weeks and 7 d/week for 5 weeks. Additional groups of 10 rats of each sex were also started for clinical pathology and tissue burden studies; additional groups of male rats were exposed for various tissue burden studies.

In the 2-year study, groups of 60 male and 60 female rats and mice were exposed to the aerosol at levels of 0, 0.03, 0.1, or 0.3 mg/m^3 for 6 h/d, 5 d/week for 22 weeks (rats) and 21 week (mice) (0.1 and 0.3 mg/m^3) groups or for 105 weeks (0 and 0.03 groups). At three months 10 animals from each group were randomly selected and evaluated. Furthermore, additional groups of 20 male rats and 20 male mice were earmarked for tissue burden studies.

9.3.3.12.3 Results

The 14-week trial led to only one death in the rats, but body weights were depressed sharply. There were significant increases in lung weights and erythrocyte counts. Mice were more susceptible

to the toxic effects, and there were deaths at the 30-mg level. The same effects on lung weights were noted.

In the 2-year study, the rats survived well, but the incidences of alveolar/bronchiolar adenomas or carcinomas were significantly higher in both sexes, even in those exposed for only 22 weeks. Adrenal pheochromocytomas increased in males at the 0.03-mg level and at the 0.3-mg stop exposure level; this tumor was increased in females at the 0.3 mg stop exposure study. Mammary gland carcinomas were significantly higher in females at 0.03 mg for 2 years, while mononuclear cell leukemia was higher in males at the 0.1 mg stop exposure level. Exposed mice in the 2-year trial had both lower survival and body weights than did the controls. Male and female mice demonstrated an increase in alveolar/bronchiolar carcinomas. All groups of exposed males had higher incidences of liver tumors; females had a significant increase only at the 0.03 mg level. Both sexes of both species showed inflammatory lesions in various organs and increased tissue burden of indium phosphide, especially in the lung.

These results led the NTP to conclude that there was clear evidence for the carcinogenicity of indium phosphide in both male and female rats and male and female mice.

The ACGIH has set a TLV of 0.1 mg/m^3 for indium and compounds to avoid pulmonary edema, bone and gastrointestinal effects (ACGIH, 2001).

9.4 DISCUSSION AND CONCLUSION

The NTP project to test selected compounds by inhalation had an impressive beginning with the decidedly positive responses from 1,2-dibromo-3-chloropropane and 1,2-dibromoethane. However, given the activity of these two compounds when administered by gavage (Olson et al., 1974), it is not surprising that they also were active by the inhalation route. Continuing on this theme, on the basis of activity, of the 44 chemicals NTP tested thus far by inhalation, seven were active in all four test systems: male rats, female rats, male mice, and female mice (Table 9.3). These active compounds included: chloroprene, 1,2-dibromo-3-chloropropane, 1,2-dibromoethane, ethylene oxide, indium phosphide, tetrafluoroethylene, and tetranitromethane. 1,3-Butadiene, tested in mice only, was a clear positive. An independent study in rats showed a moderate positive response at dose levels of butadiene manyfold that in the NTP mouse study (Owen and Glaister, 1990). The explanation may rest on differences in metabolic activation of butadiene. Half of these positive compounds were halogenated, indication that addition of halogens to a basic alkane structure can alter considerably the toxicological response.

The opposite situation, a negative animal response, occurred with eleven compounds which included: the riot control agent *o*-chlorobenzalmalononitrile, l-epinephrine hydrochloride, glutaraldehyde, hexachlorocyclopentadiene, isobutyraldehyde, methyl bromide, methyl methacrylate, propylene, nickel sulfate hexahydrate, toluene, and vinyl toluene. The epinephrine test was designated as inadequate by an NTP Report Review group since the test levels were thought to be lower than a Maximum Tolerated Dose. However, a physiological but not a carcinogenic response was noted. In addition, exposure of the animals to high doses of an active physiologic agent for two years raises the question of whether the animals could survive. Halogenated compounds and aldehydes, both considered as a basis for suspicion, were present in the 'negative' group but showed no carcinogenic effect.

Another five compounds were active in the majority of the test systems; these included cobalt sulfate heptahydrate, dichloromethane, nitromethane, propylene oxide, and tetrachloroethylene. Mice appeared more likely to show a positive response with these compounds. A group of four compounds showed no response in the majority of the test systems, and thus they can be considered negative on the basis of the weight of the evidence. These included the solvent acetonitrile, the riot control agent, 2-chloroacetophenone, gallium arsenide, and isobutene, an intermediate in polymer synthesis.

Twelve chemicals had equivocal findings, almost equally balanced between positive and negative results. In this category were: allyl glycidyl ether, bromoethane, 2-butoxyethanol, chloroethane,

Table 9.3 Summary of NTP Inhalation Studies

NTP Report No.	Compound	Male Rat	Female Rat	Male Mouse	Female Mouse
	Gases				
272	Propylene	NE	NE	NE	NE
288, 434	1,3-Butadiene	—	—	CE	CE
326	Ethylene oxide	—	—	CE	CE
346	Chloroethane	EE	EE	IS	CE
385	Methyl bromide	—	—	NE	NE
440	Ozone	NE	NE	EE	SE
450	Tetrafluoroethylene	CE	CE	CE	CE
	Liquids				
206	1,2-Dibromo-3-chloropropane	P	P	P	P
210	1,2-Dibromoethane	P	P	P	P
267	Propylene oxide	SE	SE	CE	CE
306	Dichloromethane	SE	CE	CE	CE
311	Tetrachloroethylene	CE	SE	CE	CE
314	Methyl methacrylate	NE	NE	NE	NE
329	1,2-Epoxybutane	CE	EE	NE	NE
363	Bromoethane	SE	EE	EE	CE
371	Toluene	NE	NE	NE	NE
375	Vinyl toluene	NE	NE	NE	NE
376	Allyl glycidyl ether	EE	NE	SE	EE
386	Tetranitromethane	CE	CE	CE	CE
437	Hexachlorocyclopentadiene	NE	NE	NE	NE
447	Acetonitrile	EE	NE	NE	NE
448	Isobutyl nitrite	CE	CE	SE	SE
461	Nitromethane	NE	CE	CE	CE
466	Ethylbenzene	CE	SE	SE	SE
467	Chloroprene	CE	CE	CE	CE
472	Isobutyraldehyde	NE	NE	NE	NE
475	Tetrahydrofuran	SE	NE	NE	CE
482	Furfuryl alcohol	SE	EE	SE	NE
484	2-Butoxyethanol	NE	EE	SE	SE
486	Isoprene	CE	SE	—	—
487	Isobutene	SE	NE	NE	NE
490	Glutaraldehyde	NE	NE	NE	NE
	Solids				
377	*o*-Chlorobenzalmalononitrile	NE	NE	NE	NE
379	2-Chloroacetophenone	NE	EE	NE	NE
380	L-Epinephrine hydrochloride	IS	IS	IS	IS
410, 500	Naphthalene	CE	CE	NE	SE

Table 9.3 Summary of NTP Inhalation Studies (Continued)

NTP Report No.	Compound	Male Rat	Female Rat	Male Mouse	Female Mouse
421	Talc	SE	CE	NE	NE
451	Nickel oxide	SE	SE	NE	EE
453	Nickel subsulfide	CE	CE	NE	NE
454	Nickel sulfate hexahydrate	NE	NE	NE	NE
462	Molybdenum trioxide	EE	NE	SE	SE
471	Cobalt sulfate heptahydrate	SE	CE	CE	CE
492	Gallium arsenide	NE	CE	NE	NE
499	Indium phosphide	CE	CE	CE	CE

CE, Clear Evidence; SE, Some Evidence; EE, Equivocal Evidence; NE, Negative Evidence; IS, Inadequate Study; P, Positive.

epoxybutane, furfuryl alcohol, molybdenum trioxide, naphthalene, nickel oxide, nickel subsulfide, ozone, and tetrahydrofuran. Rats showed positive responses to naphthalene and nickel subsulfide, but mice did not. Nickel subsulfide was tested partly on the basis of the overwhelming positive response in mice injected with this compound at various sites (IARC, 1990). Thus, the lack of response in mice to inhalation exposure is surprising. In contrast, rats showed no evidence of carcinogenicity from ozone, but mice had some response.

Some weak points in the NTP inhalation toxicology effort became apparent after perusing the 46 reports. There appeared to be no attention, in the reports at least, to technical developments in inhalation toxicology, to the recommendations of other agencies (EPA, 1988; 1998a, 1998b), or of literature concerning the specifics of inhalation toxicology (Salem, 1987). It seemed that aspect was left to the contractors.

Another factor, with both positive and negative aspects, was that the NTP has expanded the scope and size of the bioassay reports (Table 9.2). Comprehensive reviews on the toxicity, absorption, distribution, metabolism, and excretion of the test material are now included, as well as data on genotoxicity, reproductive, and teratogenic effects. The addition of such material increases the usefulness of any individual bioassay report. Furthermore, the scope of the histopathological examination has been expanded. The thoroughness of the histopathology, as documented in the NTP reports, can be considered both beneficial and detrimental. It is in a sense, gratifying to learn that numerous tissues were routinely sectioned, stained, and examined by the pathologists. However, there is a question about the value (and cost) of such routine procedures when a thorough necropsy by an experienced technician would likely lead to discovery of most lesions or any unusual aspects of any tissues. Further, there was no apparent effort to reduce the number of animals used. Sufficient animals are required to obtain statistically significant results, but there were instances where fewer animals could have been used. This was noted especially, in the second butadiene trial and for some of the auxiliary studies conducted with the inorganic compounds.

On the other hand, the NTP has definitely provided a valuable service for the regulatory agencies, public health professionals, industrial hygienists, safety officers, and the like. Their testing many compounds which had little long-term (two-year) toxicity studies, despite human exposure, such as butadiene, dichloromethane, or toluene, provided health professionals with reasonable data upon which to base decisions. For example, the TLVs promulgated by ACGIH are based on a

combination of factors, including exposure, acute, short- and long-term effects in both animals and exposed humans.

In conclusion, the NTP inhalation toxicology experiments have been a worthwhile enterprise. The NTP effort on testing compounds, by now 500 or more, has provided useful data, obtained in a comparable manner, in defined protocols, either by dermal inhalation, or oral routes, in well characterized animals (mice and rats). It is the largest known effort for long-term tests under comparable conditions. The data from these studies are useful for the research, regulatory, and safety communities. It is anticipated that the NTP programs will continue to be a valuable resource and will supply useful data in the coming years.

REFERENCES

ACGIH (American Conference of Governmental Industrial Hygienists), *Documentation of the Threshold Limit Values and Biological Exposure Indices*, 7th ed., Cincinnati, OH, 2001.

ACGIH (American Conference of Governmental Industrial Hygienists), TLVs® and BEIs, Cincinnati, OH, 2003.

Adkins, B., Jr., Van Stee, E.W., Simmons, J.F., and Eustis, S.L., Oncogenic response of strain A/J mice to inhaled chemicals, *J. Toxicol. Environ. Health*, 17, 311–322, 1986.

Boorman, G.A., Montgomery, C.A. Jr., Eustis, S.L., Wolfe, M.J., McConnell, E.E., and Hardisty, J.F., Quality assurance in pathology for rodent carcinogenicity testing, in *Handbook of Carcinogen Testing*, 2nd ed, Milman, H.A. and Weisburger, E.K., Eds., Noyes Publications, Park Ridge, NJ, 1994, pp. 410–422.

Burek, J.D., Nitschke, K.D., Bell, T.J., Wackerle, D.L., Childs, R.C., Beyer, J.E., Dittenber, D.A., Rampy, L.W., and McKenra, M.J., Methylene chloride: A two-year inhalation toxicity and oncogenicity study in rats and hamsters, *Fundam. Appl. Toxicol*., 4, 30–47, 1984.

Cox, D.R., Regression models and life-tables*, J. R. Stat. Soc.,* B34, 187–220, 1972.

EPA (Environmental Protection Agency), Hazard Evaluation Division, Standard evaluation procedure, inhalation toxicity tests, EPA 540–09–88–101, 1988.

EPA (Environmental Protection Agency), Health effects test guidelines, OPPTS 870.1300, Acute inhalation toxicity, EPA 712–C–98–193, 1998a.

EPA (Environmental Protection Agency), Health effects guidelines, OPPTS 870.3465. 90-Day inhalation toxicity, EPA 712–C–98–204, 1998b.

Goodman, D.G., Boorman, G.A., and Strandberg, J.D., Selection and use of the B6C3F$_1$ mouse and F344 rat in long-term bioassays for carcinogenicity, in *Handbook of Carcinogen Testing*, 2nd ed, Milman, H.A. and Weisburger, E.K., Eds, Noyes Publications, Park Ridge, NJ, 1994, pp. 347–390.

Garman, R.H., Snellings, W.M., and Maronpot, R.R., Frequency, size and location of brain tumours in F344 rats chronically exposed to ethylene oxide, *Food Chem. Toxicol.*, 24, 145–153, 1986.

Ham, T.E., Jr., Design of a long-term animal bioassay for carcinogenicity, in *Handbook of Carcinogen Testing*, 2nd ed, Milman, H.A. and Weisburger, E.K., Eds., Noyes Publications, Park Ridge, NJ, 1994, pp. 270–285.

IARC (International Agency for Research on Cancer), IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 49. Chromium, nickel and welding, Lyon, France, 1990.

IARC (International Agency for Research on Cancer), IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 60. Some industrial chemicals, Lyon, France, 1994.

IARC (International Agency for Research on Cancer), IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 71. Re-evaluation of some organic chemicals, hydrazine and hydrogen peroxide, Lyon, France, 1999.

Jameson, C.W. and Goehl, T.J., Chemistry requirements for toxicological and carcinogenicity evaluation of chemicals, in *Handbook of Carcinogen Testing*, 2nd ed., Milman, H.A. and Weisburger, E.K., Eds., Noyes Publications, Park Ridge, NJ, 1994, pp. 286–297.

Kuper, C.F., Renzel, P.G.J., Feron, V.J. and Verschuuren, H., Chronic inhalation toxicity and carcinogenicity study of propylene oxide in Wistar rats, *Food Chem. Toxicol*., 26, 159–167, 1988.

Lynch, D.W., Lewis, T.R., Moorman, W.J., Burg, J.R., Groth, D.H., Khan, A., Ackerman, L.J., and Cockrell, B.Y., Carcinogenic and toxicologic effects of inhaled ethylene oxide and propylene oxide in F344 rats, *Toxicol. Appl. Pharmacol*., 76, 69–84, 1984.

Melnick, R.L., Sills, R.C., Roycroft, J.H., Chou, B.J., Ragan, H.A., and Miller, R.A., Inhalation toxicity and carcinogenicity of isoprene in rats and mice. Comparisons with 1,3-butadiene, *Toxicology*, 113, 247–252, 1996.

NCI (National Cancer Institute), Guidelines for carcinogen bioassay in small rodents, Carcinogenesis Technical Report Series 1, 1976.

NTP (National Toxicology Program), Carcinogenesis bioassay of 1,2-dibromo-3-chloropropane (CAS 96–12–8) in F344 rats and B6C3F$_1$ mice (Inhalation study), NTP Technical Report Series 206, 1982a.

NTP (National Toxicology Program), Carcinogenesis bioassay of 1,2-dibromoethane (CAS 106–93–4) in F344 rats and B6C3F$_1$ mice (Inhalation study), NTP Technical Report Series 210, 1982b.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of 1,3-butadiene (CAS No. 106–99–0) in B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 288, 1984.

NTP (National Toxicology Program), Toxicology and carcinogensis studies of propylene (CAS 115–07–1) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 272, 1985a.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of propylene oxide (CAS 75–56–9) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 267, 1985b.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of dichloromethane (methylene chloride) (CAS 75–08–2) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 306, 1986a.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of tetrachloroethylene (Perchloroethylene) (CAS 127–18–4) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 311, 1986b.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of methyl methacrylate (CAS 80–62–6) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 314, 1986c.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of ethylene oxide (CAS 75–21–8) in B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 326, 1987.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of 1,2-epoxybutane (CAS 106–88–7) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 329, 1988a.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of chloroethane (ethyl chloride) (CAS 75–00–3) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 346, 1989a.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of bromoethane (ethyl bromide) (CAS 74–96–4) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 363, 1989b).

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of toluene (CAS No. 108–88–3) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 371, 1990a.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of vinyl toluene (mixed isomers) (65%–71% meta-isomer and 32%–35% para-isomer) (CAS 25013–15–4) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 375, 1990b.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of allyl glycidyl ether (CAS No. 106–92–3) in Osborne-Mendel rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 376, 1990c.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of tetranitromethane (CAS 509–14–8) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 387, 1990d.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of CS2 (94% *o*-chlorobenzalmalononitrile, CAS 2698–41–1) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 377, 1990e.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of 2-chloroacetophenone (CAS 532–27–4) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 379, 1990f.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of 1-epinephrine hydrochloride (CAS 55–31–2) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 380, 1990g.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of methyl bromide (CAS 74–83–9) in B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 385, 1992a.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of naphthalene (CAS 91–20–3) in B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 410, 1992b.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of 1,3-butadiene (CAS 106–99–0) in B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 434, 1993a.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of talc (CAS 14807–96–6) (non-asbestiform) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 421, 1993b.
NTP (National Toxicology Program), Comparative toxicology studies of corn oil, safflower oil, and tricaprylin (CAS 8001–30–7, 8001–23–8, and 538–23–8) in male F344/N rats as vehicles for gavage, NTP Technical Report Series 426, 1994a.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of ozone (CAS 10028–15–6) and ozone/NNK (CAS 10028–15–6/64091–91–4) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 440, 1994b.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of hexachlorocyclopentadiene (CAS 77–47–4) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 437, 1994c.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of acetonitrile (CAS 75–05–8) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 447, 1996a.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of isobutyl nitrite (CAS 542–56–3) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 448, 1996b.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of nickel oxide (CAS 1313–99–1) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), Report Series 451, 1996c.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of nickel subsulfide (CAS 12035–72–2) in F344/N rats and B6C3F$_1$ mice (Inhalation studies) NTP Technical Report Series 453, 1996d.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of nickel sulfate hexahydrate (CAS 10101–97–0) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 454, 1996e.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of tetrafluoroethylene (CAS 116–14–3) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 450, 1997a.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of nitromethane (CAS 75–52–5) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 461, 1997b.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of molybdenum trioxide (CAS 1313–27–5) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 462, 1997c.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of chloroprene (CAS 126–99–8) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 467, 1998a.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of tetrahydrofuran (CAS 109–99–9) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 475, 1998b.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of isobutene (CAS 115–11–7) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 487, 1998c.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of cobalt sulfate hexahydrate (CAS 10026–24–1) in F344/N rats and B6C3F mice (Inhalation studies), NTP Technical Report Series 471, 1998d.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of ethylbenzene (CAS 100–41–4) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 466, 1999a.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of isobutyraldehyde (CAS 78–84–2) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 472, 1999b.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of furfuryl alcohol (CAS 98–00–0) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 482, 1999c.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of isoprene (CAS 78–79–5) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 486, 1999d.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of glutaraldehyde (CAS 111–30–8) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 490, 1999e.
NTP (National Toxicology Program), Toxicology and carcinogenesis studies of 2-butoxyethanol (CAS 111–76–2) in F344/N rats and B6C3F$_1$ mice (Inhalation studies), NTP Technical Report Series 484, 2000a.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of naphthalene (CAS 91–20–3) in F344/N rats (Inhalation studies), NTP Technical Report Series 500, 2000b.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of gallium arsenide (CAS 1303–00–0) in F344/N rats and B6C3F_1 mice (Inhalation studies), NTP Technical Report Series 492, 2000c.

NTP (National Toxicology Program), Toxicology and carcinogenesis studies of indium phosphide (CAS 22398–80–7) in F344/N rats and B6C3F_1 mice (Inhalation studies), NTP Technical Report Series 499, 2002.

Nitschke, K.D., Burek, J.D., Bell, T.J., Kociba, R.J., Rampy, L.W., and McKenna, M.J., Methylene chloride: A 2-year inhalation and toxicity and oncogenicity study in rats, *Fundam. Appl. Toxicol*., 11, 48–59, 1988.

Olson, W.A., Habermann, R.T., Weisburger, E.K., Ward, J.M., and Weisburger, J.H., Induction of stomach cancer in rats and mice with halogenated aliphatic fumigants, *J. Natl. Cancer Inst*., 51, 1993–1995, 1974.

Owen, P.E. and Glaister, J.R., Inhalation toxicity and carcinogenicity of 1,3-butadiene in Sprague-Dawley rats, *Environ. Health Perspect.*, 86, 19–25, 1990.

Porter, C.J. and Bailer, A.J., Testing for increased carcinogenicity using a survival-adjusted quantal response test, *Fundam. Appl. Toxicol*., 12, 731–737, 1989.

Prejean, J.D., Conduct of long-term animal bioassays, in *Handbook of Carcinogen Testing*, 2nd ed., Milman, H.A. and Weisburger, E.K., Eds., Noyes Publications, Park Ridge, NJ, 1994, pp. 333–346.

Reznik, G., Reznik-Schuller, H., Ward, J.M., Stinson, S.F., Morphology of nasal cavity tumours in rats after chronic inhalation of 1,2-dibromo-3-chloropropne*, Br. J. Cancer*, 42, 772–781, 1980.

Reuzel, P.G., Jr., Dreef van der Meulen, H.C., Hollanders, V.M.H., et al., Chronic inhalation toxicity and carcinogenicity study of methyl bromide in Wistar rats, *Food Chem. Toxicol*., 29, 31–39, 1991.

Salem, H., Ed., *Inhalation Toxicology: Research Methods, Applications, and Evaluation*, Marcel Dekker, New York, 1987.

Snellings, W.M., Weil, C.S., and Maronpot, R.R., A two-year inhalation study of the carcinogenic potential of ethylene oxide in Fischer 344 rats, *Toxicol. Appl. Pharmacol*., 75, 105–117, 1984.

Tarone, R.E., Tests for trends in life table analysis, *Biometrika*, 62, 679–683, 1975.

Williams, D.A., The comparison of several dose levels with a zero dose control, *Biometrics*, 28, 519–531, 1972.

10 Toxicology of Fire and Smoke[1]

Barbara C. Levin and Erica D. Kuligowski

CONTENTS

10.1 INTRODUCTION

Toxicology of fire and smoke is the study of the adverse health effects caused by exposure to combustion atmospheres. In this chapter, a combustion atmosphere is defined as all of the effluents generated by the thermal decomposition of materials or products regardless of whether that effluent is produced under smoldering, nonflaming, or flaming conditions. The objectives of combustion toxicity research are to identify potentially harmful products from the thermal degradation of materials, to distinguish those materials that produce unusual or more toxic quantities of toxic combustion products, to determine the best measurement methods for the identification of the toxic products and the degree of toxicity, to determine the effect of different fire exposures on the composition of the toxic combustion products, and to establish the physiological effects of such products on living organisms. The ultimate goals of this field of research are to reduce human fire fatalities due to smoke inhalation, to determine effective treatments for survivors, and to prevent unnecessary suffering of fire casualties who have inhaled toxic combustion products.

Seventy-six percent of the people who die in fires die of the inhalation of toxic combustion products, not of burns (Hall and Harwood, 1995). This percentage has been rising by about 1% per year since 1979. Although total deaths in fires are declining, the percentage attributed to smoke inhalation has increased. Levin (1996, 1998), Purser (1988, 1995), Nelson (1995), and the National Research Council (1995) have reviewed various aspects of this subject.

[1] This chapter is a contribution of the National Institutes of Standards and Technology and is not subject to copyright. Certain commercial equipment, instruments, materials, or companies are identified in this chapter to specify the experimental procedure. Such identification does not imply recommendation or endorsement by NIST, nor does it imply that the materials or equipment identified are the best available for this purpose.

10.2 FIRE DEATH STATISTICS IN THE UNITED STATES

The latest statistics from the National Fire Protection Association (NFPA) indicate that the fire death rate in the United States was 6.1 times higher (10.1 times more if the deaths from September 11, 2001 are included) than in the United Kingdom, 1.16 times more than in Sweden, 1.3 times less than Japan (or 1.3 times more if the deaths from September 11, 2001, are included), and about the same as that in Canada (Hall, 2003). Although the reasons are still being debated, the number of fire deaths per capita since 1977 has been higher in the United States and Canada than in most of the other industrialized countries outside the former Soviet Union (Hall, 2003). Fire statistics collected by NFPA reported that approximately 1,687,500 fires occurred in the United States in 2002, the latest year for which complete statistics are available (Karter, 2003). Calculated another way, these statistics translate into a fire occurring in the United States every 19 sec, in a property located outside every 38 sec, in a structure every 61 sec, in a residence every 67 sec, and in a motor vehicle every 96 sec. These fires caused approximately 3380 civilian deaths and 18,425 reported injuries in 2002. Excluding the World Trade Center disaster of September 11, 2001, in which 2326 civilian deaths occurred, the number of deaths in 2002 decreased by almost 10% from the preceding year. However, there still was one civilian fire death every 156 min and one fire injury every 28 min. The number for injuries is believed to be less than the actual number, because many injuries are not reported. The property loss due to fires in 2002 is estimated at 10.3 billion dollars and indicates a decrease of 2.2% from the preceding year (again excluding the World Trade Center disaster).

In 2002, residential fires accounted for only 24% of the total fires but were responsible for 79% of all fire deaths and 76% of the reported injuries. Although in the years 1977 through 2002, the number of civilian fire fatalities in homes dropped from 5865 to 2670, fires in homes still cause the greatest concern to the fire community. Statistics show that children under 5 and adults over 65 years of age are the most frequent casualties of residential fires. This is attributed to their inherent difficulties in trying to escape. Statistics also show that males are more likely to die in fires than females. More fires and higher fire death rates occur in the South than any of the other geographical areas of the United States; the geographical region with the next highest fire death rate and number of fires is the Northeast.

One must distinguish between the causes of fires and the causes of fire deaths. The primary causes of residential fires have been shown to be heating and cooking (Federal Emergency Management Agency [FEMA], 1982; Runyan et al.,1992). Lack of central heat and the incorrect use of portable space heaters are two of the reasons given for the high fire and death rates in the South. Heating fires result in the highest property losses, primarily because cooking fires are usually noticed and extinguished before getting out of control. Fire deaths, however, usually result from fires ignited by cigarettes (Hall, 2003; Runyan et al., 1992). The most common fire scenario leading to fire deaths is one in which a person (usually intoxicated) falls asleep in an upholstered chair while smoking (FEMA, 1982). The cigarette falls into a crevice and starts the upholstered chair smoldering. The individual awakes and goes to bed unaware of the danger. The chair can smolder for an extended period (an hour was not unusual in laboratory tests conducted at the National Institute of Standards and Technology [NIST]) before bursting into flames. Once the flaming starts, the smoke will fill the room and escape to the other rooms. It is common to find people who have died of smoke inhalation (not burns) in or near their beds, indicating that their making little or no effort to escape was probably due to absence of warning and incapacitation from the asphyxiants in the smoke. Smoke detectors in this scenario would save many lives. Statistics have shown that working smoke detectors double one's probability of escaping alive (Hall, 2003). A recent problem is that many homes have nonfunctioning smoke detectors because the batteries were removed after a false alarm (usually from smoke produced by a kitchen or wood stove) or because old batteries were never replaced (Hall, 2003).

10.3 GENERATION OF TOXIC GASES IN FIRES: ADVERSE EFFECTS OF PARTICULATES

The adverse effects from smoke inhalation are believed to result mainly from toxic gas exposures, although the role of the particulates alone and in combination with fire gases needs further investigation. The importance, therefore, of determining the identities and concentrations of toxic gases produced from materials thermally decomposed under various fire conditions is evident. In addition, the increased variety of plastics in buildings and homes has raised the issue of whether synthetic materials may produce extremely or unusually toxic[2] combustion products. In 1975, the journal *Science* documented a case in which an experimental rigid polyurethane foam containing a fire retardant produced a very unusual toxic combustion product identified as 4-ethyl-1-phospha-2,6,7-trioxabicyclo[2.2.2]octane-1-oxide (commonly referred to as a bicyclic phosphate ester) (Petajan et al., 1975). Bicyclic phosphate compounds have been shown to cause seizures at very low concentrations. Based on these test results, this fire-retarded rigid polyurethane foam never became commercially available. To a large extent, however, it was this case that generated the burgeoning interest in the field of combustion toxicology and the widespread concern about the potential formation of "supertoxicants." Although research since the 1970s has shown that this concern is largely unfounded, the bicyclophosphate ester case and at least one other product that generated extremely toxic combustion products have indicated the need to test new formulations or materials containing new combinations of compounds to ensure that extremely or unusually toxic products are not generated. In November 2000, John Hall, Jr., indicated that the field of combustion toxicology is not producing much new data (Hall, 2000). He concluded that most of the current international debate in this area is over the interpretation and application of past research.

The gas composition of smoke depends on the chemical composition, the molecular structure and polymer formulation of the burning material, which may include a variety of additives, plasticizers, stabilizers, flame-retardants, cross-linking agents, fillers, and blowing agents. In addition, the conditions of thermal degradation, e.g., temperature, oxygen availability, and ventilation, will affect the nature of the combustion atmosphere. In a series of literature reviews by National Institutes of Standards and Technology (NIST) on the generation of combustion products and the combustion product toxicity from seven plastics (acrylonitrile-butadiene-styrenes [ABS], nylons, polyesters, polyethylenes, polystyrenes, poly(vinyl chlorides) [PVC], and rigid polyurethane foams) commonly found in materials and products, and decomposed under various thermal and atmospheric conditions, over 400 different decomposition products were noted (Rutkowski and Levin, 1986; Braun and Levin, 1986, 1987; Gurman et al., 1987; Huggett and Levin, 1987; Levin, 1987; Paabo and Levin, 1987a, 1987b). At about the same time, the Consumer Product Safety Commission reviewed the combustion products and the toxicity of the combustion products generated from acrylics, phenolics, polypropylene, and flexible polyurethane foam (Johnston et al., 1988a, 1988b; Purohit and Orzel, 1988; Orzel et al., 1989) and the National Academy of Sciences did the same type of literature review on an additional ten plastics, namely, acetal, aramid, cellulose acetate butyrate, epoxy resins, melamine-formaldehyde, polybutylene, polycarbonate, polyphenylene oxide, polytetrafluoroethylene, and urea-formaldehyde (National Research Council, 1987). Many of the combustion products were common to more than one plastic. In addition, many other combustion products probably exist that were not detected. At this time, the toxicity of most of these individual compounds is not known and little has been done to tackle the enormous problem of determining the toxicity of combinations of these compounds. The lack of detection of a specific combustion product from a material may only mean that the particular analytical techniques used were not suitable to detect that compound or that the investigator did

[2] In this chapter, the phrase "extremely toxic" is a relative term indicating that the effluent from the thermal decomposition of very small quantities of a material has been noted to cause death of experimental animals (usually rats or mice) under controlled laboratory conditions. "Unusually toxic" indicates that the toxic effect can not be totally attributable to the combustion gases (either singly or in combination) that are normally considered the main toxicants.

not specifically analyze the material for that combustion product. A limited amount of rodent testing becomes important to ensure that an unsuspected and therefore undetected toxic by-product has not formed or that synergism or antagonism of the generated products has not occurred.

Because the number of compounds one can reasonably analyze in any one test is limited, knowledge of the chemical composition, molecular structure, and formulation of the polymer can be used to provide some indication of the main gaseous products that may or may not be generated under specified experimental conditions. However, one needs to be cautious when predicting the combustion products from generic materials of unknown formulations. For example, one would expect nitrogen-containing materials (e.g., ABS, nylons, rigid and flexible polyurethanes) to produce hydrogen cyanide (HCN) and not expect HCN from a material like PVC. However, PVC containing zinc ferrocyanide[3] (an additive that was tested as a smoke suppressant) or a vinyl chloride-vinylidene chloride copolymer was found to generate HCN. In a similar fashion, based on the chemical composition, PVC is the only one of the seven plastics reviewed by NIST that would be expected to generate chlorinated combustion products. However, widespread usage of halogenated fire retardants in plastic formulations makes predicting the materials that will produce halogenated products extremely difficult. In addition, many of the fire-extinguishing agents contain halogens and can interact with the other combustion products in unpredictable ways when they are used to extinguish fires.

Temperature also plays an important role in influencing the production of decomposition products. In general, as the temperature and thus the rate of decomposition increase, the quantity of the more complex compounds and heavier hydrocarbons decreases and the concentrations of carbon monoxide (CO), carbon dioxide (CO_2), and nitrogen dioxide (NO_2) increase. The generation of HCN has also been shown to increase as a function of temperature. Another example is hydrogen chloride (HCl), the detection of which begins when stabilized PVC is heated to approximately 200°C; rapid dehydrochlorination then occurs at about 300°C (Huggett and Levin, 1987). On the other hand, more acrolein was generated from polyethylene under lower temperature, nonflaming conditions than under higher temperature flaming conditions (Paabo and Levin, 1987a).

As mentioned earlier, more work is needed to examine the adverse effects of the particulate matter that is also produced when materials are thermally decomposed. Examination of the smoke particulate and condensable matter is important for several reasons. First, many of the thermal degradation products may condense or be adsorbed by the soot particles and be transported along with the smoke into the body. Hydrogen chloride is one example of a compound that may be transported in such a fashion or can form a corrosive acid mist in moist air, such as air found in a person's lungs. One study of the particulate matter, which formed during the smoldering decomposition of rigid polyurethane foam, showed that many of the compounds detected in the soot fraction were not found in the volatile fraction (Joseph and Browner, 1980; Paabo and Levin, 1987b). Free radicals, which form in fires and are of toxicological concern due to their high reactivity (Westerberg et al., 1982; Lowry et al., 1985a, 1985b), are usually considered to have very short life spans; however, if adsorbed onto soot particles, their lifetimes can be considerably longer, and if the soot particle is the correct size, they can be inhaled deep into an individual's respiratory system. In addition, the particulate matter may interfere with the escape and rescue of individuals by causing the obscuration of vision, eye irritation (the eyes clamp shut and the victim is unable to see), and upper respiratory tract distress. An extreme case indicating the adverse effect of particulates was noted in experiments conducted at NIST. Rats exposed for 30 min to the smoke from flaming polystyrene died during the exposures and the concentration of CO in the blood, even in combination with CO_2, was too low to account for the deaths (Levin et al., 1987a). Pathological examination of these rats showed that their respiratory passages were completely blocked by soot and that suffocation was the likely cause of death (NIST, unpublished data).

Particulates have also been implicated in the toxicity of polytetrafluoroethylene (PTFE). Using the National Bureau of Standards (NBS) Cup Furnace Test Method (Figures 10.1 and 10.2; see Section 10.7), the combustion products of PTFE were found to be 300 times more toxic than other

[3] This material was never made commercially available after toxicity testing indicated that its combustion products produced very rapid deaths of experimental animals (rats).

materials decomposed in a similar fashion (Levin et al., 1982, 1983). Further studies showed that inhalation of the combustion products of PTFE decomposed in the NBS cup furnace caused focal hemorrhages, edema, and fibrin deposition in the lungs of rats (Zook et al., 1983). These same studies showed that, with time, focal interstitial thickenings developed because of hypertrophy and hyperplasia of alveolar cells and that macrophages accumulated in the alveoli. Thrombosis of pulmonary capillaries and disseminated intravascular coagulation also occurred. Renal infarcts were common. The exact reason for the unusual toxicity of PTFE's combustion products is unknown and has generated a great deal of interest (Alarie and Stock, 1984a; Birky, 1984; Williams and Clarke, 1983, 1984). The specific heating conditions of the NBS Cup Furnace Test Method[4] (Williams and Clarke, 1983; Williams et al., 1987) and the particulate fraction from PTFE have been cited (Clarke et al., 1990; Baker and Kaiser, 1991; Lee and Seidel, 1991). However, thermal decomposition in the University of Pittsburgh I (U. Pitt I) Test Method also produced their highest toxicity classification of "much more toxic than wood" (Alarie and Anderson, 1981; Alarie and Stock, 1984a). The other classifications of the U. Pitt I method are "less toxic than wood", "as toxic as wood," and "more toxic than wood." Decomposition in the German Din System produced a toxicity at least 10 times more toxic than wood (Purser, 1992). Full-scale fire tests seem to implicate hydrogen fluoride or carbonyl fluoride as the toxic agents (Clarke et al., 1992). Another compound that was suspected is perfluoroisobutylene (PFIB), which is toxic at extremely low levels and can be generated during the combustion of polymerized fluorocarbon compounds (e.g., PTFE) (Wang et al., 2001). The Occupational Safety and Health Administration (OSHA) reports that the American Conference of Governmental Industrial Hygienists (ACGIH) threshold limit value (TLV) is 0.01 ppm (0.082 mg/m^3) (www.osha.gov/dts/chemicalsampling/data/CH_260750.html, accessed April 15, 2004). However, analysis of the combustion atmospheres from PTFE at NIST did not detect any PFIB. An extensive review on the toxicology of soot was published in 1983 (Barfknecht, 1983). This review discusses the effect of soot on the induction of respiratory diseases and carcinogenicity.

10.4 TOXIC POTENCY VERSUS FIRE HAZARD VERSUS FIRE RISK

Death in a fire may be caused by:

1. Carbon monoxide (CO)
2. Toxic gases in addition to CO
3. Oxygen (O_2) at levels too low to sustain life
4. Incapacitation—either physical (inability to escape) or mental (incorrect decision making)
5. Bodily burns from flame contact
6. Very high air temperatures
7. Smoke density or irritants in smoke that affect vision and interfere with ability to escape
8. Psychological effects (e.g., fear, shock, and panic)
9. Physical insults (e.g., building or ceiling collapses, broken bones from jumping from upper floors)

Research in the field of combustion toxicology is primarily concerned with items 1 through 4, all of which are related to the toxic potency of the fire gas effluent. Toxic potency as applied to inhalation of smoke or its component gases is defined by American Society for Testing and Materials (ASTM) as "A quantitative expression relating concentration (of smoke or combustion gases) and exposure time to a particular degree of adverse physiological response, for example, death on exposure of humans or animals"(ASTM, 2004). This definition is followed by a discussion, which states, "The toxic potency of smoke from any material, product, or assembly is related to the composition of that smoke which, in turn, is dependent upon the conditions under which the smoke is generated." One should add that

[4] Further explanation of the combustion toxicity test methods are in Section 10.7.

the LC_{50}[5] is a common end point used in laboratories to assess toxic potency. In the comparison of the toxic potencies of different compounds or materials, the lower the LC_{50} (i.e., the smaller the amount of material necessary to reach the toxic endpoint), the more toxic the material is.

A toxicity assessment based on lethality due to toxic gases is only part of the total fire hazard that needs to be evaluated, especially when one is making choices as to the best material for a specific end use. ASTM defines "fire hazard" as the potential for harm associated with fire (ASTM, 2004). The discussion that follows this definition states, "A fire may pose one or more types of hazard to people, animals, or property. These hazards are associated with the environment and with a number of fire-test-response characteristics of materials, products, or assemblies including but not limited to ease of ignition, flame spread, rate of heat release, smoke generation and obscuration, toxicity of combustion products, and ease of extinguishment." Other factors that need to be evaluated when considering a material for use in a given situation include the quantity of material needed, its configuration, the proximity of other combustibles, the volume of the compartments to which the combustion products may spread, the ventilation conditions, the ignition and combustion properties of the material and other materials present, the presence of ignition sources, the presence of fire protection systems, the number and type of occupants, and the time necessary to escape.

"Fire risk" is defined as "An estimation of expected fire loss that combines the potential for harm in various fire scenarios that can occur with the probabilities of occurrence of those scenarios" (ASTM, 2004). The discussion following the definition of fire risk states, "Risk may be defined as the probability of having a certain type of fire, where the type of fire may be defined in whole or in part by the degree of potential harm associated with it, or as potential for harm weighted by associated probabilities. However it is defined, no risk scale implies a single value of acceptable risk. Different individuals presented with the same risk situation may have different opinions on its acceptability." A simple way to explain the difference between fire hazard and fire risk is to compare the fire with sky diving, a very hazardous sport; however, if one never goes sky diving, no risk is incurred.

10.5 TOXICITY ASSESSMENT: ANIMAL EXPOSURES

In most combustion toxicology experiments, the biological end point has been lethality or incapacitation of experimental animals, usually rats or mice. Incapacitation (sublethal effects) in a fire can be as perilous as lethality if an individual becomes incapable of correct decision making or physically unable to move. Under these circumstances, the ability to escape will be lost and death will occur unless the individual is rescued. Therefore, many fire scientists are concerned with the levels of combustion products or amounts of materials that, when combusted, will cause incapacitation. However, an incapacitation model for use in laboratory testing has been especially difficult to develop. Most of the tests for incapacitation that have been designed are based on the physical-motor capability of an experimental animal to perform some task (e.g., running in a motorized wheel, jumping onto a pole or lifting a paw to escape a shock, running in a maze, or pushing the correct lever to open a door to escape an irritating atmosphere) (Purser and Berrill, 1983; Kaplan and Hartzell, 1984; Kaplan et al., 1985). The concentration of toxic combustion products that cause the loss of these types of physical-motor capabilities is usually close to the concentration that is lethal and does not usually add much additional information. Other attempts to measure incapacitation have included the examination of neurological end points (e.g., measuring the increased number of errors by humans doing mathematical problems while exposed to low levels of CO [Arthur Callahan, private communication] or exposing rats and pigeons to a complete neurobehavioral battery of 25 tests) (Richie et al., 1995).

Whether one needs to examine incapacitation or lethality depends on the problem one is trying to solve. To determine the best material for a particular end use application, the lethality end point has

[5] The LC_{50} value is the result of a statistical calculation based on multiple experiments, each with multiple animals, and indicates the concentration at which 50% of the experimental animals exposed for a specific length of time would be expected to die either during the exposure time or the postexposure observation period.

proven to be more definitive and will flag the materials that produce extremely toxic combustion products better than an incapacitation end point. There are at least two reasons for this. (1) Incapacitation is only measured during the exposure that is usually 30 min or less, but lethality can also occur during the postexposure observation period, which can be two weeks or longer. A material that only causes delayed effects during the postexposure period (e.g., a material that generates HCl) can thus have an LC_{50} value that is lower (more toxic) than the incapacitation EC_{50}[6] value (i.e., the amount of thermally decomposed material necessary to cause postexposure deaths is less than the amount needed to cause incapacitation during the exposure). (2) In many cases in which the combustion products contain high concentrations of irritant gases, the animals would only appear to be incapacitated (i.e., they would stop responding to the incapacitation test indicator because of the high irritant quality of the smoke), but when removed from the combustion atmosphere, they would immediately start responding normally.

10.6 TOXICITY ASSESSMENT: PREDICTIVE MODELS

In the 1970s, there were essentially two experimental strategies to examine the issues raised by the field of combustion toxicology: (1) the analytical chemical method and (2) the animal exposure approach. In the analytical chemical method, investigators thermally decomposed materials under different experimental conditions and tried to determine every combustion product that was generated (Levin, 1987; ASTM, 2001). This approach generated long lists of compounds. The toxicity of most of these individual compounds was unknown and the concept of examining the toxicity of all the various combinations of compounds was and still is considered an impossible task. An additional problem with the analytical method was that, as mentioned earlier, one could not be certain that every toxic product was detected and identified. This approach enabled one to identify many of the multiple products that were generated but not know the toxic potency of all the identified compounds, especially when combined.

In the animal exposure approach, the animals (usually rats or mice) serve as indicators of the degree of toxicity of the combustion atmospheres (Kaplan et al., 1983; Levin et al., 1982, 1991b, 1992a). The materials of concern are thermally decomposed under different combustion conditions and the animals are exposed to the combined particulate and gaseous effluent. Multiple animal experiments (each with multiple animals) with different concentrations of material are conducted to determine an EC_{50} (incapacitation) or an LC_{50} (lethality) for a specific set of combustion conditions. Each material would then have a particular EC_{50} or an LC_{50} value that can be used to compare the toxicities of different materials decomposed under the same conditions. The lower the EC_{50} or LC_{50}, the more toxic the combustion products from that material. In this approach, one knows the relative toxicity of a material as compared with another material but does not know which of the toxic gases are responsible for the adverse effects.

In the 1980s, investigators began examining the possibility of combining the analytical chemical method with the animal exposure approach to develop empirical mathematical models to predict the toxicity (Levin, 1996; Levin et al., 1985a, 1987a, 1987b). These predictions were based on actual experiments with animals and their response to each of the main toxic combustion gases, CO, CO_2, low O_2, HCN, NO_2, HCl, and hydrogen bromide (HBr) and various combinations of these gases. The advantages of these predictive approaches are (1) the number of test animals is minimized by predicting the toxic potency from a limited chemical analysis of the smoke; (2) smoke may be produced under conditions that simulate any fire scenario of concern; (3) fewer tests are needed, thereby reducing the overall cost of the testing; and (4) information is obtained on both the toxic potency of the smoke (based on the mass of material burned) and the responsible gases (based on the primary toxic gases in the mixture). The prediction is checked with one or two animal tests to ensure that an unexpected gas or toxic combination has not formed. The results of using these empirical mathematical models indicated that, in most cases, one could predict the toxic potency of a combustion atmosphere with the main toxic gases and did not need to worry about the effects of minor or more obscure gases.

[6] The definition of the EC_{50} is essentially the same as that of the LC_{50} except incapacitation rather than lethality is the end point and incapacitation is monitored only during the exposure and not during the postexposure period.

10.6.1 Primary Toxic Combustion Gases

Complete combustion of a polymer containing carbon, hydrogen, and oxygen in an atmosphere with sufficient O_2 yields CO_2 and H_2O. It is during incomplete combustion under various atmospheric conditions in either flaming or nonflaming modes that compounds of greater toxicological concern are generated. When O_2 is limited, the primary gases formed during the combustion of most materials are CO, CO_2, and H_2O. If the materials contain nitrogen, HCN and NO_2, two principal thermo-oxidative products of toxicological concern, are also likely to be generated. Halogenated or flame-retarded materials generally produce HCl or HBr. Other commonly found fire gases include nitrogen oxides (NO_x), ammonia (NH_3), hydrogen sulfide (H_2S), sulfur dioxide (SO_2), and fluorine compounds. One also needs to consider that in fire situations, O_2 levels drop and exposure to low O_2 atmospheres will have additional adverse physiological effects. Some of these toxic combustion gases (e.g., CO, HCN, low O_2) produce immediate asphyxiant symptoms, whereas others (e.g., HCl, HBr, NO_2) fall into an irritant category and produce symptoms following the exposures.

10.6.2 The N-Gas Models

The N-Gas Models for predicting smoke toxicity were founded on the hypothesis that a small number ("N") of gases in the smoke account for a large percentage of the observed toxic potency. These predictive models were based on an extensive series of experiments conducted at NIST on the toxicological interactions of the primary gases found in fires (Levin, 1996; Levin et al., 1985a, 1987a, 1987b, 1989c, 1990a, 1991a, 1995). Both the individual gases and complex mixtures of these gases were examined. To use these models, materials are thermally decomposed by using a bench-scale method that simulates realistic fire conditions, the concentrations of the primary fire gases—CO, CO_2, low O_2, HCN, HCl, HBr, and NO_2—are measured, and the toxicity of the smoke is predicted by using the appropriate N-Gas Model. The predicted toxic potency is checked with a small number of animal (Fischer 344 male rats) tests to ensure that an unanticipated toxic gas was not generated or an unexpected toxicological effect (e.g., synergism or antagonism) did not occur. The results indicate whether the smoke from a material or product is extremely toxic (based on mass consumed at the predicted toxic level) or unusually toxic (the toxicity can not be explained by the combined measured gases). These models have been shown to correctly predict the toxicity in both bench-scale laboratory tests and full-scale room burns of a variety of materials of widely differing characteristics chosen to challenge the system (Levin et al., 1987a; Babrauskas et al., 1991). The six-gas model (without NO_2) is now included in two national toxicity test method standards—ASTM E1678-02 approved by the American Society for Testing and Materials (ASTM, 2002) and NFPA 269 approved by the National Fire Protection Association (2003). It is also included in an international standard (ISO 13344:1996) that was approved by 16 member countries of the International Organization for Standardization (ISO), Technical Committee 92 (TC92). All three of these standards were first published in 1996.

The objectives of developing the N-Gas Models were:

- To establish the extent to which the toxicity of a material's combustion products could be explained and predicted by the interaction of the major toxic gases generated from that material in the laboratory or whether minor and more obscure combustion gases needed to be considered.
- To develop a bioanalytical screening test and a mathematical model that would predict whether a material would produce extremely toxic or unusually toxic combustion products.
- To predict the occupant response from the concentrations of primary toxic gases present in the environment and the time of exposure.
- To provide data for use in computer models designed to predict the hazard that people will experience under various fire scenarios.

10.6.2.1 The Six-Gas N-Gas Model

The six-gas model (see Equation [10.1]) was based on studies at NIST on the toxicological interactions of six gases, CO, CO_2, HCN, low O_2 concentrations, HCl, and HBr. First, individual gases in air were tested to determine the concentrations necessary to cause 50% of the laboratory test animals (Fischer 344 male rats) to die either during the exposure (within exposure LC_{50}) or during the exposure plus a 14-day postexposure observation period (within plus postexposure LC_{50}). The studies on HCl and HBr were conducted at Southwest Research Institute (SwRI) under a grant from NIST (Hartzell et al., 1990). Similar measurements for various combinations of these gases indicated whether the toxicity of the mixtures of gases was additive, synergistic, or antagonistic.

Based on these empirical results, the following six-gas N-Gas model was developed:

$$\frac{m[\mathrm{CO}]}{[\mathrm{CO_2}]-b}+\frac{[\mathrm{HCN}]}{\mathrm{LC_{50}HCN}}+\frac{21-[\mathrm{O_2}]}{21-\mathrm{LC_{50}O_2}}+\frac{[\mathrm{HCl}]}{\mathrm{LC_{50}HCl}}+\frac{[\mathrm{HBr}]}{\mathrm{LC_{50}HBr}}=\text{N-Gas Value} \qquad (10.1)$$

where the numbers in brackets indicate the time-integrated average atmospheric concentrations during a 30-min exposure period [(ppm × min)/min or for O_2 (% × min)/min] (Levin, 1996, 1998; Levin et al., 1991b, 1995; International Organization for Standardization, 1996; ASTM, 2002; NFPA, 2003). The other terms are defined in the following paragraphs.

Under the experimental conditions used at NIST and with Fischer 344 male rats, the 30-min LC_{50} value of CO_2 is 47% (470,000 ppm or 846 g/m^3) with 95% confidence limits of 43 to 51% (Levin et al., 1987b,1989c, 1991a)[7]. No deaths occurred in rats exposed to 26% CO_2 for 30 min. In a real fire, the highest theoretically possible concentration of CO_2 is 21%, a concentration that could only occur if all the atmospheric O_2 were converted to CO_2, a highly improbable event. Therefore, CO_2 concentrations generated in fires are not lethal. However, CO_2 is a respiratory stimulant causing an increase in both respiratory rate and tidal volume. It also increases the acidosis of the blood. When combined with any of the other tested gases, CO_2 has a synergistic toxicological effect, i.e., the toxicity of the other gases is increased in the presence of CO_2 (Table 10.1). Empirically, however, we found that the effect of the CO_2 can only be added into the N-Gas equations once. Therefore, we included the CO_2 effect with the CO factor because we had more data on the combined effect of CO and CO_2, and CO is the toxicant most likely to be present in all fires. The results on the synergistic effect of CO_2 on CO indicated that, as the concentration of CO_2 increases (up to 5%), the toxicity of CO increases. Above 5% CO_2, the toxicity of CO starts to revert back toward the toxicity of the CO by itself. The terms m and b in Equation (10.1) define this synergistic interaction and equal −18 and 122,000, respectively, if the CO_2 concentrations are 5% or less. For studies in which the CO_2 concentrations are above 5%, m and b equal 23 and –38,600, respectively.

In rats, the 30-min LC_{50} for CO is 6600 ppm (7560 mg/m^3) and with 5% CO_2, this value drops to 3900 ppm (4470 mg/m^3). Exposure to CO in air only produced deaths during the actual exposures and not in the postexposure observation period; however, exposures to CO plus CO_2 also caused deaths in the postexposure period. Carbon monoxide is a colorless, odorless, tasteless, and nonirritating poisonous gas. The toxicity of CO comes from its binding to the hemoglobin in red blood cells and the formation of carboxyhemoglobin (COHb). The presence of CO on the hemoglobin molecule prevents the binding of O_2 to hemoglobin (O_2Hb) and results in hypoxia in the exposed individual. Because the binding affinity of hemoglobin for CO is 210 times greater than its affinity for O_2, only 0.1% CO (1000 ppm) (1150 mg/m^3) is needed to compete equally with O_2, which is normally present at 20.9% in air (20.9% ÷ 210 ≈ 0.1%). Thus, only 1000 ppm of CO in the atmosphere is enough to generate 50% COHb, a value commonly quoted (but not necessarily

[7] *Caution:* The values given for use in Equations (10.1) and (10.3) depend on the test protocol, on the source of test animals, and on the rat strain. It is important to verify these values whenever different conditions prevail and, if necessary, to determine the values that would be applicable under the new conditions.

TABLE 10.1 Synergistic Effects of CO_2

Gas[a]	LC_{50} Values[b]	
	Single Gas	With 5% CO_2
CO_2	470,000 ppm (846 g/m^3)	—
CO	6600 ppm (7560 mg/m^3)	3900 ppm (4470 mg/m^3)
NO_2	200 ppm (376 mg/m^3)	90 ppm (169 mg/m^3)
O_2	5.4%	6.4%

[a] All gases were mixed in air.

[b] Thirty-minute exposures of Fischer 344 rats. Deaths occurred during and after the exposure.

proven) as the concentration that is lethal to humans. The time to get to 50% COHb at 1000 ppm CO would be longer than 30 min.

The LC_{50} value of HCN is 200 ppm (221 mg/m^3) for 30-min exposures or 150 ppm (166 mg/m^3) for 30-min exposures plus the postexposure observation period. HCN caused deaths both during and after the exposures.

The 30-min LC_{50} of O_2 is 5.4%, which is included in the model by subtracting the combustion atmospheric O_2 concentration from the normal concentration of O_2 in air, i.e., 21%.

The LC_{50} values of HCl or HBr for 30-min exposures plus postexposure times are 3700 ppm (5450 mg/m^3) and 3000 ppm (9940 mg/m^3), respectively. HCl and HBr at levels found in fires only cause postexposure effects.

The pure and mixed gas studies showed that if the value of Equation (10.1) is 1.1 ± 0.2, then some fraction of the test animals would die. Below 0.9, no deaths would be expected and above 1.3, all the animals would be expected to die. Because the concentration–response curves for animal lethalities from smoke are very steep, it is assumed that if some percentage (not 0 or 100%) of the animals die, the experimental loading is close to the predicted LC_{50} value. Results using this method show good agreement (deaths of some of the animals when the N-gas values were above 0.9) and the good predictability of this approach.

This model can be used to predict deaths that will occur only during the fire exposure or deaths during and after the fire. To predict the deaths that would occur both during and after the exposures, Equation (10.1) is used as presented. To predict deaths only during the exposures, HCl and HBr, which only have postexposure effects, should not be included in Equation (10.1). In small-scale laboratory tests and full-scale room burns, Equation (10.1) was used successfully to predict the deaths during and after exposures to numerous materials (Braun et al., 1990; Babrauskas et al., 1991). In the case of PVC, the model correctly predicted the results as long as the HCl was greater than 1000 ppm (1470 mg/m^3); therefore, it is possible that HCl concentrations smaller than 1000 ppm may not have any observable effect on the model even in the postexposure period. More experiments are necessary to show whether a true toxic threshold for HCl does exist.

Although most of the work at NIST concentrated on deaths during or after 30-min exposures, the LC_{50}s of many of these gases both singly and mixed were determined at times ranging from 1 to 60 min and in all the cases examined, the predictive capability of Equation (10.1) holds if the LC_{50}s for the other times are substituted into the equation.

10.6.2.2 The Seven-Gas Model: Addition of NO_2 to the N-Gas Model

Nitrogen dioxide is an irritant gas that will cause lachrimation, coughing, respiratory distress, increases in methemoglobin levels, and lung edema (Lee, 1980). Single brief exposures to less than lethal concentrations can cause lung damage, emphysema, or interstitial fibrosis. Low levels have

been alleged to increase one's susceptibility to respiratory infections and aggravate one's reactions to allergens. Impairment of dark adaptation has also been noted. Delayed serious effects can be observed as late as two to three weeks following exposures. In the lungs, NO_2 forms both nitric (HNO_3) and nitrous (HNO_2) acids, which are probably responsible for the damage to the lung cells and connective tissue.

In fires, NO_2 may arise from atmospheric nitrogen fixation, a reaction that is material independent, or from the oxidation of nitrogen from nitrogen-containing materials. To examine the generation of NO_2 from nitrogen fixation, a small study was undertaken at NIST. In two full-scale fires of rooms in which the main source of fuel was polystyrene-covered walls, only low levels of NO_x (10 and 25 ppm, respectively) were found, indicating little nitrogen fixation under these conditions (Levin et al., 1989c). A real example of burning nitrogen-containing materials was the 1929 Cleveland Clinic fire in which 50,000 nitrocellulose x-ray films were consumed (Gregory et al., 1969). The deaths of 97 people in this fire were attributed mainly to NO_x. An additional 26 people died between 2 h and one month after the fire, and 92 people were treated for nonfatal injuries. In laboratory tests of nitrogen-containing materials under controlled conditions, 1 to 1000 ppm of NO_x were measured (Lieu et al., 1981; M. Paabo, NIST, unpublished data; Tsuchiya, 1984; Babrauskas et al., 1991). In military tests of armored vehicles penetrated by high-temperature ammunition, NO_2 levels above 2000 ppm (3760 mg/m^3) were found (Mayorga et al., 1995).

10.6.2.2.1 Individual and Binary Mixtures

In small-scale laboratory tests of NO_2 in air, deaths of Fischer 344 male rats occur only in the postexposure period, and the LC_{50} value following a 30-min exposure is 200 ppm (376 mg/m^3) (Levin et al., 1989c). Carbon dioxide plus NO_2 show synergistic toxicological effects (Levin et al., 1989c). The LC_{50} for NO_2 following a 30-min exposure to NO_2 plus 5% CO_2 is 90 ppm (169 mg/m^3) (postexposure deaths) (i.e., the toxicity of NO_2 doubled).

The details of the following research are provided in Levin et al., 1995. As mentioned above, CO produces only within-exposure deaths and its 30-min LC_{50} is 6600 ppm (7560 mg/m^3). In the presence of 200 ppm (376 mg/m^3) of NO_2, the within-exposure toxicity of CO doubled (i.e., its 30-min LC_{50} became 3300 ppm [3780 mg/m^3]). An exposure of approximately 3400 ppm (3890 mg/m^3) CO plus various concentrations of NO_2 showed that the presence of CO would also increase the postexposure toxicity of NO_2. The 30-min LC_{50} value of NO_2 went from 200 ppm (376 mg/m^3) to 150 ppm (282 mg/m^3) in the presence of 3400 ppm (3890 mg/m^3) of CO. A concentration of 3400 ppm of CO was used because that concentration is not lethal during the exposure, thus permitting any postexposure effects of CO on NO_2 to become evident; the LC_{50} of CO (6600 ppm) (7560 mg/m^3) would have caused deaths of the animals during the 30-min exposure.

The 30-min LC_{50} of O_2 is 5.4% and the deaths occurred primarily during the exposures. In the presence of 200 ppm (376 mg/m^3) of NO_2, the within-exposure LC_{50} of O_2 and its toxicity increased to 6.7%. In the case of O_2, increased toxicity is indicated by an increase in the value of the LC_{50} because it is more toxic to be adversely affected by a concentration of O_2 ordinarily capable of sustaining life. Exposure of the animals to 6.7% O_2 plus various concentrations of NO_2 showed that the NO_2 toxicity doubled (i.e., its LC_{50} value decreased from 200 ppm to 90 ppm [376 mg/m^3 to 169 mg/m^3]).

One of the most interesting findings was the antagonistic toxicological effect noted during the experiments on combinations of HCN and NO_2. As mentioned above, the 30-min LC_{50} for NO_2 alone is 200 ppm (376 mg/m^3) (postexposure) and the 30-min within-exposure LC_{50} for HCN alone is also 200 ppm (221 mg/m^3). These concentrations of either gas alone are sufficient to cause the death of the animals (i.e., 200 ppm HCN or 200 ppm NO_2 would cause 50% of the animals to die either during the 30-min exposure or after the 30-min exposure, respectively). However, in the presence of 200 ppm of NO_2, the within-exposure HCN LC_{50} concentration increases to 480 ppm (530 mg/m^3) or, in other words, the toxicity of HCN decreases by 2.4 times.

The mechanism for this antagonistic effect is believed to be as follows. In the presence of H_2O, NO_2 forms nitric acid (HNO_3) and nitrous acid (HNO_2) (Goldstein et al., 1980). These two acids are

the suspects most likely responsible for the lung damage leading to the massive pulmonary edema and subsequent deaths noted following exposure to high concentrations of NO_2. Nitrite ion (NO_2^-) formation occurs in the blood when the nitrous acid dissociates. The nitrite ion oxidizes the ferrous ion in oxyhemoglobin to ferric ion to produce methemoglobin (MetHb) (Equation [10.2]) (Rodkey et al., 1976). MetHb is a well-known antidote for CN^- poisoning (Klaassen, 1996). MetHb binds cyanide-forming cyanmethemoglobin, which keeps the cyanide in the blood and prevents it from entering the cells. In the absence of MetHb, free cyanide will enter the cells, react with cytochrome oxidase, prevent the utilization of O_2, and cause cytotoxic hypoxia. If, on the other hand, cyanide is bound to MetHb in the blood, it will not be exerting its cytotoxic effect. Therefore, the mechanism of the antagonistic effect of NO_2 on the toxicity of cyanide is believed to be due to the conversion of oxyhemoglobin [$O_2Hb(Fe^{2+})$] to methemoglobin [$MetHb(Fe^{3+})$] in the presence of nitrite (see Equation [10.2]).

$$2H^+ + 3NO_2^- + 2O_2Hb\left(Fe^{2+}\right) = 2MetHb\left(Fe^{3+}\right) + 3NO_3^- + H_2O \qquad (10.2)$$

10.6.2.2.2 Tertiary Mixtures of NO_2, CO_2, and HCN

Earlier work indicated that the presence of 5% CO_2 with either HCN or NO_2 produced a more toxic environment than would occur with either gas alone (Levin et al., 1987a, 1989c). The antagonistic effects of NO_2 on HCN indicate that the presence of one LC_{50} concentration of NO_2 (∽200 ppm) (376 mg/m^3) will protect the animals from the toxic effects of HCN during the 30-min exposures, but not from the postexposure effects of the combined HCN and NO_2. Thus, it was of interest to examine combinations of NO_2, CO_2, and HCN (Levin et al., 1995). In this series of experiments, the concentrations of HCN were varied from almost 2 to 2.7 times its LC_{50} value (200 ppm) (221 mg/m^3). The concentrations of NO_2 were approximately equal to one LC_{50} value (200 ppm) (376 mg/m^3) if the animals were exposed to NO_2 alone and approximately one-half the LC_{50} (90 ppm) (169 mg/m^3) if the animals were exposed to NO_2 plus CO_2; the concentrations of CO_2 were maintained at approximately 5%; and the O_2 levels were kept above 18.9%. The results indicated that CO_2 does not make the situation worse but rather provides additional protection even during the postexposure period. In each of six experiments, some or all of the animals lived through the test even though they were exposed to greater than lethal levels of HCN plus lethal levels of NO_2 and in four tests, some of the animals lived through the postexposure period even though the animals were exposed to combined levels of HCN, NO_2, and CO_2 that would be equivalent to 4.7 to 5.5 times the lethal concentrations of these gases. One possible reason that CO_2 seems to provide an additional degree of protection is that NO_2 in the presence of 5% CO_2 produces four times more MetHb than does NO_2 alone (Levin et al., 1989c).

10.6.2.2.3 Mixtures of CO, CO_2, NO_2, O_2, and HCN

The initial design of these experiments was to look for additivity of the CO/CO_2, HCN, and NO_2 factors keeping each at about one-third of its toxic level, while keeping the O_2 concentration above 19% (Levin et al., 1995). When these initial experiments produced no deaths, we started to increase the concentrations of CO up to one-third of the LC_{50} of CO alone (6600 ppm) (7560 mg/m^3), HCN was increased to 1.3 or 1.75 times its LC_{50} depending on whether the within-exposure LC_{50} (200 ppm) (221 mg/m^3) or the within- and postexposure LC_{50} (150 ppm) (166 mg/m^3) was being considered, and NO_2 was increased up to a full LC_{50} value (200 ppm) (376 mg/m^3). The results indicated that just adding a NO_2 factor (e.g., $[NO_2]/LC_{50}$ NO_2) to Equation (10.1) would not predict the effect on the animals. A new mathematical model was developed and is shown as Equation (10.3). In this model, the differences between the within-exposure predictability and the within-exposure and postexposure predictability are the following: (1) the LC_{50} value used for HCN is 200 ppm

(221 mg/m^3) for within-exposure or 150 ppm (166 mg/m^3) for within-exposure and postexposure and (2) the HCl and HBr factors are not used to predict the within-exposure lethality, only the within-exposure and postexposure lethality. According to Equation (10.3), animal deaths will start to occur when the N-Gas Value is above 0.8, and 100% of the animals will die when the value is above 1.3. Results indicated that in those few cases where the values were above 0.8 and no deaths occurred, the animals were severely incapacitated (close to death) as demonstrated by the absence of the righting reflex or the eye reflex.

$$\text{N-Gas Value} = \frac{m[\text{CO}]}{[\text{CO}_2]-b} + \frac{21-[\text{O}_2]}{21-\text{LC}_{50}(\text{O}_2)} + \left(\frac{[\text{HCN}]}{\text{LC}_{50}(\text{HCN})} \times \frac{0.4[\text{NO}_2]}{\text{LC}_{50}(\text{NO}_2)}\right) + 0.4\left(\frac{[\text{NO}_2]}{\text{LC}_{50}(\text{NO}_2)}\right) + \frac{[\text{HCl}]}{\text{LC}_{50}(\text{HCl})} + \frac{[\text{HBr}]}{\text{LC}_{50}(\text{HBr})} \tag{10.3}$$

10.6.2.2.4 The N-Gas Model Including NO_2

For an explanation of these terms, see the paragraphs following Equation (10.1). Equation (10.3) should be used to predict the within-exposure plus postexposure lethal toxicity of mixtures of CO, CO_2, HCN, reduced O_2, NO_2, HCl, and HBr. The LC_{50} values will be the same as those given for Equation (10.1) using 150 ppm (166 mg/m^3) for HCN and 200 ppm (376 mg/m^3) for NO_2. If one wishes to predict the deaths that will occur only during the exposure, the LC_{50} value used for HCN should be 200 ppm (221 mg/m^3) and the HCl and HBr factors should not be included. To predict the lethal toxicity of atmospheres that do not include NO_2, Equation (10.1) should be used.

10.7 COMBUSTION TOXICITY TEST METHODS

The toxicity of the combustion products from any new material formulation or product containing additives or new combinations of additives needs to be examined. Material and polymer chemists keep trying to develop new "fire-safe" materials (Nelson, 1995). The terms "fire safe" or "fire resistant" are not the same as noncombustible. Unless these new materials are truly noncombustible, some thermal decomposition will occur when the materials are exposed to fire conditions. Both the toxic gases and the irritants that are present in all smoke need to be considered potential dangers. The toxic products can cause both acute and delayed toxicological effects. It is the acute and extremely short-term effects that prevent escape from burning buildings by causing faulty judgment, incapacitation, and death. The irritants in the smoke can also interfere with one's ability to escape by causing severe coughing and choking and by preventing one from keeping one's eyes open long enough to find the exits. In addition, the delayed effects, such as tissue or organ injury, mutagenicity, carcinogenicity, and teratogenicity need to be studied because they may ultimately lead to permanent disability and postexposure deaths. Some of the issues involved in delayed effects are addressed in an article by Lewtas (1994) and a review by Barfknecht (1983). The current advances in the field of genetics provide investigators with new opportunities to examine the effects of combustion products at the molecular level. One objective could be to determine whether these toxic products cause DNA damage and mutations. Specific problems of interest include: Does the damage occur in nuclear DNA, or mitochondrial DNA, or both? Are certain areas of the DNA more prone to these mutations (i.e., are there hot spots?)? Can we categorize the types of mutations (e.g., transitions, transversions, deletions, insertions)? And how efficient are the repair mechanisms? Are these mutagens also known to be carcinogens?

Toxicity-screening tests for both the acute and delayed effects are needed, therefore, to evaluate the combustion products including any irritants that may be present in newly proposed materials and

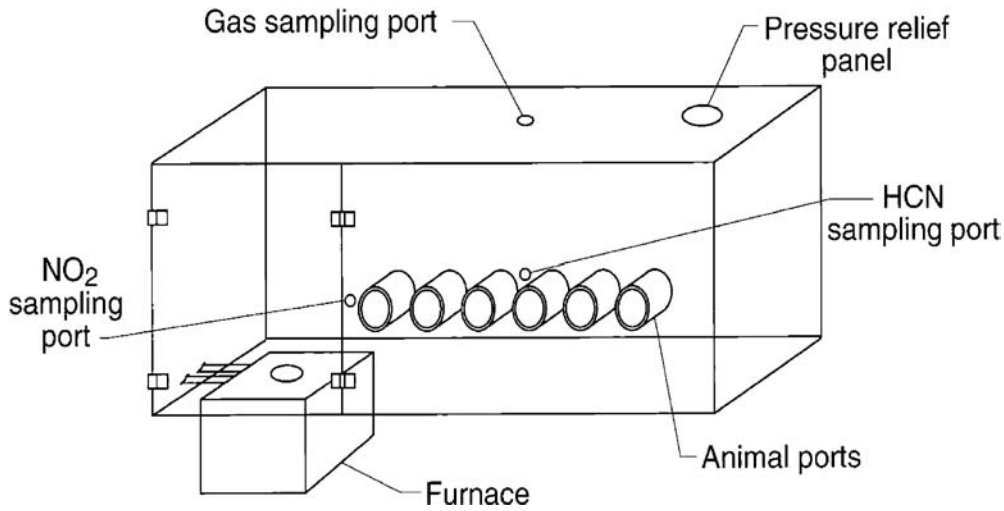


FIGURE 10.1 Schematic illustration of the NBS Cup Furnace Smoke-Toxicity Method.

products. It is imperative that the materials and products be tested under experimental conditions that simulate realistic fire scenarios of concern (e.g., flash-over conditions emanating from smoldering and then flaming of upholstered furniture in homes or smoldering fires in concealed spaces in aircraft). The ideal tests should be simple, rapid, and inexpensive; these tests should use the least amount of sample possible (because, in many cases, only small amounts of a new experimental material may be available), use a minimum number of test animals, and have a definitive toxicological end point for comparison of the multiple candidates.

Although faulty judgment and incapacitation are significant causes of worry because they can prevent escape and cause death, they are extremely difficult and complex end points to define and measure in non-human test subjects. Death of experimental animals (e.g., rats), on the other hand, is a more definitive and easily determined end point and can be used to compare the relative toxicities of alternate materials deemed suitable for the same purpose. The assumption made here is that, if the combustion products of material X are significantly more lethal than those of material Y, the combustion products of X would probably cause more incapacitation and more impairment of judgment than Y as well. The number of experimental animals can be significantly reduced by utilizing one of the predictive mathematical models developed for combustion toxicology such as the N-Gas Models previously discussed in this chapter.

Many test methods for the determination of the acute toxicity of combustion products from materials and products have been developed (Kaplan et al., 1983). In 1983, thirteen of the methods published up to that time were evaluated by Arthur D. Little, Inc. to assess the feasibility of incorporating combustion toxicity requirements for building materials and finishes into the building codes of New York State (Anderson et al., 1983). On the basis of seven different criteria, only two methods were found acceptable. These two methods were the flow-through smoke toxicity method developed at the University of Pittsburgh (U. Pitt.I Method) (Alarie and Anderson, 1979, 1981; Levin et al., 1992a) and the closed-system cup furnace smoke toxicity method (NBS Cup Furnace Method) (Figures 10.1 and 10.2) developed at NIST (known at that time as the National Bureau of Standards) (Levin et al., 1982, 1991b). In 1991 and 1992, standard reference materials were developed at NIST and made available to the users of these two methods to provide assurance that they are performing the methods correctly (Levin et al., 1991b, 1992a). Based on the results of the Arthur D. Little report, the state of New York under Article 15, Part 1120 of the New York State Fire Prevention and Building

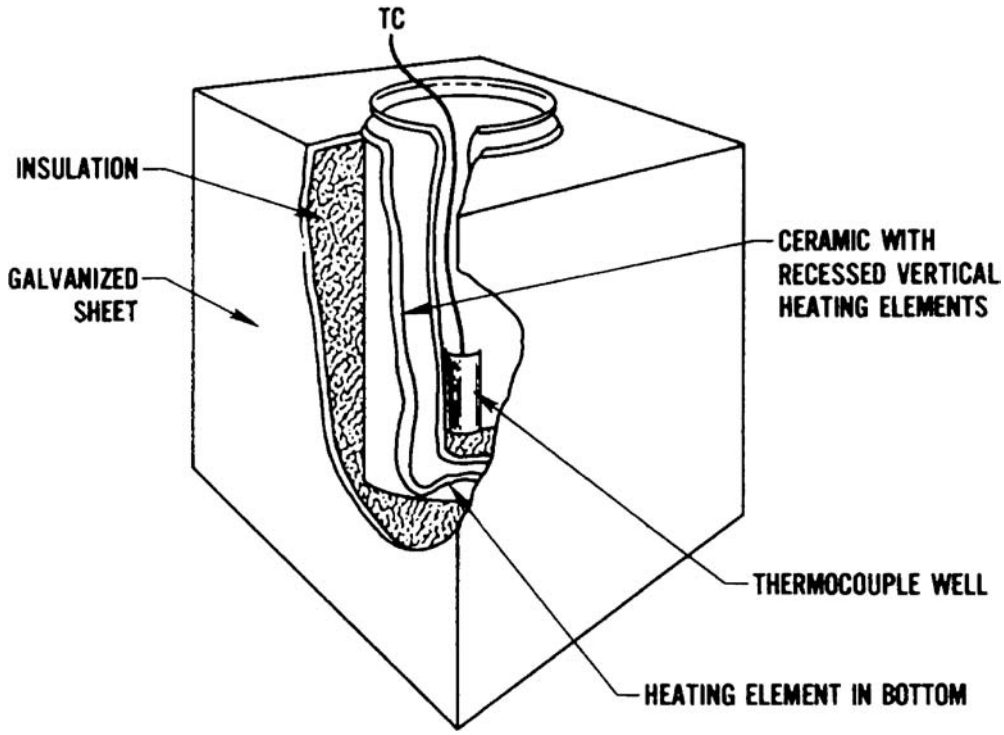


FIGURE 10.2 Pyrolysis/combustion cup furnace used in the NBS Cup Furnace Smoke-Toxicity Method.

Code, decided to require that building materials and finishes be examined by the method developed at the University of Pittsburgh and that the results be filed with the state (New York State Fire Prevention and Building Code, 1986). Note, however, that although the results are filed, the state of New York does not regulate any materials or products based on the results of this or any other toxicity test.

Since 1983, several new approaches to assess acute combustion toxicity have been examined. These approaches include a radiant furnace smoke toxicity protocol developed by NIST and SwRI (Babrauskas et al., 1991, 1998; Levin,1992a, 1992b), the University of Pittsburgh II radiant furnace method (Caldwell and Alarie, 1990a, 1990b, 1991), and the National Institute of Building Sciences (NIBS) toxic hazard test method (Norris, 1988; Roux, 1988). All three use radiant heat to decompose materials.

The NIST radiant test and the NIBS toxic hazard test use the same apparatus consisting of three components: a radiant furnace, a chemical analysis system, and an animal exposure chamber (Figure 10.3). The chemical analysis system (Figure 10.4) and animal exposure system are identical with that developed for the NBS cup furnace smoke-toxicity method. Although the apparatuses of both methods are essentially the same, they have different toxicological end points. In the NIST method, an approximate LC_{50}, based on the *mass* of material needed to cause lethality in 50% of the test animals during a 30-min exposure or a 14-day postexposure period, is the determinant of toxicity. The number of animals needed to run the test is substantially reduced by first estimating the LC_{50} by the N-Gas model and analytical tests without animals. This estimate is then verified with one or two animal tests to ensure that no unforeseen gas was generated. The toxicological end point of the NIBS toxic hazard test is the IT_{50}, the irradiation *time* (the time that the material is exposed to the radiant heat) that is required to kill 50% of the animals during a 30-min exposure or 14-day postexposure time. The actual results of the NIBS test with 20 materials indicated that the test animals died in very short periods (personal communication) and the test was unable to discriminate very well

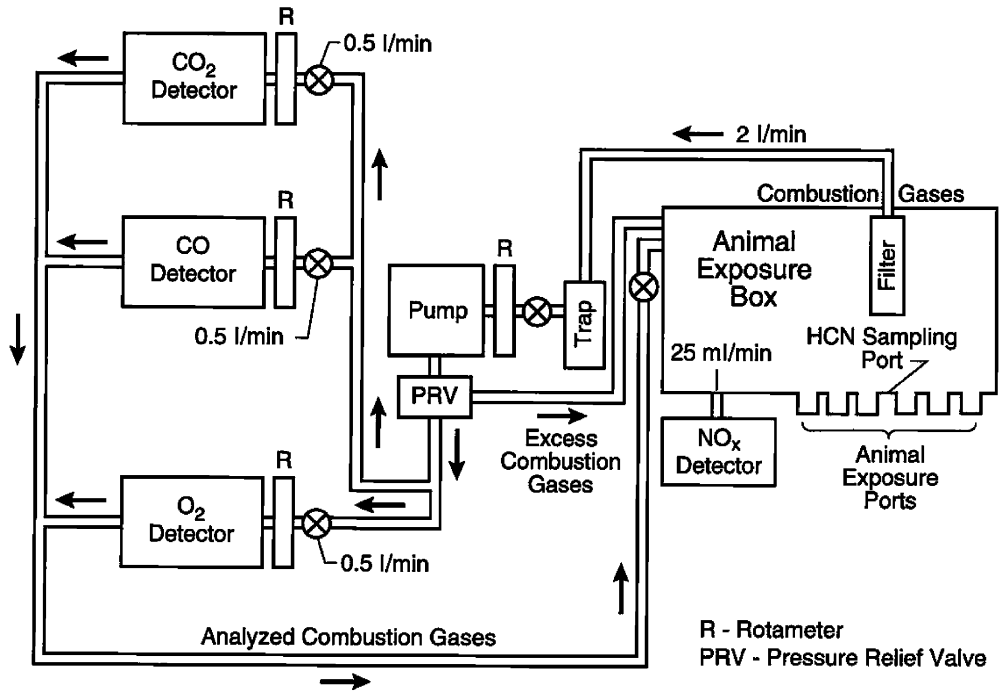


FIGURE 10.3 Schematic illustration of the NBS/NIST smoke-toxicity chemical analytical equipment in relation to the animal exposure chamber.

between materials. These results substantiate the thesis that mass (the smaller the mass necessary for an LC_{50}, the more toxic the material) is a better indicator of acute toxicity than time.

Both the NIST and NIBS test procedures are designed to simulate a postflashover scenario. The premise for simulating a postflashover fire is that most people that die of inhalation of toxic gases in *residential* fires in the United States are affected in areas away from the room of fire origin. Smoke and toxic gases are more likely to reach these distant areas following flashover. This scenario may not be relevant in certain circumstances (e.g., aircraft interior fires, where a smoldering fire in a concealed space may cause significant problems if the plane is over a large body of water and unable to land for a considerable period). In the United Kingdom, more fire fatalities are found in the room of fire origin (Hall, 2003). The reason for this difference between the two countries is not clear.

The NIST radiant test has been accepted by the ASTM as a national standard designated ASTM E1678–02 and entitled "Standard Test Method for Measuring Smoke Toxicity for Use in Fire Hazard Analysis"(ASTM, 2002). The NFPA has also adopted the NIST Radiant Test Method as a national standard called "Standard Test Method for Developing Toxic Potency Data for Use in Fire Hazard Modeling," NFPA 269 (NFPA, 2003). In 1995, the International Organization for Standardization, Technical Committee 92, Subcommittee 3 (ISO/TC92/SC3) on Toxic Hazards in Fire published an international standard for combustion toxicity after approval by 16 countries (ISO, 1996). This standard, ISO/IS 13344 entitled "Determination of the Lethal Toxic Potency of Fire Effluents," describes the mathematical models (including the N-Gas Model) available for predicting the toxic potency of fire atmospheres based on the toxicological interactions of the main combustion gases present. In the international standard, investigators have the flexibility of designing or choosing a system that will simulate conditions relevant to their fire scenario, rather than having to accept a designated combustion system.

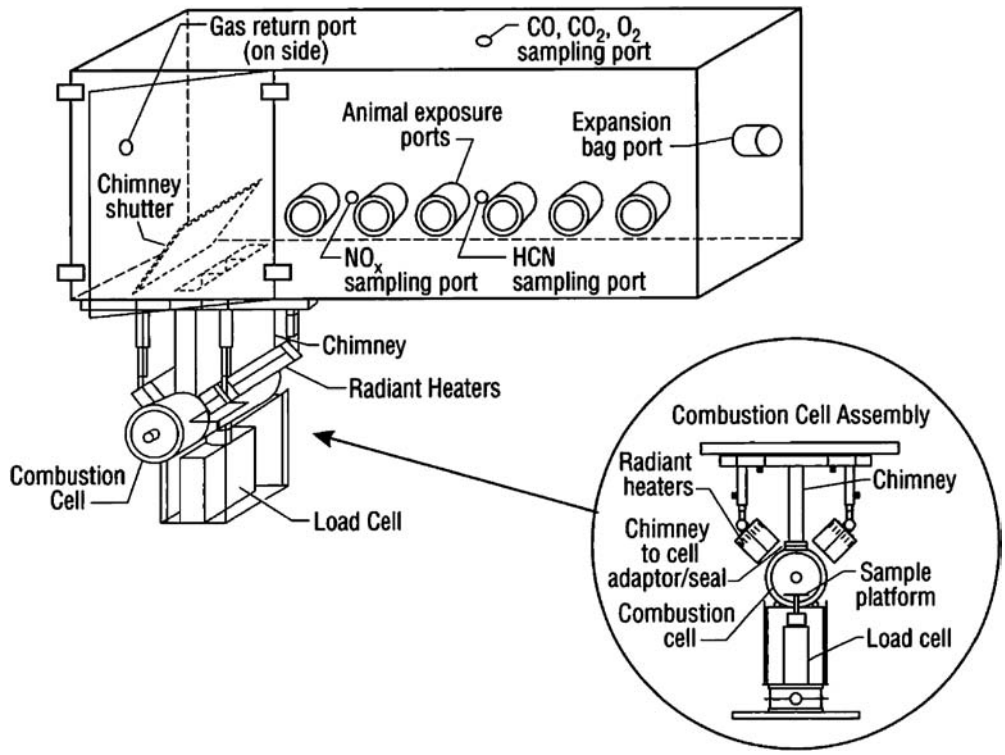


FIGURE 10.4 Schematic illustration of the NIST Radiant Smoke-Toxicity Method and the combustion cell assembly.

10.8 TOXICANT SUPPRESSANTS

Fire scientists are very familiar with fire-retardant chemicals, which are defined by ASTM as "Chemicals, which when added to a combustible material, delay ignition and combustion of the resulting material when exposed to fire" (ASTM, 2004). The discussion adds "A fire-retardant chemical can be a part of the molecular structure, an admixture or an impregnant." The term "toxicant suppressant," however, is a new expression arising from research at NIST which demonstrated that the addition of copper compounds to flexible polyurethane foam (FPU) significantly reduced the generation of hydrogen cyanide (HCN) as well as the toxicity of the combustion products when the foam was thermally decomposed (Levin et al., 1988, 1989a, 1989b, 1990b, 1992b). These experiments were designed to simulate the nonflaming and then flaming stages of a chair ignited by a cigarette (a two-phase heating system that simulates the fire scenario that results in the most fire deaths in the United States) (Levin et al., 1985b). The term "toxicant suppressant" may be defined as a chemical that, when added to a combustible material, significantly reduces or prevents one or more toxic gases from being generated when that material undergoes thermal decomposition. The resultant gas effluent should be less toxic than that from the untreated material, i.e., the toxic gas, whose concentration is being reduced, should not be converted to an equally or more toxic product.

The results of these studies at NIST indicated that:

1. HCN concentrations in the thermal decomposition products from a flexible polyurethane foam were reduced approximately 85% when the foam was treated with 0.1% or 1.0% Cu_2O and thermally decomposed via a two-phase heating system in the NBS cup furnace smoke-toxicity apparatus.

2. The copper or copper compounds could be added to the foams during or after formulation and still be operative in reducing the toxicity and HCN yield. (The BASF Corporation prepared the foams that had the Cu powder and Cu_2O added during formulation.) The addition of the copper or copper compounds during formulation did not affect the foaming process or the physical appearance of the foams except for a slight change of color.
3. Low levels of the copper compounds were effective. In particular, when cupric oxide (CuO) was used, the concentration of copper needed was only 0.08% by weight and when cuprous oxide (Cu_2O) was used, only 0.07% by weight was needed to significantly reduce the generation of HCN.
4. Full-scale room burns indicated that the presence of Cu_2O in the FPU reduced the HCN generation by approximately 50 to 70% when the experimental plan was designed to simulate a realistic scenario (the foams contained 1.0% Cu_2O, were covered with a cotton upholstery fabric, and arranged to simulate a chair; smoldering was initiated with cigarettes and flaming occurred spontaneously).
5. Under small-scale conditions, less than 3 ppm of NO_x was generated from the untreated foams, whereas a range of 3 to 33 ppm of NO_x was measured from the 0.1 to 1.0% Cu_2O-treated foams. About 6% of the HCN appeared to be converted to NO_x. In the full-scale room tests, approximately 23% of the HCN appeared to be converted to NO_x. Because we have shown in our laboratory that NO_2 acts as an antagonist to HCN, this amount of NO_x may also act to counteract the immediate toxic effects of any residual HCN.
6. Because the atmospheric oxygen (O_2) concentrations can reach very low levels in real fires, it was important to know if the reduction of HCN by copper would occur under low O_2 conditions. Small-scale tests with the ambient O_2 concentrations as low as 6% indicated that the HCN levels were reduced by as much as 82% when the FPU was treated with 0.1% Cu_2O.
7. The toxicity of the gas effluent was also reduced (an indication that the HCN was not being converted into some compound that was even more toxic). Fewer animal (Fischer 344 rats) deaths occurred during the 30-min exposures to the FPU treated with the copper and copper compounds than the untreated FPU. Toxicity based on LC_{50} values was reduced 40 to 70% in the small-scale tests with 0.1% Cu_2O-treated foams. The blood cyanide levels in the animals exposed to combustion products from the CuO-treated foams for 30 min were one-half to one-fourth those measured in the animals exposed to the smoke from the same amount of untreated foam.
8. Postexposure deaths were also reduced in the animals exposed to the combustion products from the Cu- and Cu_2O-treated FPU foams in the small-scale tests. These delayed postexposure deaths have *not* been observed in animals exposed to combustion products from flexible polyurethane foams decomposed in large-scale room fire tests. The specific cause of these postexposure deaths is not known.
9. No differences in flammability characteristics between the 0.1% Cu_2O-treated and untreated FPU foam were observed. These characteristics were examined to ensure that the positive effect on toxicity was not contradicted by negative effects on the flammability properties. The flammability characteristics examined were (1) ignitability in three systems (the cup furnace smoke-toxicity method, the Cone Calorimeter, and the Lateral Ignition and Flame Spread Test [LIFT]); (2) heat release rates under small-scale (Cone Calorimeter) and medium-scale (furniture calorimeter) conditions; (3) heats of combustion under small-scale (Cone Calorimeter) and medium-scale (furniture calorimeter) conditions; (4) CO/CO_2 ratios under small-scale (Cone Calorimeter) and medium-scale (furniture calorimeter) conditions; (5) smoke obscuration (Cone Calorimeter); and (6) rate of flame spread (LIFT).
10. Research conducted at the BASF Corporation indicated that the physical properties of the 1.0% Cu_2O-treated FPU were not significantly different from the comparable untreated

FPU. The physical properties examined were tensile strength, elongation, tear strength, resilience, indentation force deflection, support factor, compression sets, and airflow.

11. The use of melamine-treated FPU is becoming more common (Weil and Choudhary, 1995; Weil and Zhu, 1995); it is one of two FPU foams currently allowed in Great Britain. Small-scale tests indicated that a melamine-treated FPU generated six times more HCN than an equal amount of a non-melamine-treated foam. The presence of Cu_2O reduced the HCN from the melamine foam by 90%.

Jellinek and coworkers in the late 1970s also showed that the concentrations of HCN generated from the thermal decomposition of a polyurethane at 300 and 400°C decreased when flowed through copper compounds (Jellinek and Takada, 1977; Jellinek et al., 1978). In their studies, the polyurethane films were very thin (15 μm thick and 50 mg in weight). In some experiments, the metal powder was mixed with the polymer and, in others, copper metal films of 400 to 1000A were deposited on top of the polymer films. In most cases, the percent of copper was 10% or greater. The lowest concentration that they tested was a 2.6% copper film that inhibited the evolution of HCN by 66%. Their experiments indicated that the copper is probably acting as an oxidative catalyst that would decompose gaseous HCN into N_2, CO_2, H_2O, and small amounts of nitrogen oxides. Further research is needed to determine whether this is the actual molecular mechanism that allows copper to act as a HCN toxicant suppressant.

The research of Levin and her coworkers differed from that of Jellinek in that much larger samples of FPU (including full-scale room burns of cushions and simulated chairs), much smaller concentrations of copper were used, and the toxicity of the combustion products from the copper-treated FPU was also examined.

Unpublished data of Levin also indicated that the combustion products from a wool fabric treated with copper would generate 50% less HCN than the untreated fabric. These results demonstrate a potentially more universal effect, namely that treating nitrogen-containing materials with copper compounds will reduce the HCN generated when that material is exposed to fire conditions. Taking these results one step further, one could develop other toxicant suppressants that, when added to materials and products, would now prevent or significantly reduce the toxic effluents that are generated when they are thermally decomposed.

10.9 CONCLUSIONS

The field of toxicology of combustion products has come a long way since the early 1970s when the National Commission on Fire Prevention and Control submitted their report entitled "America Burning" to President Nixon (National Commission on Fire Prevention and Control, 1973). That report, which included the statement that "Appallingly, the richest and most technologically advanced nation in the world leads all the major industrialized countries in per capita deaths and property loss from fire," provided the impetus to determine the cause of and to try to solve this major problem in the United States. It was shortly thereafter that Petajan et al. (1975) published their paper in *Science* entitled "Extreme Toxicity from Combustion Products of a Fire-Retarded Polyurethane Foam" and raised the specter of "supertoxicants" emanating from combustion atmospheres. In the following years, the field of combustion toxicology expanded greatly and many materials and products were tested by various methods. With the exception of the material described in the Petajan et al. article and one other product described in section 10.3, the concern about supertoxicants has proven to be largely unfounded. However, the potential that the combustion products from new formulations or combinations of compounds may prove to be extremely or unusually toxic has encouraged manufacturers to continue to test their new products. The state requirements of New York have also been a motivating force for manufacturers to test the building products they wish to sell in New York (New York State Uniform Fire Prevention and Building Code, 1986). This testing may have prevented dangerous products from reaching the marketplace.

After reaching a peak in the 1980s, the amount of research in the area of combustion toxicology has declined significantly. With the exception of some military laboratories, very few laboratories are still doing this type of research. The Building and Fire Research Laboratory at NIST, where much of the research quoted in this chapter was conducted, abolished its combustion toxicology research program in 1992. This does not mean, however, that additional research is not needed. Some of the areas that should be examined include: the effects of the addition of more gases and heat to the N-Gas Model; the effects of particulates alone and in combination with the N-gases; the effects of chronic exposures to fire atmospheres (e.g., those experienced by fire fighters); sublethal effects on the neurological and other organ systems; mutagenic, teratogenic, and carcinogenic effects of acute and chronic exposures; the development of improved therapeutic methods for the treatment of victims of smoke (including particulates) inhalation; and additional research on toxicant suppressants with the ultimate aim of developing fire-safe materials (i.e., materials that do not produce toxic gases even when they are thermally decomposed). Because approximately 80% of fire deaths are the result of smoke inhalation, a less toxic smoke could significantly increase the time available for escape and reduce the number of injuries and deaths from fire.

REFERENCES

Alarie, Y.C. and Anderson, R.C., Toxicologic and acute lethal hazard evaluation of thermal decomposition products of synthetic and natural polymers, *Toxicol. Appl. Pharmacol.,* 51, 341–362, 1979.

Alarie, Y.C. and Anderson, R.C., Toxicological classification of thermal decomposition products of synthetic and natural polymers, *Toxicol. Appl. Pharmacol.,* 57, 181–188, 1981.

Alarie, Y. and Stock, M.F., Toxicity of Teflon®, similar results obtained with radiant-convective heat sources or in the hot fire gases, *Fire Mater.,* 8, 54–55, 1984a.

Alarie, Y. and Stock, M.F., Letters to the Editor, *Fire Mater.,* 8, 56, 1984b.

American Society for Testing and Materials (ASTM), *Annual Book of ASTM Standards*, ASTM E 800-01, Standard guide for measurement of gases present or generated during fires, American Society for Testing and Materials, West Conshohocken, PA, 2001.

American Society for Testing and Materials (ASTM), *Annual Book of ASTM Standards*, ASTM E1678-02, Standard test method for measuring smoke toxicity for use in fire hazard analysis, American Society for Testing and Materials, West Conshohocken, PA, 2002.

American Society for Testing and Materials (ASTM), *Annual Book of ASTM Standards*, ASTM E176–04, Standard terminology of fire standards, American Society for Testing and Materials, West Conshohocken, PA, 2004.

Anderson, R.C., Croce, P.A., Feeley, F.G., III, and Sakura, J.D., Study to assess the feasibility of incorporating combustion toxicity requirements into building material and furnishing codes of New York State, Final Report to Department of State, Office of Fire Prevention and Control, Albany, New York, from Arthur D. Little, Inc., Cambridge, MA, 1983.

Annual Report to the Secretary of Health and Mental Hygiene and the General Assembly of Maryland, submitted by the Advisory Council to the Center for the Study of Health Effects of Fire, June 30, 1997.

Babrauskas, V., Gann, R.G., Levin, B.C., Paabo, M., Harris, R.H., Peacock, R.D., and Yusa, S., A methodology for obtaining and using toxic potency data for fire hazard analysis, *Fire Saf. J.,* 31, 345–358, 1998.

Babrauskas, V., Levin, B.C., Gann, R.G., Paabo, M., Harris, R.H., Jr., Peacock, R.D., and Yusa, S., Toxic potency measurement for fire hazard analysis, NIST Special Publication 827, National Institute of Standards and Technology, Gaithersburg, MD, 1991.

Baker, B.B., Jr. and Kaiser, M.A., Understanding what happens in a fire, *Anal. Chem.,* 63, 79A–83A, 1991.

Barfknecht, T.R., Toxicology of soot, *Prog. Energy Combust. Sci.,* 9, 199–237, 1983.

Birky, M.M., Reply to "Combustion product toxicity: dependence on the mode of product generation" by S.J. Williams and F.B. Clarke and "Toxicity of Teflon®, similar results obtained with radiant-convective heat sources or in the hot fire gases." Letters to the Editor, *Fire Mater.,* 8, 56, 1984.

Braun, E., Davis, S., Klote, J.H., Levin, B.C., and Paabo, M., Assessment of the Fire Performance of School Bus Interior Components, NISTIR 4347, National Institute of Standards and Technology, Gaithersburg, MD, 1990.

Braun, E. and Levin, B.C., Polyesters: a review of the literature on products of combustion and toxicity, *Fire Mater.,* 10, 107–123, 1986.

Braun, E. and Levin, B.C., Nylons: a review of the literature on products of combustion and toxicity, *Fire Mater.,* 11, 71–88, 1987.

Caldwell, D.J. and Alarie, Y.C., A method to determine the potential toxicity of smoke from burning polymers. I. Experiments with douglas fir, *J. Fire Sci.,* 8, 23–62, 1990a.

Caldwell, D.J. and Alarie, Y.C., A method to determine the potential toxicity of smoke from burning polymers. II. The toxicity of smoke from douglas fir, *J. Fire Sci.,* 8, 275–309, 1990b.

Caldwell, D.J. and Alarie, Y.C., A method to determine the potential toxicity of smoke from burning polymers. III. Comparison of synthetic polymers to douglas fir using the UPitt II flaming combustion/toxicity of smoke apparatus, *J. Fire Sci.,* 9, 470–518, 1991.

Clarke, F.B., Seidel, W.C., Scherer, K.V., Cline, D., Olson, A. and Bonesteel, J., Formation, identity, and coagulation of fluoropolymer-derived smoke aerosols. The relationship between aerosol behavior and observed toxicity of fluoropolymer smoke, in *Interflam 90,* Fifth International Fire Conference, Interscience Communications Limited, London, U.K., 1990, pp. 297–304.

Clarke, F.B., Van Kuijk, H., Valentine, R., Makovec, G.T., Seidel, W.C., Baker, B.B., Kasprzak, D.J., Bonesteel, J.K., Janssens, M., and Herpol, C., The toxicity of smoke from fires involving perfluoropolymers: full-scale fire studies, *J. Fire Sci.,* 10, 488–527, 1992.

Federal Emergency Management Agency, *Fire in the United States*, 2nd ed., Report FEMA–22, FEMA, Jessup, MD, 1982.

Goldstein, E., Goldstein, F., Peek, N.F., and Parks, N.J., Absorption and transport of nitrogen oxides, in *Nitrogen Oxides and Their Effects on Health,* Lee, S.D., Ed., Ann Arbor Science, Ann Arbor, MI, 1980, pp. 143–160.

Gregory, K.L., Malinoski, V.F., and Sharp, C.R., Cleveland Clinic fire survivorship study, 1929–1965, *Arch. Environ. Health,* 18, 508–515, 1969.

Gurman, J.L., Baier, L., and Levin, B.C., Polystyrenes: a review of the literature on the products of combustion and toxicity, *Fire Mater.,* 11, 109–130, 1987.

Hall, J.R., Jr., Overview of research on people and fire in the U.S., in *Fifteenth Meeting of the UJNR Panel on Fire Research and Safety, March 1–7, 2000*, Vol. 1. Bryner, S.L., Ed., NISTIR 6588, National Institute of Standards and Technology, Gaithersburg, MD, 2000.

Hall, J.R., Jr., Fire in the U.S. and Canada. National Fire Protection Association, Quincy, MA (www.nfpa.org), 2003.

Hall, J.R., Jr. and Harwood, B., Smoke or burns—which is deadlier? *NFPA J.,* January/February, 38–43, 1995.

Hartzell, G.E., Grand, A.F., and Switzer, W.G., Toxicity of smoke containing hydrogen chloride, in *Fire and Polymers: Hazard Identification and Prevention*, Nelson, G.L., Ed., ACS Symposium Series 425, American Chemical Society, Washington, DC, 1990, pp. 12–20.

Huggett, C. and Levin, B.C., Toxicity of the pyrolysis and combustion products of poly(vinyl chlorides): a literature assessment, *Fire Mater.,* 11, 131–142, 1987.

International Organization for Standardization (ISO), Determination of the lethal toxic potency of fire effluents, TC 92/SC3, ISO 13344:1996 (www.bre.co.uk/iso/ and www.iso.ch/cate/d21617.html) (accessed 16 Apr. 2004), 1996.

Jellinek, H.H.G., Chaudhuri, A., and Takada, K., Inhibition of hydrocyanic acid evolution from polyurethanes during oxidative degradation, *Polymer J.,* 10, 253–268, 1978.

Jellinek, H.H.G. and Takada, K., Toxic gas evolution from polymers: evolution of hydrogen cyanide from polyurethanes, *J. Polymer Sci.,* 15, 2269–2288, 1977.

Johnston, P.K., Doyle, E., and Orzel, R.A., Acrylics: a literature review of thermal decomposition products and toxicity, *J. Am. Coll. Toxicol.,* 7, 139–200, 1988a.

Johnston, P.K., Doyle, E., and Orzel, R.A., Phenolics: a literature review of thermal decomposition products and toxicity, *J. Am. Coll. Toxicol.,,* 7, 201–219, 1988b.

Joseph, K.T. and Browner, R.F., Analysis of particulate combustion products of polyurethane foam by high performance liquid chromatography and gas chromatography-mass spectrometry, *Anal. Chem.,* 52, 1083–1085, 1980.

Kaplan, H.L., Grand, A.F., and Hartzell, G.E., *Combustion Toxicology, Principles and Test Methods,* Technomic Publishing Co., Lancaster, PA, 1983.

Kaplan, H.L., Grand, A.F., Switzer, W.G., Mitchell, D.S., Rogers, W.R., and Hartzell, G.E., Effects of combustion gases on escape performance of the baboon and the rat, *J. Fire Sci.,* 3, 228–244, 1985.

Kaplan, H.L. and Hartzell, G.E., Modeling of toxicological effects of fire gases. I. Incapacitating effects of narcotic gases, *J. Fire Sci.,* 2, 286–305, 1984.

Karter, M.J., Jr., 2002 U.S. fire loss, *NFPA J.,* September/October 97, 59–63, 2003.

Klaassen, C.D., Nonmetallic environmental toxicants, in Goodman & Gilman's *The Pharmacological Basis of Therapeutics,* 9th ed., Hardman, J.G., Limbird, L.E., Molinoff, P.B., and Ruddon, R.W., Eds., McGraw-Hill, New York, 1996. pp. 1673–1696.

Lee, S.D., *Nitrogen Oxides and Their Effects on Health,* Ann Arbor Science, Ann Arbor, MI, 1980.

Lee, K.P. and Seidel, W.C., Pulmonary response to perfluoropolymer fume and particles generated under various exposure conditions, *Fundam. Appl. Toxicol.,* 17, 254–269, 1991.

Levin, B.C., A summary of the NBS literature reviews on the chemical nature and toxicity of the pyrolysis and combustion products from seven plastics: acrylonitrile-butadiene-styrenes (ABS), nylons, polyesters, polyethylenes, polystyrenes, poly(vinyl chlorides) and rigid polyurethane foams, *Fire Mater.,* 11, 143–157, 1987.

Levin, B.C., The development of a new small-scale smoke toxicity test method and its comparison with real-scale fire tests, *Toxicol. Lett.,* 64/65, 257–264, 1992a.

Levin, B.C., The development of a new small-scale smoke toxicity test method and its comparison with real-scale fire tests, in *Toxicology from Discovery and Experimentation to the Human Perspective,* Chambers, P.L., Chambers, C.M., Bolt, H.M., and Preziosi, P., Eds., Elsevier Press, New York, 1992b. pp. 257–264.

Levin, B.C., New research avenues in toxicology: 7-gas N-gas model, toxicant suppressants, and genetic toxicology, *Toxicology,* 115, 89–106, 1996.

Levin, B.C., Combustion toxicology, in *Encyclopedia of Toxicology,* Vol. 1, Wexler, P., Ed., Academic Press, San Diego, 1998. pp. 360–374.

Levin, B.C., Fowell, A.J., Birky, M.M., Paabo, M., Stolte, A., and Malek, D., Further development of a test method for the assessment of the acute inhalation toxicity of combustion products, NBSIR 82–2532, National Bureau of Standards (now National Institute of Standards and Technology), Gaithersburg, MD, 1982.

Levin, B.C., Paabo, M., and Birky, M.M., An interlaboratory evaluation of the National Bureau of Standards Test Method for assessing the acute inhalation toxicity of combustion products, NBSIR 83–2678, National Bureau of Standards (now National Institute of Standards and Technology), Gaithersburg, MD, 1983.

Levin, B.C., Paabo, M., Bailey, C., Harris, S.E., and Gurman, J.L., Toxicological effects of the interactions of fire gases and their use in a toxic hazard assessment computer model, *The Toxicologist,* 5, 127, 1985a.

Levin, B.C., Paabo, M., Fultz, M.L., and Bailey, C.S., Generation of hydrogen cyanide from flexible polyurethane foam decomposed under different combustion conditions, *Fire Mater.,* 9, 125–133, 1985b.

Levin, B.C., Paabo, M., Gurman, J.L., and Harris, S.E., Effects of exposure to single or multiple combinations of the predominant toxic gases and low oxygen atmospheres produced in fires, *Fundam. Appl. Toxicol.,* 9, 236–250, 1987a.

Levin, B.C., Paabo, M., Gurman, J.L., Harris, S.E., and Braun, E., Toxicological interactions between carbon monoxide and carbon dioxide, *Toxicology,* 47, 135–164, 1987b.

Levin, B.C., Gurman, J.L., Paabo, M., Harris, R.H., Clark, H.M., and Yoklavich, M.F., Reduction of both atmospheric hydrogen cyanide and acute inhalation toxicity of combustion products from flexible polyurethane by copper additives, First Annual Report to the International Copper Research Association, Inc. from the National Institute of Standards and Technology, Gaithersburg, MD, 1988.

Levin, B.C., Paabo, M., Harris, R.H., Eller, N., and Highbarger, L., Copper additives decrease the acute inhalation toxicity of the combustion products of flexible polyurethane by inhibiting hydrogen cyanide generation, Second Annual Report to the International Copper Association, Ltd. from the National Institute of Standards and Technology, Gaithersburg, MD, 1989a.

Levin, B.C., Paabo, M., Harris, R.H., Clark, H.M., Yoklavich, M.F., Eller, N., and Highbarger, L., The effect of copper additives on atmospheric hydrogen cyanide and acute inhalation toxicity from the combustion products of flexible polyurethane, paper presented at the Proceedings of the Fire Retardant Chemicals Association Fall Conference, Scottsdale, AZ, 1989b, pp. 107–112.

Levin, B.C., Paabo, M., Highbarger, L., and Eller, N., Synergistic effects of nitrogen dioxide and carbon dioxide following acute inhalation exposures in rats. NISTIR 89–4105, National Institute of Standards and Technology, Gaithersburg, MD, 1989c.

Levin, B.C., Paabo, M., Highbarger, L., and Eller, N., Toxicity of complex mixtures of fire gases, *The Toxicologist,* 10, 84, 1990a.

Levin, B.C., Braun, E., Shields, J.R., and Lowe, D., Reduction of hydrogen cyanide concentrations and acute inhalation toxicity from flexible polyurethane foam combustion products by the addition of copper compounds. Part III. The effect of copper additives on the flammability characteristics of flexible polyurethane foam. NISTIR 4441, National Institutes of Standards and Technology, Gaithersburg, MD. 1990b.

Levin, B.C., Paabo, M. and Navarro, M., Toxic interactions of binary mixtures of NO_2 & CO, NO_2 & HCN, NO_2 & reduced O_2, and NO_2 & CO_2, *The Toxicologist.* 11, 222, 1991a.

Levin, B.C., Paabo, M. and Schiller, S.B., A standard reference material for calibration of the cup furnace smoke toxicity method for assessing the acute inhalation toxicity of combustion products, *J. Res. Natl. Inst. Stand. Technol.,* 96, 741–755, 1991b.

Levin, B.C., Alarie, Y., Stock, M.F., and Schiller, S.B., The development of a standard reference material for calibration of the University of Pittsburgh smoke toxicity method for assessing the acute inhalation toxicity of combustion products, *J. Res. Natl. Inst. Stand. Technol.,* 97, 245–252, 1992a.

Levin, B.C., Braun, E., Paabo, M., Harris, R.H., and Navarro, M., Reduction of hydrogen cyanide concentrations and acute inhalation toxicity from flexible polyurethane foam combustion products by the addition of copper compounds. Part IV: Effects of combustion conditions and scaling on the generation of hydrogen cyanide and toxicity from flexible polyurethane foam with and without copper compounds, NISTIR 4989, National Institute of Standards and Technology, Gaithersburg, MD, 1992b.

Levin, B.C., Braun, E., Navarro, M., and Paabo, M., Further development of the N-Gas Model: an approach for predicting the toxic potency of complex combustion mixtures, in *Fire and Polymers II: Materials and Tests for Hazard Prevention,* Nelson, G.L., Ed., ACS Symposium Series 599, American Chemical Society, Washington, DC, 1995, pp. 293–311.

Lewtas, J., Human exposure to complex mixtures of air pollutants, *Toxicol. Lett.,* 72 163–169, 1994.

Lieu, P.J., Magill, J.H., and Alarie, Y.C., Flammability—toxicity ratings of some polyphosphazene and polyurethane foams, *J. Combust. Toxicol.,* 8, 242–259, 1981.

Lowry, W.T., Juarez, L., Petty, C.S., and Roberts, B., Studies of toxic gas production during actual structural fires in the Dallas area, *J. Forensic Sci.,* 30, 59–72, 1985a.

Lowry, W.T., Peterson, J., Petty, C.S., and Badgett, J.L., Free radical production from controlled low-energy fires: toxicity considerations, *J. Forensic. Sci.,* 30, 73–85, 1985b.

Mayorga, M.A., Januszkiewicz, A.J., and Lehnert, B.E., Environmental nitrogen dioxide exposure hazards of concern to the U.S. Army, in *Fire and Polymers II: Materials and Tests for Hazard Prevention,* Nelson, G.L., Ed., ACS Symposium Series 599, American Chemical Society, Washington, DC, 1995, pp. 323–343.

National Commission on Fire Prevention and Control, *America Burning,* Superintendent of Documents, U.S. Government Printing Office, Washington, DC, 1973.

National Fire Protection Association (NFPA), Standard test method for developing toxic potency data for use in fire hazard modeling, NFPA 269, NFPA National Fire Codes, National Fire Protection Association, Quincy, MA, 2003, pp. 269-1–269-18.

National Research Council, *Reviews of Combustion- and Pyrolysis-Product Toxicology of Ten Plastics*, Board on Environmental Studies and Toxicology, Commission on Life Sciences, Washington, DC, 1987.

National Research Council, *Fire- and Smoke-Resistant Interior Materials for Commercial Transport Aircraft,* National Materials Advisory Board, Publication NMAB-477-1, National Academy Press, Washington, DC, 1995.

Nelson, G.L., *Fire and Polymers II; Materials and Tests for Hazard Prevention,* Nelson, G.L., Ed., ACS Symposium Series 599, American Chemical Society, Washington, DC, 1995.

New York State Uniform Fire Prevention and Building Code, Article 15, Part 1120, Combustion toxicity testing and regulations for implementing building materials and finishes; fire gas toxicity data file, Office of Fire Prevention and Control, Department of State, New York State, Albany, NY, December 16, 1986.

Norris, J.C., National Institute of Building Sciences combustion toxicity hazard test, in *Proceedings of the Joint Meeting of the Fire Retardant Chemicals Association and the Society of Plastics Engineers,* Technomic Publishing, Lancaster, PA, 1988, pp. 146–155.

Orzel, R.A., Womble, S.E., Ahmed, F., and Brasted, H.S., Flexible polyurethane foam: A literature review of thermal decomposition products and toxicity, *J. Am. Coll. Toxicol.,* 8, 1139–1175, 1989.

Paabo, M., National Institute of Standards and Technology, Gaithersburg, MD, unpublished data.

Paabo, M. and Levin, B.C., A literature review of the chemical nature and toxicity of the thermal decomposition products of polyethylenes, *Fire Mater.,* 11, 55–70, 1987a.

Paabo, M. and Levin, B.C., A review of the literature on the gaseous products and toxicity generated from the pyrolysis and combustion of rigid polyurethane foams, *Fire Mater.,* 11, 1–29, 1987b.

Petajan, J.H., Voorhees, K.J., Packham, S.C., Baldwin, R.C., Einhorn, I.N., Grunnet, M.L., Dinger, B.G., and Birky, M.M., Extreme toxicity from combustion products of a fire-retarded polyurethane foam, *Science,* 187, 742–744, 1975.

Purohit, V. and Orzel, R.A., Polypropylene: A literature review of thermal decomposition products and toxicity, *J. Am. Coll. Toxicol.,* 7, 221–242, 1988.

Purser, D.A., Recent developments in understanding the toxicity of PTFE thermal decomposition products, *Fire Mater.,* 16, 67–75, 1992.

Purser, D.A., Toxicity assessment of combustion products, in *The SFPE Handbook of Fire Protection Engineering,* Beyler, C.L., Ed., National Fire Protection Association, Quincy, MA, pp. 200–245, 1988.

Purser, D., Smoke toxicity, in *Improved Fire and Smoke-Resistant Materials for Commercial Aircraft Interiors, A Proceedings,* National Materials Advisory Board, National Research Council, Publication NMAB-477–2, National Academy Press, Washington, DC, pp. 175–195, 1995.

Purser, D.A. and Berrill, K.R., Effects of carbon monoxide on behavior in monkeys in relation to human fire hazard, *Arch. Environ. Health,* 38, 308–315, 1983.

Ritchie, G.D., Rossi, J., III, and Macys, D.A., Application of the Naval Medical Research Institute Toxicology Detachment neurobehavioral screening battery to combustion toxicology, in *Fire and Polymers II, Materials and Tests for Hazard Prevention,* Nelson, G.L., Ed., ACS Symposium Series 599, American Chemical Society, Washington, DC, 1995, pp. 343–365.

Rodkey, F.L., A mechanism for the conversion of oxyhemoglobin to methemoglobin by nitrite, *Clin. Chem.,* 22, 1986–1990, 1976.

Roux, H.J., The NIBS SMOTOX WG program, paper presented at Proceedings of the 37th International Wire and Cable Symposium, Reno, NV, 1988, pp. 543–545.

Runyan, C.W., Bangdiwala, S.I., Linzer, M.A., Sacks, J.J., and Butts, J., Risk factors for fatal residential fires, *N. Engl. J. Med.,* 327, 859–863, 1992.

Rutkowski, J.V. and Levin, B.C., Acrylonitrile-butadiene-styrene copolymers (ABS): combustion products and their toxicity—a review of the literature, *Fire Mater.,* 10, 93–105, 1986.

Tsuchiya, Y., Significance of NO_2 in fire gas toxicity, paper presented at Proceedings of the Canada-Japan-USA Trilateral Cooperative Study on Fire Gas Toxicity, Third Expert Meeting, Ottawa, Canada, 1984.

Wang, H., Ding, R., Ruan, J., Yuan, B., Sun, X., Zhang, X., Yu, S., and Qu, W., Perfluoroisobutylene-induced acute lung injury and mortality are heralded by neutrophil sequestration and accumulation, *J. Occup. Health,* 43, 331–338, 2001.

Weil, E. and Choudhary, V., Flame-retarding plastics and elastomers with melamine, *J. Fire Sci.,* 13, 104–126, 1995.

Weil, E. and Zhu, W., Some practical and theoretical aspects of melamine as a flame retardant, in *Fire and Polymers II: Materials and Tests for Hazard Prevention,* Nelson, G.L., Ed., ACS Symposium Series 599, American Chemical Society, Washington, DC, 1995, pp. 199–216.

Westerberg, L.M., Pfaffli, P., and Sundholm, F., Detection of free radicals during processing of polyethylene and polystyrene plastics, *Am. Ind. Hyg. Assoc. J.,* 43, 544–546, 1982.

Williams, S.J., Baker, B.B. and Lee, K.P., Formation of acute pulmonary toxicants following thermal degradation of perfluorinated polymers: Evidence for a critical atmospheric reaction, *Food Chem. Toxicol.,* 25, 177–185, 1987.

Williams, S.J. and Clarke, F.B., Combustion product toxicity: dependence on the mode of product generation, *Fire Mater.,* 7, 96–97, 1983.

Williams, S.J. and Clarke, F.B., Reply to "Toxicity of Teflon®, similar results obtained with radiant-convective heat sources or in the hot fire gases," Letters to the Editor, *Fire Mater.,* 8, 55–56, 1984.

Zook, B.C., Malek, D.E., and Kenney, R.A., Pathologic findings in rats following inhalation of combustion products of polytetrafluoroethylene (PTFE), *Toxicology,* 26, 25–36, 1983.

Part II

Inhalation Toxicology Methods

11 Toxicogenomics of Low-Level Nerve Agent Vapor Exposure

Janet Weeks Sekowski

CONTENTS

11.1 INTRODUCTION

To date, a great paucity of information exists regarding the molecular level effects of inhaled toxicants of military interest. Historically, research on inhaled military agents has focused on the overt physiological and biochemical responses. A great proportion of the research carried out on inhalation exposure to chemical warfare agents (CWAs) has been directed toward military operational requirements and has been focused toward questions of survivability and immediate treatment, not longer-term effects of low-level exposures.

Gene expression, the process whereby genes are transcribed from the DNA template into messenger RNA (mRNA) and protein expression, the translation of that mRNA into the finish polypeptide product, are highly regulated and sensitive cellular events. The internal signals that trigger induction (or repression) of gene transcription and subsequent translation can respond within seconds to a toxic insult. Furthermore, this process is sensitive to the degree that alteration of gene expression can occur at toxicant exposure levels far below those that produce physiological symptoms. Most importantly, changes in gene expression have been demonstrated to precede toxicant-associated injury or disease by weeks, months, and even years. The advent of high-quality DNA microarray technology has made it possible to simultaneously measure the expression level of thousands of genes. The use of microarrays to study the response of genes to toxic insults has created a new subfield of toxicology called toxicogenomics. Toxicogenomic and proteomic techniques hold great promise to reveal new mechanisms of action and open new avenues of investigation toward understanding the molecular underpinnings of inhalation exposures to CWAs and other compounds of military interest.

11.1.1 Background on Organophosphate Nerve Agent Toxicology

Over the past more than 50 years, much has been learned about the acute effects of organophosphate (OP) nerve agents. The most common OP agents are the "G" series agents and VX. The G agents were synthesized and developed by the Germans just prior to World War II. Initially synthesized as pesticides, they were found to be acute inhibitors of the enzyme acetylcholinesterase (AchE) in humans, causing death within minutes from accumulation of synaptic acetylcholine. Soon after the war ended, the United States and its allies discovered the stockpiles and began synthesizing and testing the nerve agents. Although the only known battlefield use of nerve agents has been in the Iran–Iraq War, many countries and non-state-sponsored political groups have possession of and knowledge to manufacture organophosphate nerve agents and nerve agent munitions.

Since the Gulf War, interest has been increasing in understanding the possible longer lasting effects of low-level nerve agent exposure. In the United States this new research focus, led primarily by the U.S. Veterans Administration, has engendered much of the recent work in the area of low-level agent toxicology. The terrorist attacks in Japan in the mid-1990s raised much awareness about the lack of information regarding the possibility of mass exposure to low-level chemical agent plumes. The Aum Shinrikyo cult of Japan used sarin in the two terrorist attacks. The first attack was in an apartment complex in Matsumoto, Japan (in 1994) and the second (1995) was in the Tokyo subway. In these attacks, 5500 people were treated and 13 people died. Many of those treated were exposed to low levels of sarin vapor (Sidell et al., 2002). From these tragic events, however, at least one positive outcome has been (and continues to be) gleaned. The experience of the patients and the physicians who treated them are able to give science and medicine new, invaluable insights into the human response to low-level sarin exposure. The operational insight and the overall greater awareness of the problem hopefully will soon lead to the development of better detection, protection, and treatment measures.

Currently, we are attempting to understand, using cell culture and model animal systems, what molecular events occur during and after low-level exposure to OP nerve agents, particularly GB (sarin) and GF (cyclosarin). By correlating the molecular level alterations with changes in organ-level biochemistry, we hope to answer questions that will spur the development of the best detection, decontamination, and protective measures possible. In doing so, we hope to prevent the suffering of both military personnel and civilians.

11.1.2 Properties and Mechanisms of Action of Sarin and Cyclosarin

Sarin (GB; isopropyl methylphosphonofluoridate, $CH_3PO(F)OCH(CH_3)_2$) is an acutely toxic, odorless (when in the pure state) OP nerve agent. It has a relatively high volatility (approximately that of water), making it relatively nonpersistent in materials that have absorbed the liquid and vapors. It also undergoes relatively rapid hydrolysis outside the pH range 4 to 7, resulting in the production of two less toxic compounds, hydrogen fluoride and methyl phosphonic acid. Cyclosarin (GF; cyclohexyl methylphosphonofluoridate; $CH_3PO(F)OC_6H_{12}$) is just as acutely toxic as sarin but is approximately 38 times less volatile than sarin at 25°C (GF = 581 mg/m^3; GB = 22,000 mg/m^3). It also is reported to have a slightly sweet odor resembling peach, musk, or shellac.

The primary toxic mechanism of action for both compounds is the inhibition of butyrylcholinesterase in serum and acetylcholinesterase in red blood cells and at cholinergic receptors in tissue. After either cholinesterase enzyme is bound by the agent, it is unable to hydrolyze the neurotransmitter acetylcholine, resulting in accumulation of acetylcholine at the receptors. This causes hyperstimulation of both muscarinic and nicotinic acetylcholine receptors. Symptoms of hyperstimulation at muscarinic receptors include miosis (pinpointed pupils), eye pain, respiratory distress (tightness of chest and dyspnea), exocrine gland stimulation (tearing, sweating, salivation), gastrointestinal disturbances (nausea, vomiting, cramping, diarrhea), and bradycardia. Stimulation of the nicotinic receptors causes a range of effects including twitching, convulsions, peripheral and respiratory muscle weakness, flaccid or rigid muscle tone, and ultimately paralysis. Sarin and cyclosarin have also been reported to demonstrate noncholinergic effects, such as disturbing other enzymatic activities, altering

other neurotransmitter systems, and binding directly to other receptors. Certain aspects of the central nervous system (CNS) are directly affected by sarin and cyclosarin exposure. CNS effects include headache, drowsiness, giddiness, excessive dreaming, fatigue, forgetfulness, anxiety, dyspnea, cyanosis, and death. For all of the above, the type and severity of the symptoms depend on the level and duration of exposure and the route of exposure. Despite more than 50 years' worth of research, large data gaps exist in the body of OP agent literature. Particularly lacking is consensus regarding the near- and long-term clinical significance of the noncholinergic effects and the overall effects of acute and chronic low-level exposure.

11.1.3 Known Effects of Chronic Low-Level and Cyclosarin Exposure

The largest hindrance in reaching consensus in understanding the effects of low-level exposure is the lack of human data from well-designed, controlled studies. Researchers have been able to draw certain conclusions, however, from several studies that used very short-term exposures of OP agents on humans, from several accidental high-dose human exposures, and from studies using animal models. There is also a considerable body of literature regarding the effects of OP insecticides. Although many of the data are relevant, it is dangerous to draw too close a parallel to OP nerve agents. The behavior and toxicology of the various OP compounds vary widely between the particular compounds. Overall, the studies that have been carried out to examine the effects of chronic exposure to OP compounds have revealed many general insights toward understanding the effects of OP nerve agents on the various physiological systems of the body.

11.1.4 Organophosphate-Induced Delayed Polyneuropathy (OPIDN)

The best-known notable area of research in assessing whether chronic low-level sarin exposure can cause lasting neuronal damage is the syndrome known as organophosphate-induced delayed neuropathy (OPIDN). For more than 100 years, acute OP compound exposure has been linked to the development of OPIDN. In general, the neuropathy presents as muscle weakness and ataxia, especially in the lower limbs. This weakness typically occurs between 8 and 14 days after an acute exposure, depending on the type of compound and route and extent of exposure. Occasionally, the neuropathy progresses in severity toward true paralysis. The neuropathy is associated with demyelination of peripheral nerve axons and certain parts of the CNS (Lotti, 1992). It is commonly believed that the demyelination relies on the OP compound's ability to phosphorylate and irreversibly inhibit the enzyme neuropathy target esterase (NTE) (Johnson, 1993). The role NTE plays in the initiation of OPIDN remains unclear. Most of the OPIDN studies have been carried out using the hen, rat, or mouse as a model. In two studies using humans exposed to symptomatic doses of GB (Munro et al., 1994), no findings of OPIDN were reported. Thus, given the human data available, it does not appear that humans develop OPIDN from GB exposure, even at acute doses.

11.1.5 Other Neurological Changes

Many other symptoms of chronic OP exposure are even less well defined than OPIDN. These include behavioral and psychological changes, deficits in memory and cognition. Reports of changes in behavior and psychological state after exposure to OPs have been circulating for more than 50 years (Buccofusco, 1998). Some of the manifestations reported included altered electrocephalogram (EEG) measures, reduced concentration and memory, reduced information processing and psychomotor speed, linguistic deficits, depression, anxiety, sleep disturbance, and easy fatigability (Burchfiel and Duffy, 1983; Ecobichon and Joy, 1994; Sidell et al., 2002). The existing body of data deals primarily exclusively with levels of exposure that cause symptoms. As such, it is likely that most of these reported changes are due primarily to inhibition of ChE and the neurochemical imbalances that result from it. However, because of the extreme amount of individual variability in the

type, degree, and duration of the physiological response to an OP exposure, it is possible that these symptoms could occur in individuals exhibiting few or no other signs of OP exposure (Sidell et al., 2002; Ecobichon and Joy, 1994). Overall, there is very little direct and convincing data suggesting that low levels of GB exposure lead to consistent EEG, psychological, or behavioral alterations. More research is needed in this area.

11.1.6 General Health Effects

Other pathological effects of low-level OP exposure have been investigated over the past 50 years, yet hardly any additional data have been revealed. Carcinogenicity and genotoxicity have been measured in many studies by using high doses of OP compounds, resulting in no findings of either pathogenic mechanism. OP exposures have also been assessed for teratogenicity and other reproductive effects, yet potential involvement of either has not been demonstrated.

11.2 TOXICOGENOMICS—MOVING BEYOND CLASSICAL ORGANOPHOSPHATE TOXICOLOGY

The body of OP toxicological research revealed over the past 50 or more years is predominantly classical toxicology. The primary goal of these toxicological investigations has been and continues to be to measure physical parameters (e.g., overt symptoms of exposure, physical activity, enzymatic activity), histological features of tissue samples (histopathology), and blood chemistry (e.g., ChE measures) as indicators to identify adverse effects of exposure. These data have been and continue to be very useful diagnostic indicators of acute levels of OP exposure, but they are less useful for understanding the molecular mechanisms of toxicity or to gain insight into the potential for longer-term impacts of OP exposure. In addition, classical toxicological methods are often not sufficiently sensitive to detect very-low-level toxicity. Tools and methods that evaluate toxicity and pathology at the mechanistic level are essential to establish meaningful estimates for molecular-level lowest observable adverse effect levels (LOAELs) for OP nerve agents. Of the tools available, one of the most exciting and useful ones is the DNA microarray. A microarray allows one to simultaneously measure the expression of tens to thousands of gene transcripts. Microarray-based investigations have already begun to make an impact on the field of toxicology, engendering a new subfield termed toxicogenomics. A toxicogenomic approach to a question that includes DNA microarray-generated gene expression data that are verified by using reverse transcriptase-polymerase chain reaction (RT-PCR) or another technique and is complemented with an investigation into protein expression (proteomics) begins to build a compelling foundation for the discovery of new molecular toxicological mechanisms.

11.2.1 Background on Gene Expression Profiling

DNA microarray-initiated research has been used in the past several years to discover new molecular mechanisms and to establish gene expression profiles (or signatures) that can reliably reflect the molecular status or phenotype of cell and tissues. By building databases of these signatures, it is possible to build test sets that can be used to identify, by their gene expression profile, samples of unknown composition or disease state, or that have been exposed to unknown compounds. A terrific example of this possibility has been demonstrated by the laboratory of Golub et al. (1999). By clustering gene expression profiles from acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) cells, they were able to correctly classify 29 of 34 bone marrow or peripheral blood samples as being either AML or ALL. Similarly, a study carried out by Ross et al. (2000) demonstrated the ability to accurately classify 60 diverse types of tumor cell lines based solely on gene expression profiles.

11.2.2 Use of Gene Expression Profiling in Toxicology

Several recently published studies have also shown that gene expression profiles from cells treated with a toxicant or drug treatment are indicative of the mechanism of action of the xenobiotic. A study conducted by Scherf et al. (2000) demonstrated that gene expression profiling could indeed correctly classify the mechanism of action of anticancer drugs. The cellular responses (measured by gene expression profiles) to the 118 anticancer therapeutic agents clustered accurately according to the mechanism of action of the therapeutic agent to which the cell type was exposed, regardless of the cell type. Another study, by Waring et al. (2001), addressed the question of whether gene expression profiling could be used to predict a toxic response. Using rats exposed to a battery of 15 known hepatotoxins, they successfully demonstrated a strong correlation between the rat liver gene expression profiles, histopathogical profiles, and clinical chemistry. Overall, these two studies make a compelling case for the utility of gene expression profiling to predict the mechanism of toxicity of exogenous compounds.

11.2.3 Use of Gene Expression Profiling in Inhalation Toxicology

The past year alone has seen a tremendous increase in the use and publication of inhalation toxicology studies employing transcriptomics and proteomics profile gene expression changes in lung tissues. Gene expression profiling has proved to be a fruitful method to better understand the molecular mechanism of action in the lung tissue and cells exposed to particulates such as diesel exhaust and coal dusts. A study by Risom et al. (2003) contributed an important advance in the understanding of the antioxidant mechanisms that combat the oxidative stress induced by diesel exhaust particles (DEP) in lung tissue. They found that the levels of 8-oxo-7, 8-dihydro-2-deoxyguanosine (8-oxodG), a DNA mutation induced by oxidative stress, were mirrored by an increase in the repair protein OGG1 gene expression in the lung tissue. This work suggests that repeated low-level exposures to DEP might up-regulate the OGG1 defense mechanism, thereby preventing the accumulation of 8-oxodG mutations in exposed lung cells. In another particulate inhalation toxicology study, Hu et al. (2003) measured the gene expression changes (using the Affymetrix human array platform) from primary lung epithelial cells exposed to two types of coal dust. Through gene expression profiling they discovered a unique mechanism of action that correlates both with the severity of coal dust–induced coal workers' pneumoconiosis (CWP) and the amount of iron in the coal dust. Their study is one of the first to use gene expression profiling to reveal specific gene expression responses following exposure to dusts from various coal seams. Gene expression measurement has also been a useful technique to illuminate the molecular toxic mechanisms of inhaled drugs of abuse. Because inhalant nitrates have been shown to be associated with increased tumor incidence in mice and Karposi's sarcoma in humans, Tran et al. (2003) asked whether the angiogenic factor, the vascular endothelial growth factor gene (VEGF), might be mediating some of the toxic effects. By measuring the transcript and protein levels of VEGF and VEGF receptors in mice exposed to isobutyl nitrate, they were led to the discovery that the VEGF and its receptors were up-regulated in the liver, but not the lungs, of these animals. Because significant changes occurred in gene expression for VEGF and its receptors in the liver, these results suggest that the toxic effects of inhaled nitrate might be due in part to angiogenic mechanisms in the liver and potentially other exposed organs. Last, gene expression has recently been successfully used to reveal novel molecular mechanisms of action for the inhaled threat toxin, Ricin. The study authored by DaSilva et al. (2003) used cDNA arrays to examine to the transcript changes in lung tissue of mice exposed to aerosolized Ricin. By mining the gene expression array data, they uncovered the up-regulation of a list of important genes involved with tissue healing, inflammatory response, cell growth, apoptosis, and DNA repair. This investigation opened many new avenues of investigation for potential biomarkers of exposure and targets for therapeutic intervention.

11.3 DEVELOPMENT OF THE OP NERVE AGENT DNA MICROARRAY

11.3.1 Objective

The objective of this work is to develop a DNA microarray containing the genetic expression signatures of chemical warfare agents. The resulting "CWA Signature DNA Microarray" will enhance CWA detection and protect the soldier in *three important ways:* (1) it will provide a "before and after" genetic snapshot of soldiers who enter a potentially contaminated environment and give unambiguous verification of exposed individuals, (2) identification of genes altered by CWA exposure may aid in the improvement of preventive or therapeutic measures, and (3) it will provide a means to help identify individuals illegally manufacturing or working with CWA. The CWA Signature DNA Microarray will be used in the laboratory to screen CWAs, mixtures of CWAs and Toxic Industrial Chemicals (TICs), and other potential toxicants for their mechanism of action and similarity to other CWAs and toxicants. It is anticipated that data obtained through the development and use of this assay will fill many critical data gaps in support of developing CB defensive detection, protection, and decontamination material. Overall, this long-range project is expected to contribute tremendously to the missions of the Low-Level Operational Toxicology and the Deployment Toxicology Programs.

11.3.2 Approach

We obtain low-level CWA (i.e., GB and GF)-exposed rat blood, brain, and liver tissue through an ongoing collaboration with the Toxicology Team at the Edgewood Chemical Biological Center (ECBC) (Mioduszewski, Whalley, and Benton). The toxicology team at ECBC is world renowned for their whole-body (rat) inhalation model of chemical agent exposure. This exposure model utilizes the most widely accepted mammalian model in the field of toxicology, provides the most relevant route of low-level chemical agent exposure, and provides extremely accurate dosing information.

With the advent of high-quality, commercially available gene expression arrays, array technology has become a widely accessible functional genomics tool for the molecular toxicologist. The transcript processing and hybridization is carried out using the method as described by the Vahey laboratory (Vahey et al., 2002). In brief, mRNA is extracted from the tissue, is converted to cDNA, and then to fluorescently labeled cRNA. The labeled cRNA is hybridized to the DNA array, stained, and then visualized by using a fluorometer. The raw expression level data are translated by using bioinformatics software and databases into meaningful biological information such as relative induction/repression ratios, complex pattern analyses, association with biochemical pathways, and participation in disease processes. We have chosen to use the Rat Toxicology U34 and the Rat Whole Genome U34A GeneChip from Affymetrix in our investigations of GB and GF in the rat model. The Rat Neurobiology GeneChip, RN-U34, contains more than 1200 known genes and expressed sequence tags (ESTs), and the Rat Genome GeneChip, RG-U34A, contains more 6000 known genes and ESTs. The genes included in the arrays encode polypeptides critical for cellular functions such as stress response, apoptosis, DNA replication/repair, recombination, transcription, and cell–cell communication and for such structures as ion channels and cell surface receptors, to name a few. Of the thousands of genes on either array, only a small subset are significantly (greater than 2-fold) induced or repressed compared with the control animals. Once verified by RT-PCR, the significantly altered genes will be included in our database and considered for integration in the design of the CWA Signature DNA Microarray.

11.3.3 Preliminary Data

To date, we have analyzed and compiled significant data from our gene expression studies on chlorpyrifos and GB. Our initial pilot study revealed that rats injected with an LD_{30} dose of the OP pesticide,

chlorpyrifos, had significantly altered gene expression patterns compared with the control animals (Figure 11.1). Furthermore, we observed that several of the genes remained altered at 24 h post-exposure. The arrows in Figure 11.1 indicate that the expression level for the glutamate aspartate transporter (GLAST) increased to more than 4-fold above the level observed in the control condition. Overall, the pilot study demonstrated the utility of the microarray approach and demonstrated to our satisfaction that gene expression analysis could provide a valuable window through which we could reveal new avenues of research relating to genetic mechanisms of OP compound toxicity (Sekowski et al., 2001, 2003). Our more recent studies, examining the toxicogenomic effects of GB (sarin) and GF (cyclosarin) whole-body inhalation exposure in rats, have resulted in a more significant body of data. Not only do the more recent studies contain statistically meaningful numbers of animals, but the animals have also been exposed to actual OP nerve agents via an operationally relevant route of exposure. Thus, we were able to collect a tremendous amount of statistically significant gene expression data and begin to reveal some potential toxic mechanisms of action that appear to occur even at these low levels of CWA exposure (Sekowski et al., in press).

Significance of our gene expression data is based on several layers of metrics. First, the overall quality of the data is vetted. By using Affymetrix's software tool, Microarray Suite, the raw image of the processed GeneChip is examined for flaws, bubbles, etc. Next, the background noise, consistency in scaling factors, and the sets of positive (BioB, BioC, GAPDH, etc.) control genes are examined. If the data look consistent within the experiment and are of high quality, the data are transferred to other bioinformatics platforms. Currently, we are using Gene Spring from Silicon Genetics and Data Mining Tool from Affymetrix. With these programs, the data are run through statistical tests such as Mann–Whitney (Wilcoxin Sign Rank Test) and ANOVA to demonstrate the consistency within and difference between sets of data. The data are also easily visualized by using either one of these programs. Trends and outliers are easily picked out. Clustering analysis (e.g., K-means and functional) and principle components analysis (PCA) can be performed on the data. These software tools have allowed gene lists to be honed from thousands of genes to just a handful of genes that are significantly altered.

By measuring gene expression in the brain, liver, and blood from both male and female animals exposed to different doses and durations of GB and GF vapor we have observed several important trends. The first is that significant differences in gene expression can be measured even after a one-week "recovery period" following exposure. This is a highly significant finding and has exciting implications for the persistence of gene expression signatures in the body. The second important trend observed was that there appears to be a dose–response relationship to the level of OP agent exposure and the range and average of gene expression changes (Figures 11.2 and 11.3).

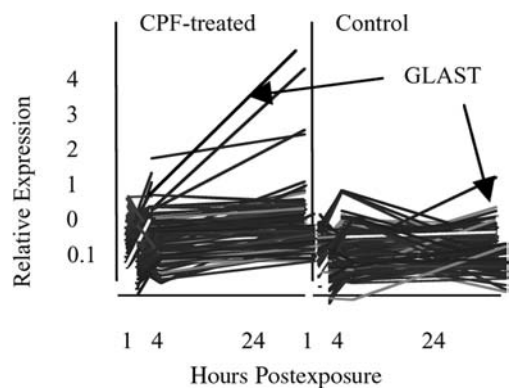


FIGURE 11.1 Altered gene expression patterns of the chlorpyrifos (CPF)-treated animals compared with control animals at 1-, 4-, and 24-hour postexposure.

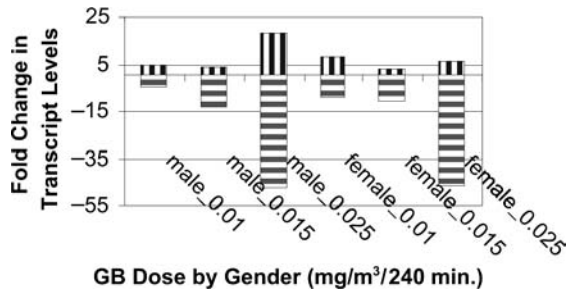


FIGURE 11.2 Dose–response relationship of GB exposure and range of gene expression changes. The ranges of fold changes in gene expression increased with dose, suggesting a general dose–response in gene expression.

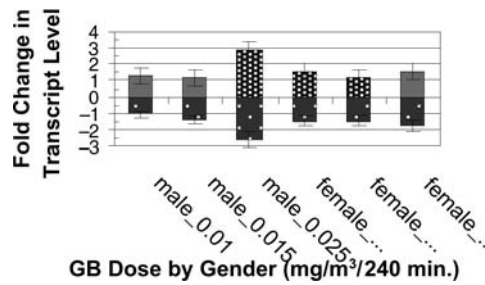


FIGURE 11.3 Dose–response relationship of GB exposure and average fold change in gene expression. The average fold changes also increased slightly with dose. This trend is more pronounced in the male rats.

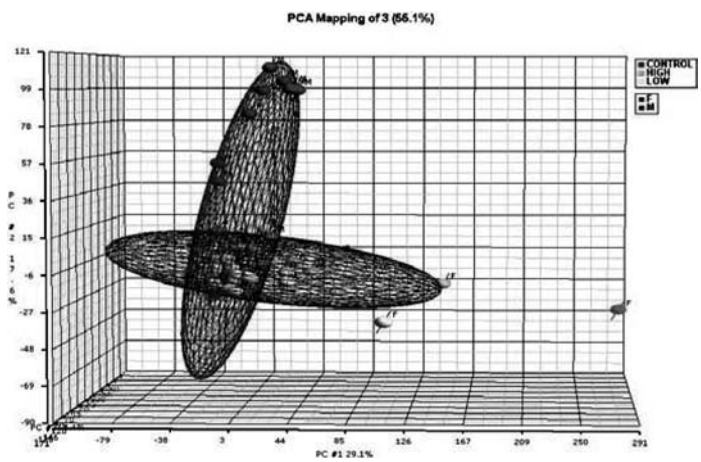


FIGURE 11.4 Male-female differences in brain gene expression after low-level GF exposure. Principal Component Analysis data points caged to show the gene expression profiles for male and female rats shows a significant difference in gene expression trends.

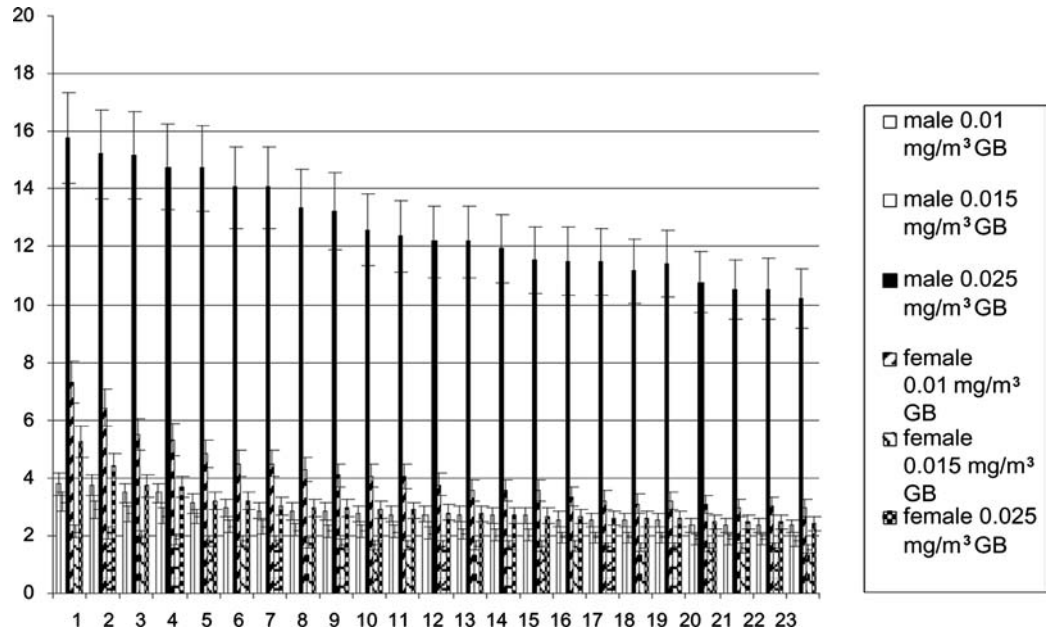


FIGURE 11.5 Gene expression up-regulation one week after low-level GB exposure. A partial list of highly significant ($p \leqslant 0.001$) gene expression alterations remaining after a one-week recovery period following exposure to GB vapor at concentrations near the levels that caused miosis in these animals. Key to genes in Fig. 6: 1, rc_AI227660 EST224355; 2, M84488 Rat vascular cell adhesion molecule-1; 3, rc_AI230404 EST227099 R; 4, AF030087UTR#1 neurotransmitter-induced early gene 2 (ania-2); 5, U88324 RNU88324 G protein beta-1 subunit (rGb1); 6, AF020758 P2X2-5 receptor (P2X2); 7, AF044910 survival motor neuron (smn); 8, U31554 limbic system-associated membrane protein; 9, rc_AI101255 EST210544; 10, X62839 potassium channel protein (3120 bp); 11, D84450 Na^+, K^+-ATPase beta-3 subunit; 12, M24852 neuron-specific protein PEP-19; 13, U69882 RRU69882 calcium-activated potassium channel rSK2 (SK); 14, AF078779 putative four-repeat ion channel; 15, J05510 inositol-1,4,5-triphosphate receptor; 16, M84725 neuronal protein (NP25); 17, X16623 neuraxin; 18, U03470 RFas antigen ligand; 19, rc_AI237836 EST234398; 20, AF014365 CD44; 21, AF056704 synapsin IIIa; 22, D32249 RATNDAP1 neurodegeneration-associated protein 1; 23, M38061 glutamate receptor (GluR-B).

The third trend observed to date has been that apparently significant differences exist in the gene expression patterns in the males versus the female rats following GB exposures (Figure 11.4). This finding, together with the finding from the Toxicology Team that the females are nearly twice as sensitive to the miotic effects of GB (Mioduszewski et al., 2002) and GF (Whaley et al., person communication, 2003) suggests that the response to certain CWAs may be different in men and women. As a result, it was concluded that all future studies should incorporate both male and female animals.

The most recent data in this study surround the identity of the genes whose expression was significantly altered after exposure GB and GF. The gene expression investigation using the animals exposed to GB utilized the Rat Neurobiology GeneChip. This GeneChip is composed of a 1324 gene and EST subset of the whole rat genome. These are genes that Affymetrix has designated as related to neuronal processes. We were able to glean many promising leads from the GB-exposed rat brain RNA by using this array. Figure 11.5 represents a sampling of the highest up-regulated genes that were detected with the array. Lesser up- and down-regulated genes may also be important functionally (and may be investigated later), but for the sake of brevity only the data displayed in Figure 11.5 will be discussed in this proposal. In Figure 11.5, for example, it is apparent that the largest change in gene expression was measured for the males at the 0.025 mg/m^3 level. The authors doubt that this

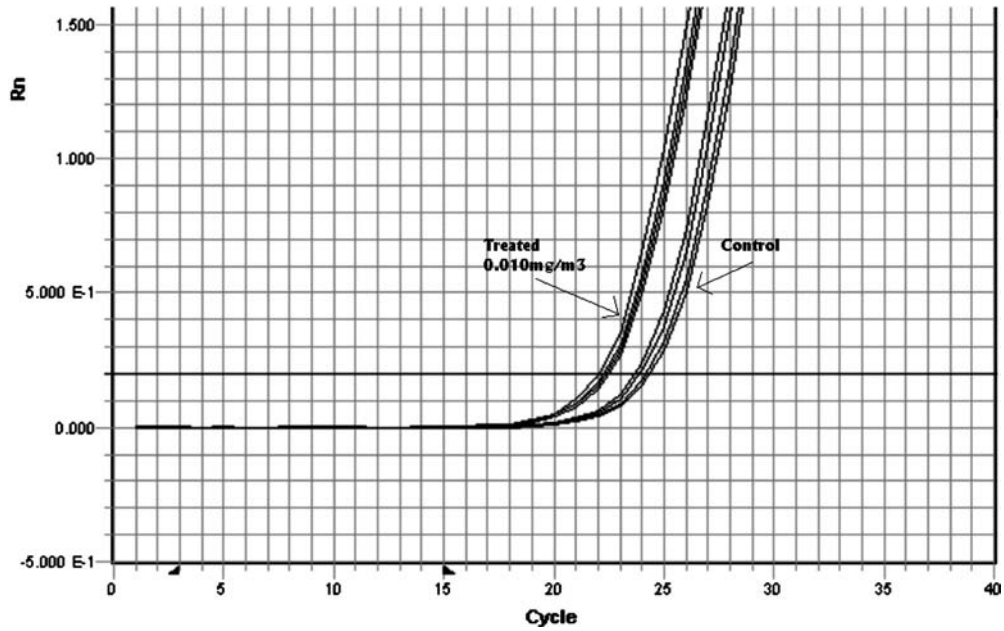


FIGURE 11.6 RT-PCR of the transcript for (U53927) brain astroglial high-affinity cationic amino acid transporter (RCAT2) from male rat brain exposed to GB. The CT_{50} for the exposed male rats (0.01 mg/m^3 for 4 h) was 22.5 and the controls were 24.5 cycles. A difference of two cycles translates to a 4-fold difference in transcript level. Although the Rat Neurobiology GeneChip measured the transcript level as a 7-fold up-regulation, the fold level change is considered in the same range as that measured by RT-PCR. Rn is the number of reactions based on the fluorescent signal above the threshold limit.

greatly enhanced response occurring near the ECT_{50} (for miosis for a 4-h GB vapor whole-body inhalation exposure in male Sprague–Dawley rats) is coincidental. The ECT_{50} (miosis) was reported to be approximately 0.012 mg/m^3 for females and 0.024 mg/m^3 for males (Mioduszewski et al., 2002). Thus, the large change in gene expression at 0.025 mg/m^3 in males likely reflects some of the neurochemical changes that comprise the environment in which miosis occurs. However, it is not clear why the females do not show this same trend in gene expression near the level of the measured female ECT_{50} (0.012 mg/m^3 for 240 min).

The graph in Figure 11.5 shows only the genes most significantly altered in expression (greater than 2-fold) after the rats' exposure to GB vapor. In this list are several ion and amino acid receptor proteins, including two potassium channel proteins (X62839, U69882), a sodium channel protein (M22253), the P2X2–5 receptor (ion-gated purinergic receptor) (AF020758), a glutamate receptor (M38061), and a putative ion channel protein (AF078779). Also up-regulated, synapsin III (AF056704) is a synaptic vesicle protein essential for normal Ca^{2+} regulation of neurotransmitter release. An up-regulation of the channel proteins and the synaptic vesicle protein at one week after exposure could signify that the brain is still restoring homeostasis after the injury caused by GB exposure. The induction of the neurotransmitter induced early gene 2 (part of the ania family of genes) (AF03008) is known to be stimulated within minutes of neurotransmitter receptor activation. This class of genes encodes transcription factors that regulate late-response genes. This class of gene has been implicated in response to hypoxic and other types of brain injury (Raghavendra et al., 2003). Up-regulation may signify continuing recovery from neuronal injury. Similarly, a few cell-damage-repair and plasticity-associated proteins were also found to be up-regulated. These include the neurodegeneration-associated protein (involved in repair/plasticity) (D32249), neuroaxin (involved with neuron-microtubule associations) (C16623), neuron-specific

protein (PEP19) (highest in cerebellum) (M24852), and neuronal protein 25 (NP25) (highest in cerebellum; related to calponin) (M84725). We found also two inflammation/immune-related genes: CD44 (a lymphocyte-stimulating protein) (AFO14365) and vascular cell adhesion molecule-1 (a member of the immunoglobulin superfamily and an important factor involved in the process of leukocyte recruitment in inflammation) (M84488).

Last, the down selection of the significant genes that appear to be the most interesting or most promising of revealing biochemical mechanism appear are currently being verified using real time RT-PCR. RT-PCR is the most widely used method to verify gene expression array results. In Figure 11.6 is an example of the output from the real-time RT-PCR assay.

ACKNOWLEDGMENTS

The author thanks Dr. James J. Valdes for his many valuable suggestions. The contents of this chapter are in a large part shared in a chapter by the same author in *Toxicogenetics and Proteonomics,* J.J. Valdes and J.W. Sekowski, eds., CRC Press, Boca Raton, 75–86.

REFERENCES

Buccofusco, J.J., Chronic Organophosphorus Exposure and Cognition, *Government Reports, Announcements & Index* (GRA&I), Issue 06, 1998.

Burchfiel, J.L. and Duffy, F.H., Organophosphate neurotoxicity: chronic effects of sarin on the electroencephalogram of monkey and man, *Neurobehav. Toxicol. Teratol.*, 4, 767–778, 1983.

DaSilva, L., Cote, D., Roy, C., Martinez, M., Duniho, S., Pitt, M.L., Downey, T., and Dertzbaugh, M., Pulmonary gene expression profiling of inhaled ricin, *Toxicon*, 41, 813–822, 2003.

Ecobichon, D.J. and Joy, R.M., *Pesticides and Neurological Diseases*, 2nd ed., CRC Press, Inc., Boca Raton, FL, 1994.

Golub T.R., Slonim, D.K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J.P., Coller, H., Loh, M.L., Downing, J.R., Caligiuri, M.A., Bloomfield C.D., and Lander, E.S., Molecular classification of cancer: class discovery and class prediction by gene expression monitoring, *Science*, 286, 531–537, 1999.

Hu, W., Zhang, Q., Su ,W.C., Feng, Z., Rom, W., Chen, L.C., Tang, M., and Huang, X., Gene expression of primary human bronchial epithelial cells in response to coal dusts with different prevalence of coal workers' pneumoconiosis, *J. Toxicol. Environ. Health. A*, 66, 1249–1265, 2003.

Johnson, K.M., Symposium introduction: retrospect and prospect for neuropathy target esterase (NTE) and the delayed polyneuropathy (OPIDN) induced by some organo-phosphorus esters, *Chem. Biol. Interact*, 87, 339–346, 1993.

Lotti, M., The pathogenesis of organophosphate induced delayed polyneuropathy, *Crit. Rev. Toxicol.,* 21, 465–487, 1992.

Mioduszewski, R.J., Manthei, J.H., Way, R., Burnett, D.C., Gaviola, B.P., Muse, W.T., Sommerville, D.R., Crosier, R.B., Scotto, J.A., McCaskey, D.A., Crouse, C.L., Matson, K.L., and Thomson, S.A., Low-Level Sarin Vapor Exposure in Rats: Effect of Exposure Concentration and Duration on Pupil Size, U.S. Army Technical Report, ECBC-TR-235, May 2002.

Munro, N.B., Ambrose, K.R., and Watson, A.P., Toxicity of the organophosphate chemical warfare agents GA, GB, and VX: implications for public protection, *Environ. Health Perspect*., 102, 18–38, 1994.

Raghavendra Rao, V.L., Dhodda, V.K., Song, G., Bowen, K.K., and Dempsey, R.J., Traumatic brain injury-induced acute gene expression changes in rat cerebral cortex identified by GeneChip analysis, *J. Neurosci. Res.*, 71, 208–219, 2003.

Risom, L., Dybdahl, M., Bornholdt, J., Vogel, U., Wallin, H., Moller, P., and Loft, S., Oxidative DNA damage and defence gene expression in the mouse lung after short-term exposure to diesel exhaust particles by inhalation, *Carcinogenesis*, 24, 1847–1852, 2003. E-pub 2003 Aug 14.

Ross, D.T., Scherf, U., Eisen, M.B., Perou, C.M., Rees, C., Spellman, P., Iyer, V., Jeffrey, S.S., Van de Rijn, M., Waltham, M., Pergamenschikov, A., Lee, J.C., Lashkari, D., Shalon, D., Myers, T.G., Weinstein, J.N., Botstein, D., and Brown, P.O., Systematic variation in gene expression patterns in human cancer cell lines, *Nat. Genet*., 24, 227–235, 2000.

Scherf, U., Ross, D.T., Waltham, M., Smith, L.H., Lee, J.K., Tanabe, L., Kohn, K.W., Reinhold, W.C., Myers, T G., Andrews, D.T., Scudiero, D.A., Eisen, M.B., Sausville, E.A., Pommier, Y., Botstein, D.,

Brown, P.O., and Weinstein, J.N., A gene expression database for the molecular pharmacology of cancer, *Nat. Genet*., 24, 236–244, 2000.

Sekowski, J.W., O'Connell, K.P., Khan, A.S., Vahey, M., Nau, M., Kahlil, M., Eldefrawi, M.E., and Valdes, J.J., Changes in gene expression after exposure to the organophosphorus insecticide chlorpyrifos, U.S. Army Technical Report, ECBC-TR-210, September 2001.

Sekowski, J.W., O'Connell, K.P., Khan, A.S., Vahey, M., Nau, M., Kahlil, M., Eldefrawi, M.E., and Valdes, J.J., Changes in gene expression after exposure to an organophosphorus (OP) agent, in *Proceedings from* "*Alternative Methods in Toxicological Methods for the New Millenium: Science and Application,*" National Institutes of Health, Bethesda, MD, 2003, pp. 413–427.

Sekowski, J.W., Vahey, M., Nau, M., Orehek, M.A., Mahmoudi, S.F., Bucher, J., Horsmon, M., Menking, D., Whalley, C., Benton, B., Mioduszewski, R., Thomson, S., and Valdes, J.J., Changes in gene expression following whole body exposure to GB vapor in rats, U.S. Army Technical Report, in press.

Sidell, F.R., Takafuji, E.T., and Franz, D.R., Medical Aspects of Chemical and Biological Warfare, Government Reports Announcements & Index (GRA&I), Issue 11, 2002.

Tran, D.C., Yeh, K.C., Brazeau, D.A., and Fung, H.L., Inhalant nitrite exposure alters mouse hepatic angiogenic gene expression, *Biochem. Biophys. Res. Commun.*, 310, 439–445, 2003.

Valdes, J.J., Changes in gene expression after exposure to an organophosphorus (OP) agent, in *Proceedings from* "*Alternative Methods in Toxicological Methods for the New Millenium: Science and Application,*" National Institutes of Health, Bethesda, MD, pp. 413–427, 2003.

Vahey, M., Nau, M., Jogodzinski, J., Yalley-Ogunro, M., Taubman, N., Micjeal, N., and Lewis, M., Impact of viral infection on the gene expression profiles of proliferating normal human peripheral blood mononuclear cells infected with HIV-RF, *AIDS Res. Hum. Retrovir.*, 18, 179–192, 2002.

Waring, J.F., Jolly, R.A., Ciurlionis, R., Lum, P.Y., Praestgaard, J.T., Morfitt, D.C., Buratto, B., Roberts, C., Schadt, E., and Ulrich, R.G., Clustering of hepatotoxins based on mechanism of toxicity using gene expression profiles, *Toxicol. Appl. Pharmacol*., 175, 28–42, 2001.

12 Respiratory Sensitization

Malcolm Blackwell

CONTENTS

12.1 BACKGROUND

Health effects resembling respiratory sensitization (allergy) have been recognized for well over 2000 years but recently the condition, known as asthma, has become more prevalent, in particular, in affluent countries, and has been identified as the cause of debilitating and potentially fatal disease. Allergy may be defined as the adverse health effects resulting from a specific immune response. More specifically, chemical respiratory allergy can be defined as the adverse health effects that result from the stimulation by chemicals, drugs, or proteins of specific immune responses (Kimber and Dearman, 1997). Such responses tend to occur only in a minority of individuals after exposure to a substance to which they have become sensitized. Furthermore, the adverse effects occur at exposure levels well below those that would have induced effects previously in the same individuals or would be expected to occur as a result of nonimmunologically mediated mechanisms, such as irritation.

A number of varying and overlapping definitions are available for key terms, such as hypersensitivity, respiratory sensitization, allergy, and asthma. Medical, regulatory, industrial, and academic scientists may each have their own understanding of these terms. Nevertheless, the terms "asthma" and "respiratory sensitization" may be regarded as synonymous for the purposes of this chapter.

Respiratory sensitization is widely reported following exposure to environmental proteins such as pollen and proteins present in the feces of the house dust mite. There is, perhaps, less public awareness of similar effects occurring because of exposure to chemicals in the workplace. In 1989 a scheme for the Surveillance of Work Related and Occupational Respiratory Disease (SWORD) was initiated in the United Kingdom, the results of which have been reported periodically. During the first year of this project it was discovered that the incidence of acute occupational respiratory disease was some three times greater than had previously been appreciated. The most frequent new diagnosis was of asthma, which represented 26.4% of all cases (Meredith et al., 1991). Five years later asthma remained the most common form of occupational respiratory disease (Ross et al., 1995). In recent years asthma has become more prevalent and now affects 1 in 7 children in Britain. In the United States more than 14 million people are affected by asthma; one third of these are children. The incidence of asthma increased by 42% between 1982 and 1992, and mortality has increased by 40%, accounting for 5400 deaths per year in the United States alone (U.S. Chemical Manufacturers Association, personal communication, 2000). The causes of these increases are not known and are likely to be multiple. Nevertheless, it is widely believed, in particular, by the public and the media, that air pollution and industrial chemicals are significant contributing factors.

In asthma, mast cells in the bronchial lining become sensitized to inhaled material after exposure (normally prolonged exposure). A further episode of exposure then causes the mast cells to release inflammatory mediators that initiate an immune response. This results in an immediate narrowing of the airways, tissue swelling, and increased mucous production. It also starts a local inflammatory reaction with prominent eosinophil participation, which causes the release of additional mediators, damages the cells lining the airways, and helps to explain why the manifestations of asthma may be so prolonged.

A wide range of substances has been identified as causing occupational asthma, based on epidemiological data, by initiating respiratory allergy. Although only about 5% of adult asthma cases are considered to be of direct occupational origin, this is an important and potentially avoidable problem (U.S. Chemical Manufacturers Association, personal communication, 2000). Certain classes of chemicals are commonly associated with occupational respiratory allergy, but there is insufficient information to predict respiratory sensitization potential from analysis of structure alone. Reactivity with proteins is likely to be relevant but, as yet, no validated experimental methods are available for predicting which chemicals might be expected to induce respiratory sensitization. In view of the importance of chemical-induced asthma as an industrial disease and the possible implication of such chemicals affecting the general public, as pollutants, an urgent need exists for predictive tests. Such tests would enable the identification of chemicals with potential to cause respiratory sensitization at an early stage in development. This could result either in abandonment of the chemical in favor of a safer alternative or implementation of effective safety precautions to control its use and prevent human exposure. In addition, predictive tests could be used to determine the respiratory sensitization potential of existing chemicals that give cause for concern.

12.2 REGULATORY FRAMEWORK

Most developed countries around the world have a regulatory system of control for new and existing chemical substances. In general, these systems require a prescribed set of safety studies to be performed including toxicology and physicochemical properties. These studies are used to determine hazards associated with the chemical in question. This hazard information can then be used to assess the risks involved in the intended use of the chemical and, where appropriate, to exert controls to minimize that risk. The regulatory systems vary throughout the world in terms of the specific testing that is required and the nature of the regulatory procedures involved, but the overall purpose of

the testing remains the same: to protect the public, workers, and the environment from the hazards associated with new or existing chemicals.

The European system of regulatory control will be used to illustrate a typical regulatory mechanism but the general principles can be extrapolated to the rest of the world.

12.2.1 New Chemicals

In 1979 a European Community Directive (79/831/EEC; EEC, 1979) established a notification scheme for new substances. New substances were defined as chemicals not on the European Community (EC) market between January 1971 and September 1981. The directive did not apply to chemical products already covered by other legislation, such as pharmaceuticals and agrochemicals. Before placing a new substance on the market, the manufacturer or importer must notify the appropriate member state. One of the requirements for notification is the provision of a technical dossier, which includes a description of various toxicological and physicochemical tests together with a classification proposal based on the results of those tests. In many cases the notifier will also prepare a formal risk assessment for approval by the competent authority.

For a new substance that is to be supplied at greater than 1 tonne per annum a, so-called, "base set" of tests is required. For smaller supply amounts a reduced notification can be made, which carries a less onerous testing requirement. The base-set testing includes the following:

A range of physicochemical tests
Acute oral and dermal (or inhalation) toxicity tests
A repeated-dose oral toxicity test (other routes such as inhalation may sometimes be appropriate)
Skin and eye irritation tests
A skin sensitization test
In vitro genotoxicity tests (Ames and chromosome aberration—an *in vivo* test may be required immediately if either of these is positive)
A range of ecotoxicity tests.

At higher supply levels (greater than 10 tonnes per annum or 100 tonnes cumulative) further testing is required. The specific tests that are needed must be discussed and agreed upon in advance with the competent authority in the appropriate member state. They may include longer-term toxicity studies (including carcinogenicity testing), further genotoxicity and ecotoxicity tests, and reproductive toxicity studies.

Clearly, a comprehensive set of hazard tests is required for notification of new chemicals in the European Union (EU). However, no requirement exists for a test of respiratory sensitization potential, which reflects the absence of an internationally accepted or convenient experimental model. This clearly represents a significant gap in the data needed by manufacturers and regulators to ensure safety. The issue is not ignored in the European notification scheme, and classification as a possible respiratory sensitizer may be required, if the substance is structurally similar to other known sensitizers. A contact (skin) sensitization study is required as part of the base-set package of tests and, although fewer chemicals are thought to be responsible for inducing respiratory sensitization than is the case for contact sensitization, respiratory sensitization represents a significant contribution to risk. Furthermore, it may be easier to protect workers and the public against contact sensitization, by the use of gloves and protective clothing, than it is to prevent exposure to respiratory allergens. Once an individual is sensitized, inhalation exposure to very low concentrations may result in a hypersensitivity response. This gap in the available testing package is well recognized by regulatory authorities who eagerly await the development of a suitable test for respiratory sensitization (P. Evans, U.K. Health and Safety Executive, personal communication, 1999).

The hazardous properties of specific substances are identified in the EU by a system of classification and labeling, which is a statutory requirement in each member state. Criteria used to derive the

appropriate classification and labeling are given in Annex VI to the Dangerous Substances Directive (67/548/EEC; EEC, 1967), an annex commonly referred to as "the labeling guide" (2001/59/EEC; EC, 2001). Hazards are identified by a system of warning symbols and risk phrases. Manufacturers or importers are expected to assign appropriate classification and labeling themselves, in the first instance. Eventually, the competent authority in the member state in which the notification was made will make a formal recommendation for classification and labeling. This recommendation may be different from that proposed by the manufacturer or importer. The recommendation is then approved at European level and will eventually appear in Annex I to the Dangerous Substances Directive. This annex is a compilation of several thousand approved classification and labeling entries and is published in member states.

The guidance given in the EU labeling guide regarding respiratory sensitization is rudimentary. It states that substances (and preparations) should be classified in the category of "danger sensitising" and assigns the symbol Xn, with the indication of "danger harmful" and the risk phrase "R42" (may cause sensitization by inhalation), if at least one of the following criteria apply: if practical evidence is available that shows the substances and preparations are capable of inducing a sensitization reaction in humans by inhalation, at a greater frequency than would be expected from the response of a general population, and if the substance or preparation is an isocyanate, unless there is evidence that the substance or preparation does not cause sensitization by inhalation.

As a result of the application of these criteria, a total of 23 individual substances assigned with the R42 risk phrase have been included in Annex I to the Dangerous Substances Directive (HSE, 1997). These substances included isocyanates, anhydrides, cobalt, nickel sulfate, butadiene epoxide, glycidol, methenamine, and several complex dyes. In some of these cases, the toxicological evidence for assignment of R42 was not altogether convincing and difficulties arose when classification was required of chemicals with more complex data available, such as: glutaraldehyde, ethylenediamine, and methylmethacrylate (Evans, 1997). A decision was made in 1993 to develop more detailed criteria and the revised criteria were formally adopted by EU member states in May 1996, with the intention that they came into effect in national law by 31 May 1998. The revised criteria were officially published in September 1996 as an annex to Commission Directive 96/54/EC (EC, 1996) and are reproduced as follows.

12.2.2 Sensitization by Inhalation

Substances and preparations shall be classified as sensitizing and assigned the symbol "Xn," the indication of danger "Harmful" and the risk phrase R42 in accordance with the criteria given below.

12.2.2.1 R42 May Cause Sensitization by Inhalation

If evidence shows that the substance or preparation can induce specific respiratory hypersensitivity

Where there are positive results from appropriate animal tests

If the substance is an isocyanate, unless evidence shows that the substance does not cause respiratory hypersensitivity

A series of comments follows the criteria to aid in their interpretation.

12.2.2.2 Comments Regarding the Use of R42

12.2.2.2.1 Human Evidence

Evidence that the substance can induce specific respiratory hypersensitivity will normally be based on human experience. In this context, hypersensitivity is normally seen as asthma, but other hypersensitivity reactions such as rhinitis and alveolitis are also considered. The condition will have the

clinical character of an allergic reaction. However, immunological mechanisms do not have to be demonstrated.

When considering the evidence from human exposure, in addition to the evidence from the cases, a decision on classification must necessarily take into account the size of the population exposed and the extent of the exposure.

The evidence referred to above could be

- clinical history and data from appropriate lung function tests related to exposure to the substance, confirmed by other supportive evidence that may include:
- a chemical structure related to substances known to cause respiratory hypersensitivity
- *in vivo* immunological test (e.g., skin-prick test)
- *in vitro* immunological test (e.g., serological analysis)
- studies that may indicate other specific but nonimmunological mechanisms of action, e.g., repeated low-level irritation, pharmacologically mediated effects
- data from positive bronchial challenge tests with the substance conducted according to accepted guidelines for the determination of a specific hypersensitivity reaction.

Clinical history should include both medical and occupational history to determine a relationship between exposure to a specific substance and development of respiratory hypersensitivity. Relevant information includes aggravating factors both in the home and workplace, the onset and progress of the disease, family history, and medical history of the patient in question. The medical history should also note other allergic or airway disorders from childhood and smoking history.

The results of positive bronchial challenge tests are considered to provide sufficient evidence for classification on their own. It is recognized, however, that in practice many of the examinations listed above will already have been carried out.

Substances that elicit symptoms of asthma by irritation only in people with bronchial hyperreactivity should not be assigned R42.

12.2.2.2.2 Animal Studies

Data from tests that may be indicative of the potential of a substance to cause sensitization by inhalation in humans may include IgE measurements (e.g., in mice) and specific pulmonary responses in guinea pigs.

Several important concepts are contained within this guidance that are worthy of mention. The first paragraph states that, although the condition will have the clinical character of an allergic reaction, immunological mechanisms do not have to be demonstrated. Later, however, it is made clear that substances that elicit symptoms of asthma by irritation only in people with bronchial hyperreactivity should not be assigned R42. Thus, asthmatic responses occurring in individuals with preexisting conditions such as congenital asthma or bronchitis should be disregarded. In paragraph two there is a statement designed to ensure that human exposure evidence is taken on a case-by-case basis with regard to the size of the exposed population. This is because a few cases of asthma related to exposure to a high production volume chemical, where large numbers of people have been exposed, would not necessarily trigger labeling with R42. Conversely, a small cluster of cases among a smaller workforce exposed to a specialty chemical might give rise to a legitimate concern. Data obtained from positive bronchial challenge tests are considered to be conclusive but it is clear that more than one positive test is needed and these must be conducted to a standardized, preferably published, protocol.

The guidance also refers to positive results from appropriate animal tests, and two examples are given. This part of the guidance has been deliberately left vague in recognition of the absence of any generally accepted or fully validated methods. It seems, perhaps, reasonable to assume that positive results from even an unvalidated animal test should be taken as indicative of potential to cause respiratory sensitization. Negative results, of course, could not be given the same credence. It is worth commenting here, however, that positive results obtained from studies that do not include an inhalation challenge phase, such as the IgE assay, could be misleading. In these studies, the ability of the chemical to reach the respiratory tract in an active form will not have been demonstrated (see later in this chapter.)

The regulatory guidance discussed above forms part of the regulations directed primarily at new chemicals. It is obviously unlikely that new chemicals would have any significant human data available, unless they have a use history in another part of the world. In this respect, much of the guidance is likely to be more useful in the context of existing chemicals.

12.2.3 Existing Chemicals

In the EU an existing substance is defined as one of the approximately 110,000 chemicals on the EC market at some time between January 1971 and September 1981. These substances, by definition, have not been subjected to the notification procedure and the hazard data available are variable. This situation has been addressed by the Existing Substances Regulation (EEC, 1993), which allows for the prioritization of high-tonnage chemicals, collection of data, and subsequent risk assessment. A technical guidance document for risk assessment has been produced (EEC, 1994), which again recognizes the difficulties in assessing potential for respiratory sensitization. It refers to the mouse IgE test and guinea pig models as being potentially useful but, clearly, the most data here are likely to be derived from studies of human exposure. In these cases, the exposure conditions that resulted in sensitization may not be fully understood, but comparisons can be made of prevailing conditions in the historical cases and those that apply to the population under consideration. "Population" in this context may relate to workers, consumers, or members of the public indirectly exposed via the environment.

A possible outcome of the risk assessment procedure is that there is a need for further information and testing. The guidance states that, in the case of respiratory sensitization, it is not expected that further testing in animals would be required. Again, this represents a recognition of the absence of an internationally recognized test. It is noted, however, that *in vitro* testing (such as an investigation of protein binding) or further exposure information may be useful in refining the risk assessment.

The European Commission has recently proposed wide ranging changes to chemical control regulation within the European Union. These proposals were detailed in a White Paper: "Strategy for a Future Chemicals Policy" (European Commission, 2001) and, for the first time, aim to bring together regulation of new and existing chemicals. Central to these proposals is an integrated system for the Registration, Evaluation and Authorisation of Chemicals (REACH). At the time of writing the legislation is in draft form but places a duty on companies that produce, import, and use chemicals to assess the risks that may arise from their use. Where justified, this may necessitate generation of new data for existing chemicals. A phased system is envisaged in which priority is given to chemicals of highest concern and those produced in the largest quantities. It is possible that the registration requirements under this new scheme will highlight concern over possible respiratory sensitizing chemicals and, hence, further demonstrate the need for a reliable predictive test.

12.2.4 Regulation in the United States

In the United States the system of chemical regulation is rather different and is defined under the Toxic Substances Control Act (TSCA). The government agency responsible for its implementation is the Environmental Protection Agency (EPA). Companies are required to submit a "premanufacture notice" to the EPA 90 days before they manufacture or import any industrial chemical that is not on an EPA list of commercial chemicals. In contrast to the European system, companies are not expected to submit any hazard data at this stage. On receipt of the premanufacture notice, the EPA conducts its own screen of the new substance by comparing it with structurally similar substances. For this purpose the EPA maintains a database of "categories of concern" (EPA, subject to review) and, on the basis of this information, may impose subsequent testing requirements. Structural alerts for respiratory sensitization in the EPA categories of concern include isocyanates and anhydrides. In the experience of the author, a requirement for respiratory sensitization testing has sometimes been imposed by the EPA for such chemicals, the recommended test being a guinea pig respiratory parameter test (Karol, 1995) (see later in this chapter). The suitability of this test for the intended purpose, however, is questionable.

12.2.5 Regulation in Japan

In Japan, new chemicals are controlled by the Japanese Chemical Substance Control Law (MITI/MHW, 1986). In common with Europe, this system requires a formal notification prior to manufacture or import with a requirement for certain test results. The impetus for development of the Japanese system was environmental rather than a concern over worker or consumer safety. Its principles are, therefore, fundamentally different from the European or American systems.

The starting point for testing is a biodegradation study and substances that biodegrade completely do not require further testing. Nonbiodegradable substances, and any stable degradants that are produced by biodegradation, are subjected to a set of screening studies. The tests are limited to bioaccumulation (in fish), repeated-dose oral toxicity, and genotoxicity studies. Significant bioaccumulation triggers a requirement for extensive further testing including long-term carcinogenicity testing in animals. In reality, few bioaccumulative chemicals progress any further in development. For those chemicals that do not bioaccumulate, unfavorable results in toxicity or genotoxicity studies result in designation of the chemical and imposition of controls on its manufacture and use. In these latter cases, there is no requirement for any further testing.

A separate regulatory control concerned with the safety of workers exposed to new chemicals exists in Japan. This system is the responsibility of the Japanese Ministry of Welfare and the only testing requirement is for a single genotoxicity test (the Ames test for mutagenicity). In Japan no regulations require testing industrial chemicals for their potential to cause skin or respiratory sensitization. A revision to the Japanese testing requirements was announced on May 28, 2003, which retains the focus on nonbiodegradable, bioaccumulative, and chronically toxic substances; although tonnage levels at which testing is triggered have been altered. No mention is made of sensitization but additional data may be required for particular categories of chemical substance.

12.3 CHEMICAL SENSITIZERS

The ability of certain protein antigens encountered in the environment and workplace to cause allergic asthma is well established, and a wide range of different proteins has been implicated. Perhaps the most important are a group of proteins present in the feces of the house dust mite *Dermatophagoides pteronysimus* many of which are naturally occurring proteases. In addition to these, protein components of pollens from grasses, weeds, and trees, mold spores *(Aspergillus)*, bird feathers, and animal danders and urine are known to induce respiratory sensitization. Apart from these environmental allergens, exposure to protein aerosols may also occur in the workplace. These include animal and plant proteins associated with farming, laboratory animals, brewing, and baking, as well as bacterial enzymes used in detergents.

High-molecular-weight proteins are not the only substances implicated in respiratory sensitization and many reports investigate occupational asthma due to low-molecular-weight chemicals. Clearly these are not, in themselves, large enough molecules to cause an immune response and they can only do this by haptenating with macromolecules (proteins). Most of the information about low-molecular-weight respiratory sensitizing chemicals is derived from index case reports, cross-sectional surveys, or epidemiological studies in the workplace, such as the SWORD initiative in the United Kingdom. From data such as these, lists can be derived of chemicals that are implicated in human respiratory allergy. Some examples of low-molecular-weight sensitizers are given in Table 12.1. In addition, the U.K. Health and Safety Executive have published a critical assessment of the evidence for agents implicated in occupational asthma (HSE, 1997). This includes three lists of low- and high-molecular-weight substances:

1. Substances considered to meet the EU criteria, revised in 1996, for classification as a respiratory sensitizer (a cause of asthma) and labeling with R42.
2. Substances considered *not* to meet the EU criteria, revised in 1996, for classification as a respiratory sensitizer (a cause of asthma) and labeling with R42.

TABLE 12.1 Examples of Low-Molecular-Weight Respiratory Sensitizers

Chemical	Reference
Acid anhydrides	
Phthalic anhydride	Maccia et al., 1976
Tetrachlorophthalic anhydride	Howe et al., 1983
Trimellitic anhydride	Bernstein et al., 1982
Hexahydrophthalic anhydride	Moller et al., 1985
Maleic anhydride	Topping et al., 1986
Diisocyanates	
Diphenylmethane diisocyanate	Zeiss et al., 1980
Hexamethylene diisocyanate	Vandenplas et al., 1993
Toluene diisocyanate	O'Brien et al., 1979
Reactive dyes	Docker et al., 1987
Plicatic acid	Cartier et al., 1986
Carmine	Quirce et al., 1994
Platinum salts	Murdoch et al., 1986

TABLE 12.2 Summary of HSE List of Agents Implicated in Occupational Asthma

List 1 Respiratory Sensitisers	List 2 Not Respiratory Sensitisers	List 3 Chemicals of Concern
Azodicarbonamide	Formaldehyde	Acetic anhydride
Carmine	Hydralazine	Ethyl cyanoacrylate
Castor bean dust	Methyl methacrylate	Methyl cyanoacrylate
Chloramine-T		Flour dust
Chloroplatinates and other haloplatinates		Proteolytic enzymes
Chromium (VI) compounds		
Cobalt (metal and compounds)		
Cow epithelium/urine		
Crustacean proteins		
Diazonium salts		
Ethylenediamine		
Glutaraldehyde		
Some hardwood dusts		
Isocyanates		
Laboratory animal excreta/secreta		
Latex		
Maleic anhydride		

TABLE 12.2 Summary of HSE List of Agents Implicated in Occupational Asthma (Continued)

List 1 Respiratory Sensitisers	List 2 Not Respiratory Sensitisers	List 3 Chemicals of Concern
Methyl-tetrahydrophthalic anhydride		
Papain		
Penicillins		
Persulfates		
Phthalic anhydride		
Piperazine		
Some reactive dyes		
Rosin-based solder flux fume		
Some softwood dusts		
Spiramycin		
Tetrachlorophthalic anhydride		
Trimellitic anhydride		

Source: HSE (1995)

3. Substances on the ACTS/WATCH program on account of concerns over respiratory sensitization.

The content of these lists is summarized in Table 12.2 and the HSE publication provides data summaries and full references to primary data sources.

A low-molecular-weight chemical (hapten) can only induce an immune response if complexed with a carrier molecule (a protein). Haptens usually only bear one antigenic determinant, or epitope, which must be at least 1 kDa to elicit an antibody response. The response to hapten conjugates requires two populations of lymphocytes: T and B cells. The cells producing the antibodies, which are derived from B cells and T cells, act as helpers in this process. B-cell preparations, depleted of T cells, cannot respond to hapten conjugates. The T cells are responsive to the carrier portion of the conjugate, although in some cases they also recognize the hapten (Cruse and Lewis, 1995).

12.4 MECHANISMS OF SENSITIZATION

12.4.1 Hypersensitivity

Hypersensitivity has been classified into four distinct types, each having a different immunological mechanism (Coombs and Gell, 1975). Although numerous modifications to this classification system have been proposed it remains a useful nomenclature:

12.4.1.1 Type I: Immediate Hypersensitivity

This represents the classical allergic reaction utilizing reaginic or allergic antibody, primarily Immunoglobulin E (IgE). The antibody binds to and thus sensitizes mast cells. After sensitization, inhalation of antigen results in cell-bound IgE antibody being cross-linked on tissue mast cells, resulting in membrane changes, degranulation, and subsequent release of soluble mediators to produce a rapid reaction. Respiratory hypersensitivity is usually considered as being dependent on IgE antibody and is regarded, primarily, as a type I, immediate, hypersensitivity reaction. Nevertheless, the subsequent inflammatory response also produces delayed and prolonged effects.

12.4.1.2 Type II: Cytotoxic Antibody Hypersensitivity

An example of type II hypersensitivity is autoimmune hemolytic anemia.

12.4.1.3 Type III: Immune Complex Reactions

An example of type III hypersensitivity is serum sickness.

12.4.1.4 Type IV: Delayed Hypersensitivity

In this type of hypersensitivity immune recognition occurs due to T lymphocytes with cytokines as mediators. Contact allergy is a type IV response occurring because of clonal expansion of allergen-reactive T lymphocytes, in the draining lymph node(s), after an encounter with a skin-sensitizing chemical. These memory T lymphocytes act as effector cells following subsequent exposure to the inducing allergen. The local accumulation of T lymphocytes and their subsequent activation and release of cytokines and other mediators will result in the inflammatory reaction characterized as allergic contact dermatitis (Kimber, 1996).

12.4.2 Immunobiology of Respiratory Sensitization

In respiratory hypersensitivity, synthesis of IgE is T cell dependent and the cytokine interleukin-4 (IL-4) is of overriding importance in the production of IgE (Finkleman et al., 1988a). Kuhn et al. (1991) showed that mice lacking the gene for IL-4 also lack IgE and do not produce IgE antibody even in response to strong stimuli. On the other hand, transgenic mice carrying an IL-4 transgene, and therefore expressing high levels of this cytokine, show increased serum concentrations of IgE and stronger IgE antibody responses than wild-type controls (Tepper et al., 1990; Burstein et al., 1991). Mature T lymphocytes are classified on the basis of their surface-protein markers, such as CD4 and CD8. CD4+ T lymphocytes recognize antigens, presented by antigen-presenting cells, in the context of major histocompatibility complex (MHC) class II molecules. $CD8^+$ T lymphocytes recognize antigen in the context of class I MHC histocompatibility molecules and are involved in cytotoxicity responses against cells such as those infected by viruses, as well as various other immune reactions. The $CD4^+$ T cells participate in the afferent limb of the immune response to exogenous antigen, which stimulates the synthesis of IL-2. This, in turn, activates $CD8^+$ T cells, natural killer (NK) cells, and B cells, thereby orchestrating an immune response to the antigen. Thus, they are termed helper T lymphocytes. In mice, two types of $CD4^+$ T-helper cells (Th) have been identified, designated as Th1 and Th2, which differ with respect to the spectra of cytokines they secrete following stimulation (Mossman et al., 1986). Both produce interleukin 3 (IL-3) and granulocyte macrophage-colony stimulating factor (GM-CSF), but only Th1 cells secrete interleukin 2 (IL-2), interferon γ (IFN-γ) and tumor necrosis factor β (TNF-β), whereas only Th2 cells secrete IL-4, IL-5, IL-6, and IL-10 (Mossman and Coffman 1989; Mossman et al., 1991). Other types of Th cells also exist which may represent transient stages in development, with Th1 and Th2 cells being the most differentiated forms, which appear after prolonged exposure to an antigenic stimulus or to strong immunogens. Similar populations of $CD4^+$ cells can be recognized in humans (Romagnani, 1991, 1992) and this has important implications for the induction of chemical allergy.

Considerable evidence shows that different classes of chemical allergens provoke immune responses in mice characteristic of selective Th-cell activation. It has been shown in mice that chemicals known to cause occupational respiratory sensitization induce responses consistent with the preferential activation of Th2-type cells. Conversely, a number of chemicals that are contact sensitizers but are known or suspected not to cause respiratory hypersensitivity induce Th1-type responses (Dearman and Kimber, 1991, 1992; Dearman et al., 1992a). Furthermore, there is evidence for differential Th responses in human allergic conditions (Kapsenburg et al., 1991). Apart

from increasing IgE production, stimulation of Th2 reactivity favors, in other ways, development and expression of immediate hypersensitivity. IL-3, IL-4, and IL-10 are all mast cell growth factors or cofactors (Smith and Rennick, 1986; Thompson-Snipes et al., 1991), whereas IL-5 is an important eosinophil growth and differentiation factor (Yokota et al., 1987). Cytokine products of Th2 cells, therefore, favor the induction of acute allergic responses and immediate-type hypersensitivity, with IL-4 and IL-5 promoting, respectively, the production of IgE antibody and the development localization and function of eosinophils (Finkelman et al., 1988a; Coffman et al., 1989; Iwami et al., 1993). In contrast, Th1 cells produce IFN-γ and thereby inhibit the stimulation of IgE antibody responses and the elicitation of immediate-type allergic reactions, favoring instead the development of cell-mediated immunity and delayed hypersensitivity (Finkelman et al., 1988b; Cher and Mossman, 1987; Fong and Mossman, 1989; Iwamoto et al., 1993). However, such cytokines do not necessarily derive exclusively from $CD4^+$ Th cells. In fact, a similar functional difference exists among $CD8^+$ T lymphocytes, and two populations, known as Tc1 and Tc2, have been described which show selective cytokine production comparable with Th1 and Th2 cells, respectively (Croft et al., 1994; Kemeny et al., 1994; Sad et al., 1995; Mossman and Sad, 1996). $CD4^+$ T lymphocytes seem to be the source of the type 2 cytokines (IL-4 and IL-10) responses stimulated in mice by exposure to either contact or respiratory chemical allergens. A different situation exists with respect to production of IFN-γ. Both $CD4^+$ and, to a lesser extent, $CD8^+$ T lymphocytes appear to contribute to the production of this cytokine by certain contact sensitizers, but in respiratory allergy $CD8^+$ cells are the major source of the low levels of IFN-γ produced (Dearman et al., 1996). This implies that the selective stimulation of Th2-type cells by respiratory sensitizers may be more absolute than suspected previously, with the induction of only Th2- and Tc1-type responses.

The characteristics of contact and respiratory sensitization can be summarized as follows.

12.4.3 Contact Sensitization

Application of a contact-sensitizing chemical to the skin results in activation and clonal expansion of T lymphocytes in the draining lymph nodes. This activation is made possible by the network of Langerhans cells in the epidermis, which serves as a cellular trap for environmental antigens. In response to skin sensitization, local Langerhans cells are induced to migrate from the skin, via afferent lymphatics, to the draining lymph nodes. A significant proportion of these Langerhans cells bear high levels of antigen, and once in the lymph nodes these cells transform into dendritic cells that act as antigen-presenting cells. The memory T lymphocytes that result act as effector cells following subsequent exposure to the inducing antigen. These memory cells migrate to the dermis from the vasculature and then to the epidermis, probably directed by chemoattractants. The Th-cell clones are differentiated toward the Th1 type and produce a characteristic array of cytokines, including IL-3, GM-CSF, IL-2, IFN-γ, and TNF-β, which result in the inflammatory reaction.

12.4.4 Respiratory Sensitization

Specific antibodies usually effect respiratory sensitization, and two major classes of pulmonary allergic reactions can be distinguished.

Hypersensitivity pneumonitis results from inflammatory reactions caused by short- or long-term intermittent exposure to certain protein antigens. Examples include farmer's lung caused by inhalation of *Saccharopolyspora recivirgula (Micropolyspora faeni)* antigens and cheese worker's lung caused by *Penicillin roqueforti* antigens. The disease is associated characteristically with the presence of antigen-precipitating IgG antibody. Pathogenesis may, however, also involve the activation of complement- and cell-mediated immunity.

Allergic asthma and rhinitis are most commonly immediate-onset reactions (within 1 h and often within minutes of exposure) and result from the local release of inflammatory mediators following degranulation of sensitized mast cells in the bronchial lining. These reactions are primarily

effected by IgE antibody. IgE-specific antibody has been demonstrated for all recognized chemical respiratory allergens and a clear association exists for some chemical allergens between the presence of specific IgE antibody and the development of respiratory symptoms. Nevertheless, a clear link between allergic responses and serum-IgE antibody has, in some instances (notably with some diisocyanates), failed to emerge (Kimber, 1996). The immediate reactions involve bronchoconstriction, tissue swelling, and increased mucous production together with the onset of an inflammatory response. Histopathologically, the changes include mucous plugging, epithelial damage, goblet cell hyperplasia, basement membrane thickening, and eosinophil infiltration. The inflammatory response results in prolonged effects in the airways such that asthmatic reaction may be persistent or have a late onset. It is possible here that other types of immune processes are involved (O'Byrne et al., 1987; Corrigan and Kay, 1992). The airways even of mild asthmatics are chronically inflamed, with infiltration of activated eosinophils and T lymphocytes, degranulation of mast cells, epithelial damage, deposition of collagen below the basement membrane, and goblet cell hyperplasia (Beasely et al., 1989; Jeffrey et al., 1989; Djukanovic et al., 1990).

The synthesis of IgE antibody is T cell dependent and a characteristic array of cytokines is produced, including GM-CSF, IL-3, IL-4, IL-5, IL-6, and IL-10. These cytokines both promote production of IgE and induce the inflammatory response. IFN-γ serves to antagonize this process and to inhibit IgE production (Finkelman et al., 1988b). Immunohistochemical analysis of bronchial mucosal biopsies has recently identified the mast cell as a source of preformed characteristic cytokines (Bradding et al., 1992, 1994; Okayama et al., 1995). TNF-α, GM-CSF, IL-3, IL-4, IL-6, IL-8, IL-10, and IL-13 are all stored in mast cell granules and may be rapidly released on IgE-dependent stimulation.

Mast cells are ideally placed to initiate a rapid and robust response to allergen exposure. They are numerous on and beneath the bronchial epithelium and adjacent to bronchial smooth muscle and blood vessels. Every mast cell is likely to have at least some allergen-specific IgE bound to its surface Fcε receptors. In contrast, very few T cells in the lung are specific for any one allergen, and cytokine release upon activation may require several hours to become significant (Sampson and Holgate, 1997).

Although the immediate-hypersensitivity response must be triggered by mast cells, the T cells are crucial in ultimately defining the quality or specificity of the immune response by fine tuning the production of specific IgE by B lymphocytes. Without this specific IgE mast cells would not be able to respond effectively and rapidly to allergen exposure. Allergen-specific T-cell clones are of the Th2 type in acute asthma, but in ongoing chronic asthma the T-cell cytokine repertoire is more diverse, suggesting that the majority of T cells have functions other than allergen recognition (Sampson and Holgate, 1997). The severity of T-cell activation is related to the severity of disease. Mild disease may be mast cell driven but in more severe, chronic cases the pathology in the asthmatic lung is thought to be due to a T-cell-driven eosinophilic inflammation.

12.5 THE ROLE OF EOSINOPHILS IN RESPIRATORY SENSITIZATION

Since the early part of this century, it has been known that asthma is associated with eosinophilia of the blood and lung (Ellis, 1908). Horn et al. (1975) demonstrated that peripheral blood eosinophils are inversely correlated with the severity of asthma as measured by forced expiratory volume (FEV). Eosinophils are polymorphonuclear leukocytes, which can be identified in Wright- or Giemsa-stained preparations by staining of secondary granules in the leukocyte cytoplasm as brilliant reddish or orange refractile granules. Cationic peptides are released from these secondary granules when an eosinophil interacts with a target cell and may lead to death of the target. Eosinophils make up 2 to 5% of the total white blood cells in humans and the proportion is not substantially different in other species. After a brief residence in the circulation, eosinophils marginate, becoming adherent to the endothelium of blood vessel walls. They subsequently migrate into tissues by passing between the lining endothelial cells. It is believed that they do not return to the circulation.

The mechanism by which leukocytes negotiate the endothelial barrier is complex and involves cell adhesion molecules (CAMs). Carbohydrate ligands on leukocytes initially interact loosely with

lectin-binding regions of P, L, and E selectin, causing the leukocyte to roll along the endothelial wall. One of the effects of allergen challenge is to modulate endothelial selectin expression. Release of autocoids including histamine, leukotrienes B_4 and C_4, and platelet-activating factor (PAF) results in elevation of P-selectin on endothelial cells within an hour of exposure. The inflammatory cytokines IL-1, TNF-α, and IFN-γ produce a slower expression of E-selectin (Gundel et al., 1991; Vonderheide and Springer, 1992). Inhibition of leukocyte rolling occurs because of interaction of leukocyte integrins (found on neutrophils, eosinophils, and lymphocytes) with intercellular adhesion molecule 1 (ICAM-1) found on the endothelium (Montefort et al., 1994). The leukocyte becomes flattened on the endothelial wall before migrating across the endothelium. Endothelial expression of ICAM-1 is up-regulated by the same cytokines that up-regulate E-selectin. Blockade of ICAM-1 and E-selectin by using neutralizing antibodies attenuates eosinophil and neutrophil influx in sensitized and subsequently challenged monkeys and reduces the late asthmatic response and the associated increase in responsiveness (Gundel et al., 1991).

The distribution of eosinophils corresponds mainly to areas exposed to the external environment, such as skin, mucosa of the bronchi, and gastrointestinal tract. Besides being involved in asthmatic responses, eosinophils also have elevated levels in individuals with parasitic infections. Presumably, this latter phenomenon represents their evolutionary purpose.

Occupational asthma shows the characteristic features of airway smooth muscle contraction, edema, and fluid accumulation, resulting, presumably, from the local release by mast cells of inflammatory mediators such as histamine and leukotrienes. Chronic inflammation plays an important role in asthma and is associated with infiltration of the bronchial mucosa by inflammatory cells, mucous production, the destruction and sloughing of airway epithelial cells, and subepithelial fibrosis secondary to collagen deposition (Roche et al., 1989; Beasely et al., 1989). Of particular importance in the development of bronchial mucosal inflammation is the eosinophil acting together with infiltrating T lymphocytes. Indeed, pulmonary eosinophilia is characteristic of allergic asthma and differentiates it from other inflammatory conditions (Briatico-Vangosa et al., 1994). Although the role of eosinophils in respiratory hypersensitivity is not fully understood, increased numbers of eosinophils have been shown in bronchoalveolar lavage (BAL) fluid and in bronchial mucosal biopsies approximately 24 h after the initial asthmatic response (De Monchy et al., 1985; Frew et al., 1995). This eosinophil influx is thought to play a major role in the so-called late asthmatic response with eosinophils being selectively recruited into the lung from the vasculature and replenished by an increased proliferation or release from the bone marrow. There is no doubt that the eosinophilia associated with respiratory hypersensitivity is influenced markedly by cytokines, in particular, IL-5, which is a product of Th2 cells (Chand et al., 1992; Gulbenkian et al., 1992; Iwami et al., 1992, 1993).

The eosinophil is a terminally differentiated cell that produces an array of inflammatory mediators, including toxic oxygen radicals and basic proteins. Of particular importance are the cysteinyl-leukotrienes that seem to have a bronchoconstrictor role in acute asthma and after allergen challenge. In fact cysteinyl-leukotriene antagonists are entering clinical practice as oral prophylatic agents to reduce significantly the incidence and severity of acute exacerbations, preventing late-phase bronchoconstriction and bronchial hyperresponsiveness and reducing the inflammatory response (Sampson and Holgate, 1997; Lin et al., 2002). The four principal cationic proteins in the eosinophil granule are the major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN), and eosinophil cationic protein (ECP). MBP, EPO plus H_2O_2 plus halide, EPO alone, and ECP are all toxic in varying degrees to guinea pig tracheal epithelium, producing exfoliation and ciliostasis (Gleich, 1990). MBP, in particular, is highly cytotoxic to bronchial epithelium and is elevated in asthmatic sputum (Frigas and Gleich, 1986). In addition, the eosinophil is the predominant cell implicated in bronchial epithelial damage in asthma. At its most severe this damage can include stripping of the pseudostratified ciliated epithelium down to the basal cell layer. In mild to moderate asthma, the epithelial cleavage occurs along the line of desmosomes between the columnar and basal cells (Montefort et al., 1992a, 1992b). Proteins released by eosinophils may be responsible for damage to these desmosomes, including metalloendopeptidases, ECP, MBP, or EPO.

TABLE 12.3 Eosinophil Products Implicated in the Pathophysiology of Asthma

Eosinophil Products	Effect
LTC4, 15-HETE	Mucous hypersecretion
IL-5, IL-3, GM-CSF	Eosinophil priming
LTD4, PAF, IL-5, 15-HETE	Eosinophil recruitment
LTD4, PAF	Bronchoconstriction, mucosal edema
LTE4, MBP	Bronchial hyperresponsiveness
LTD4, TGF-β	Epithelial proliferation, airway remodeling
MBP, EPO, ECP	Epithelial damage

LTC4, cysteinyl-leukotriene; 15-HETE, hydroxyeicosatetraenoic acid.

In patients with marked asthma, MBP concentrations are elevated in BAL fluid and the elevations of both MBP and eosiniphils can be related to bronchial smooth muscle hyperreactivity to inhaled methacholine. Tests of the hypothesis that the eosinophil is responsible for bronchial hyperreactivity have demonstrated that application of MBP to tracheal rings causes smooth muscle hyperreactivity to histamine and acetylchloine. Direct injection into the epithelium *in vivo* also causes smooth muscle hyperreactivity (Gleich, 1990).

Cysteinyl-leukotrienes have been shown to act as potent and specific chemoattractants of eosinophils (Hay et al., 1995), which may be associated with their effect in inducing bronchial hyperresponsiveness. Leukotriene antagonists and synthesis inhibitors seem to reduce eosinophil and basophil influx after allergen challenge in humans and to show significant steroid-sparing effects in severe asthma (Sampson and Holgate, 1997). Cysteinyl-leukotrienes may therefore have a proinflammatory function, possibly involving positive feedback, so that their production by eosinophils results in further eosinophil influx. Furthermore, the eosinophil is also a source of proinflammatory cytokines that may be chemoattractive for eosinophils and may enhance their survival.

Clearly, pulmonary infiltration of eosinophils is characteristic of respiratory sensitization (asthma). It is a direct result of the IgE-mediated Th2-type response seen in asthma, occurring largely because of the chemoattractant properties of cytokines produced during this response, in particular, IL-5. Furthermore, eosinophils are crucially involved in the pathogenesis of the asthmatic response itself, in particular, the late phase and prolonged effects, but possibly also the immediate bronchoconstriction response. A summary of the eosinophil products implicated in the pathophysiology of asthma and their effects is given in Table 12.3.

12.6 PREDICTIVE METHODS FOR IDENTIFYING RESPIRATORY SENSITIZERS

Currently no well-validated or widely applied methods exist for determining which chemicals may induce respiratory sensitization. The nature of the immune response is such that *in vitro* methods are unlikely to provide a complete picture, although techniques have been described to determine the potential of low-molecular-weight chemicals to interact with protein (Wass and Berlin, 1990). Interaction with protein is a necessary prerequisite for allergenicity because low-molecular-weight compounds are incomplete antigens (haptens). Such protein-binding studies may be useful as a prescreen for new chemicals or complex mixtures with unknown properties (Sarlo and Clark, 1992). They cannot, however, be regarded as models for the induction of an immune response and they do not address issues such as absorption and metabolism. Perhaps the best hope for *in vitro* alternatives is the use of structure–activity relationships for which some powerful relational databases are now being developed. Indeed some progress has been reported in identifying structural alerts for

respiratory sensitization in a structure–activity model (Graham et al., 1997). Nevertheless, without a reliable *in vivo* predictive test, it is difficult to develop or validate the necessary "rule base" for such a system. Several *in vivo* models have been described of which the most promising are the guinea pig respiratory parameter tests, the mouse IgE assay, cytokine-profiling studies, and the eosinophil peroxidase assay.

12.6.1 Respiratory Parameter Tests

Guinea pig respiratory parameter tests have been developed by using whole-body plethysmography techniques to assess changes in respiratory function, tidal volume, and respiratory rate (Karol et al., 1980, 1981). These techniques have been successfully used to demonstrate respiratory sensitization after inhalation of isocyanates. However, protein conjugation was required for the inhalation challenge exposure to induce a response and this process has been reported to be poorly reproducible (Botham et al., 1988). Nevertheless the model has been adapted to evaluate responses to other chemicals.

In this model, guinea pigs are housed in whole-body plethysmographs and exposed to chemical atmosphere for 3 h per day for 5 consecutive days. Animals are exposed to chemical or chemical-carrier conjugate aerosols 17 days after the last exposure to determine pulmonary reactivity. The model utilizes changes in respiratory rate as the predominant indication of a hypersensitivity response, which is measured by use of pressure-sensitive transducers attached to whole-body plethysmographs. Respiratory frequency and plethysmograph pressure are monitored continuously from 2 h before allergen provocation challenge through to 24 h postchallenge. Body temperature is also measured because fever has been detected accompanying the late asthmatic response. The criteria used to identify a hypersensitivity response are:

Respiratory rate: 35% or greater increase from baseline
Fever: 0.6°C or greater increase from baseline.

Serum is collected at selected time intervals to measure antibodies to the chemical (Karol, 1995).

This model has been used to examine antibodies and pulmonary responses to chemicals such as toluene diisocyanate (TDI), diphenylmethane diisocyanate (MDI) (Karol and Thorne, 1988), trimeric hexamethylene diisocyanate (Des-N) (Pauluhn and Eben, 1991), trimellitic anhydride (TMA) (Pauluhn and Eben, 1991; Botham et al., 1988), phthalic anhydride (PA) (Sarlo and Clark, 1992), reactive black B dye (Sarlo and Clark, 1992), and procion yellow mx4r dye (Botham et al., 1988).

A review by Sarlo (1997) summarized the results of these studies as follows. Pulmonary reactivity (immediate and/or delayed onset) was detected in animals exposed to the isocyanates and the anhydrides when challenged, by inhalation, with the appropriate chemical-carrier conjugate. Pulmonary reactivity, elicited by inhalation challenge with the chemical alone could be demonstrated with TDI, MDI, and TMA. No pulmonary reactivity was detected in the animals exposed to the reactive dyes after inhalation challenge with dye or dye-carrier conjugate.

Allergic and nonallergic antibodies were found to all the chemicals (the major allergic antibody in guinea pigs is IgG1 whereas that in humans is IgE). The presence of antibodies and the amount of antibody produced were not predictive of pulmonary reactivity. Those animals with pulmonary responses had antibodies to the chemical but not all animals with antibodies experienced pulmonary reactions upon challenge. Also the development of antibodies occurred at exposure concentrations lower than those needed to establish pulmonary reactivity.

The Karol inhalation model has the advantages of allowing exposure via the respiratory tract and evaluation of dose–response relationships, but it is a time- and labor-intensive model, requiring specialized equipment and facilities. In addition, changes in respiratory pattern can occur as a result of respiratory tract irritation and, therefore, need careful interpretation. Exposure to nonirritant concentrations is desirable but in practice it is difficult to achieve.

Similar respiratory parameter tests have been developed incorporating intradermal injections of a high dose of chemical to induce an immune response (Pauluhn and Eben, 1991; Botham et al.,

1989; Rattray et al., 1994). Either one or two intradermal injections were used followed by serology and inhalation challenge, with free chemical or chemical-carrier conjugate, 2 weeks after the injection. TMA, Des-N, and MDI were evaluated. Allergic antibodies to all three chemicals were detected but pulmonary reactivity could only be elicited by inhalation challenge with TMA or MDI. In a further injection model, guinea pigs were injected subcutaneously with several low doses of free chemicals over a period of 4 weeks followed by a boost injection at 6 weeks and evaluation for antibody and respiratory reactivity at week 8 (Sarlo and Karol, 1994; Sarlo and Clark, 1992). A total of six chemicals have been assessed using this model: TDI, TMA, PA, reactive black B dye, MDI, and hexachloroplatinic acid. Respiratory reactivity was assessed by a single intratracheal challenge with chemical-carrier conjugate. Results indicated dose–response relationships between the induction dose of chemical and antibody titers or respiratory reactivity. The dose–responses in respiratory reactivity, however, were not always clearly related to induction dose or to the titer levels of chemical-specific antibodies. The injection models are faster to conduct and less expensive than the inhalation model but still require specialized equipment and facilities. More recently a combined intradermal injection/repeated inhalation sensitization with subsequent inhalation challenge protocol has been developed (Pauluhn and Mohr, 1998). In this study TDI was used as the test chemical with challenge to the free chemical and TDI-protein conjugate. Respiratory responses, antibody analysis, and lung histopathology were evaluated. The outcome of the test was less dependent on the exposure concentration used for sensitization of the animals when the combined protocol was used, which improved the robustness of the test.

A major disadvantage of the injection and inhalation models is the need for a chemical-carrier conjugate, which is necessary to measure chemical-specific allergic and nonallergic antibodies. There are no standard methods for preparing or analyzing chemical conjugates and the quality of the conjugate can affect the result of the serological assay (Botham et al., 1988). In addition, all the respiratory parameter tests suffer from the problem of confounding results caused by irritant effects of the test chemical. It is not possible, in these tests, to distinguish between respiratory changes caused by irritation and those induced by an immunologically mediated mechanism, unless antibody analysis is also conducted. This necessitates exposure to nonirritant concentrations, which may be too low to elicit a response.

12.6.2 The Mouse IgE Test

The mouse IgE test was developed to differentiate between contact and respiratory sensitizers and is based on the different induced immune responses characteristic of differential activation of T-helper cells. Dearman and Kimber (1991) and Dearman et al. (1991, 1992b) showed that a respiratory sensitizer, trimellitic anhydride, induced a characteristic rise in serum IgE levels whereas a contact sensitizer, 2,4-dinitrochlorobenzene (DNCB), did not. Subsequent studies have revealed similar results with other respiratory sensitizers, diphenylmethane diisocyanate and phthalic anhydride (Dearman and Kimber, 1992; Dearman et al., 1992c). The basis of the model rests on the theory that all chemical respiratory allergens are also contact allergens and that dermal exposure can lead to either a contact sensitization response or to a respiratory allergy. In addition, the skin may be a relevant route of exposure for sensitization to chemical respiratory allergens. Several studies have examined antibody and pulmonary responses of guinea pigs after dermal exposure to various isocyanates. One or two dermal applications of 25 or 100% TDI led to the generation of TDI-specific allergic antibody and one third of the animals experienced pulmonary reactions upon inhalation exposure to TDI or TDI-protein conjugate (Karol et al., 1981). Preferential stimulation of Th1 or Th2 cells will determine whether contact allergy or respiratory allergy ensues. A chemical with the potential to be a respiratory allergen will induce stimulation of Th2-dominated immune responses resulting in elevated IgE levels and development of chemical-specific IgE antibodies. A high-IgE-responding strain of mouse, the BALB/c strain, is used to maximize the effect. Dearman et al. (1992c) showed that dermal exposure of BALB/c strain mice to known chemical respiratory sensitizers, but not to chemicals considered only to be contact sensitizers, induced an increase in the serum concentration

of IgE. Groups of mice received 50 μl of a single concentration of the test chemical on each shaved flank followed, 7 days later, by 25 μl of a 1:1 dilution of the same chemical applied to the dorsum of both ears. At various periods following exposure mice were exsanguinated by cardiac puncture and serum prepared. The concentration of IgE in serum samples was measured by using a sandwich enzyme-linked immunosorbent assay (ELISA) calibrated with a monoclonal mouse IgE antibody. Results were compared with historical control values for serum IgE levels in untreated mice. In subsequent investigations the serum IgE concentration was measured 14 days after initiation of exposure to the test material and studies were conducted to examine dose–response relationships. It was found that each of four chemical respiratory allergens, TMA, TDI, MDI, and hexamethylene diisocyanate, induced a dose-dependent increase in serum IgE concentration compared with concurrent vehicle-treated control groups and historical control values. No similar increases were observed when mice were treated in the same way with the contact-sensitizing chemicals DNCB or oxazolone (Hilton et al., 1995). With this method a chemical is considered to have potential to induce sensitization of the respiratory tract if it provokes, at one or more test concentrations, a statistically significant elevation in the serum concentration of IgE, compared with values derived from control mice treated with the vehicle alone (Hilton et al., 1996). These authors proposed the use of DNCB and TMA as negative and positive controls, respectively.

The IgE test appears, therefore, to be capable of demonstrating a specific immune response but it does not demonstrate elicitation of that response as asthma. It is not suitable for measuring the respiratory sensitization potential of proteins because protein sensitizers do not appear to produce, in mice, an increase in serum IgE concentration comparable with that induced by chemical allergens (Hilton et al., 1994). It has also been shown recently that respiratory sensitization induced by certain acid anhydrides may occur by an IgE-independent mechanism (Dearman et al., 2002). Furthermore, this method cannot be used to examine dose–response relationships for the elicitation of respiratory allergic reactions in previously sensitized animals.

12.6.3 Cytokine Fingerprinting

The divergent immune responses seen with contact and respiratory sensitizing chemicals have also been utilized in another proposed method for screening chemicals known as cytokine fingerprinting. It appears that the phenotype of selective cytokine secretion develops as the response to a chemical allergen evolves. Cytokines isolated from draining lymph nodes early in the immune response, after initial exposure of mice, produce a similar profile of cytokines irrespective of the chemical allergen used. After more chronic treatment, however, selective cytokine secretion becomes apparent. Several studies have been conducted to examine mitogen-inducible IL-4, IL-10, and IFN-γ as cytokines indicative of the differential immune response. Chemicals were applied to mice as in the IgE assay by initial application to the shaved flanks on two occasions, 5 days apart. A further 5 days later, chemicals were applied to the dorsum of both ears daily for three consecutive days. The draining auricular lymph nodes were then excised and lymph node cell (LNC) preparations were prepared. These LNC preparations were maintained in culture and cytokine analysis was conducted on the culture supernatants by using sandwich ELISA techniques (Dearman et al., 1994, 1995, 1996). In these studies, primary exposure of BALB/c mice to TMA or oxazolone produced similar amounts of mitogen-inducible IL-4, but after more prolonged exposure the production of IL-4 was significantly greater in mice treated with TMA (Dearman et al., 1994, 1995). Treatment with TMA was also found to favor the production of IL-10 whereas oxazolone or DNCB preferentially induced IFN-γ (Dearman et al., 1995). In a further study, exposure to TDI and DNFB (a respiratory and contact sensitizer, respectively) also stimulated distinct cytokine patterns. LNCs from mice treated with TDI were found to secrete high levels IL-10 and mitogen-inducible IL-4, but only comparatively low concentrations of IFN-γ. Exposure to DNFB resulted in the converse profile of cytokine secretion.

In some respects this proposed method is particularly attractive. It would be possible to use this technique to identify chemicals with sensitization potential by measuring stimulation of cytokine production and to identify the quality of the immune response likely to develop by the profile of

cytokines produced. Thus, a sensitizer could be identified and defined as a respiratory or contact sensitizer in the same assay. Unfortunately this method suffers from some of the same disadvantages as the IgE method. It does not demonstrate elicitation of the immune response as asthma, providing only an indication that a particular type of immune response has been induced. It might be possible, for example, for a chemical to be shown as positive for respiratory sensitization by the IgE test or by cytokine fingerprinting but to lack the ability to gain access to, or to elicit hypersensitivity reactions in, the respiratory tract. It would not be possible to develop the cytokine-fingerprinting technique for use with inhalation exposure because of the absence of a single draining lymph node for the respiratory tract.

12.6.4 The Eosinophil Peroxidase Assay

As a result of the importance of pulmonary eosinophilia in the pathogenesis of asthma, several animal models of this condition have been investigated including sheep, primates, guinea pigs, and rats (Abraham et al., 1988; Ishida et al., 1989; Gundel et al., 1990; Elwood et al., 1991; Foster and Chan, 1991; Pauluhn and Mohr 1998). All these studies rely on the histological detection of pulmonary eosinophils in BAL fluid or tissue sections. Such methodology is particularly time consuming (its accuracy depending on the number of cells and fields counted), susceptible to artifacts of tissue processing and fixation, and unamenable to automation (Tagari et al., 1993). A biochemical method has been described that utilizes the preference of eosinophil peroxidase (EPO) for bromide ion (Br^-) as a substrate to quantify numbers of eosinophils (Bozeman et al., 1990). This assay may provide the basis for a simple, quantitative, and reproducible method for identifying respiratory sensitizers.

The assay is based on the natural function of EPO, which is to use H_2O_2 to oxidize halide ions to produce an array of antimicrobial oxidizing and halogenating agents. EPO in conjunction with H_2O_2 and halide kills a variety of microorganisms *in vitro* (Gleich and Adolphson, 1986). In general, the preferred halide is iodide (I^-), but EPO is effective at, and preferentially uses, the concentrations of bromide that are present in physiological fluids (Weiss et al., 1986). A similar function is shared by the myeloperoxidase (MPO) enzyme present in neutrophils and monocytes, but MPO primarily catalyzes the oxidation of chloride by H_2O_2 to yield the highly reactive oxidizing and chlorinating agent hypochlorous acid. The EPO found in cytoplasmic granules of human eosinophilic leukocytes is much less active in chloride oxidation but highly active with Br^-, I^-, and the pseudohalide ion thiocyanate (SCN^-) (Bozeman et al., 1990). MPO and EPO also share the ability to catalyze the oxidation of phenols and aromatic amines. Many assays based on measuring the rate of H_2O_2-dependent oxidation of phenols and aromatic amines have been developed to measure peroxidase activity. Bozeman et al. (1990) selected the spectrophotometric assay of tetramethylbenzidine (TMB) oxidation as having a number of advantages, including its high sensitivity (enzyme concentrations of 1 n*M* are readily assayed) and the fact that TMB is less hazardous than many other peroxide substrates.

Bozeman et al. (1990) showed that EPO activity is preferentially stimulated by the presence of Br^-. The presence of potassium bromide (KBr) or the bromide salt of a detergent (CETAB) caused a dramatic increase, up to 7-fold, in the activity of purified EPO enzyme. In contrast, the same concentration (3 m*M*) KBr caused a much smaller, only 1.5-fold, increase in the activity of purified MPO enzyme. This factor was used to develop an assay that differentiates between EPO and MPO activity. The assay uses spectrophotometry to quantify the H_2O_2-dependent oxidation of TMB with Br^- as a substrate. The reaction can be stopped by addition of H_2SO_4 and the yellow product absorbs at approximately 450 nm.

The assay was further developed by Tagari et al. (1993) for use with homogenized lung tissue and they showed that, by using appropriate dilutions of lung homogenate, protein quenching of the reaction was insignificant. The same workers showed that guinea pig eosinophils, purified by centrifugation over Ficoll 400 containing sodium diatrizoate, showed greater peroxidase activity than unpurified polymorphonuclear leukocytes (containing 3 to 5% eosinophils). These findings indicate that guinea pig EPO is preferentially stimulated by Br^- similarly to that derived from human eosinophils.

The work was conducted on peritoneal eosinophils derived from guinea pigs after three times weekly injections of polymixin sulfate. With these cells, both endogenous and Br^--stimulated peroxidase activities were found to be directly proportional to the number of eosinophils. EPO could be completely inhibited by 3 μ*M* azide, which further confirms the biochemical homology of the guinea pig and human enzymes (Bozeman et al., 1990). Preliminary studies indicated that high concentrations of lung homogenate inhibited the oxidation of TMB, presumably by protein binding of the TMB, but a 1:500 final dilution of tissue was found to have minimal effects on the EPO activity of added eosinophils. Furthermore, this dilution did not affect the stimulatory effect of 3 m*M* KBr. This latter effect was quantified in the presence of lung homogenate and showed a log-linear relationship.

Tagari et al. (1993) went on to demonstrate that pulmonary eosinophilia could be identified in sensitized guinea-pigs by using this assay. The study used a protein sensitizer, ovalbumin, to which animals were sensitized by intraperitoneal injection followed by inhalation challenge with an ovalbumin aerosol. The assay was conducted by using homogenized lung tissue obtained from these animals. Inhalation of 3% ovalbumin by unsensitized animals resulted in a modest (35%) increase in EPO activity similar to that observed after inhalation of saline or bovine serum albumin (BSA). Animals that had been presensitized by intraperitoneal injection of ovalbumin showed visible rhinitis, respiratory abnormalities, and a 2-fold increase in EPO activity. Double sensitization of guinea pigs with two injections of ovalbumin 2 weeks apart, and ovalbumin inhalation 1 week after the second injection, increased the EPO activity to more than 3-fold greater than controls.

More recent studies have confirmed that the EPO assay can be used in guinea pigs to quantify the pulmonary eosinophil influx that typifies the respiratory sensitization response. The assay, originally described by Tagari et al. (1993), has been refined and developed as a potential predictive test for chemical respiratory sensitizers. An experimental protocol has been described involving induction of immune responses by intraperitoneal injection followed by inhalation challenge exposures. Approximately 17 h after inhalation exposure, samples of blood-free lung parenchyma were homogenized and subjected to the EPO assay. Substantial elevations in pulmonary EPO activity were detected after induction and challenge with both a protein sensitizer (ovalbumin) and a low-molecular-weight sensitizer (trimellitic anhydride). The immunological basis of these responses was confirmed because similar increases were not observed when inhalation exposure was conducted without presensitization or in ovalbumin-presensitized animals subsequently exposed by inhalation to a different protein (BSA) (Blackwell, 1998).

A further study (Blackwell, 1999) involved six test groups treated with the three test chemicals (TDI, PA, and DNCB), with either single or repeated exposure inhalation challenge. Induction of an immune response was obtained by two intraperitoneal injections of the test chemicals, mixed with aluminum hydroxide as an adjuvant, 2 weeks apart. One week after the second injection animals were exposed, by nose-only inhalation, to aerosol atmospheres of the test chemicals either once only or once per day for three consecutive days. The test groups, which received intraperitoneal induction and a single inhalation challenge exposure to either TDI or PA, showed slightly higher group mean absorbance values in the EPO assay than controls. The increase, however, was not statistically significant with only one or two animals in each group showing a response in the EPO assay that could be considered indicative of a hypersensitivity reaction. This muted response was not unexpected, in particular, for TDI, because demonstration of respiratory sensitization in guinea pigs with this chemical has proved problematic in other studies unless challenge exposures were conducted using a TDI protein conjugate (Karol et al., 1980; Botham et al., 1988). Previous studies had produced unequivocal sensitization reactions with trimellitic anhydride and ovalbumin (Blackwell, 1998) by using this protocol, so it was considered unlikely that the problem lay in failure to induce an antibody response. The previous studies, by others, on TDI had identified the need for challenge exposure to a protein conjugate, so the evidence suggested the need for an enhanced inhalation challenge procedure. It was hoped that this could be accomplished without using protein conjugation. Accordingly, further groups were treated with each of the three test chemicals (TDI, PA, and DNCB) with the same protocol but modified to include repeated inhalation challenge exposures (up to 1 h per day for 3 consecutive days).

The impact of the repeated-challenge procedure on the outcome of the EPO assay was dramatic. The animals sensitized to, and repeatedly challenged with, PA showed an approximately 8-fold increase in EPO activity compared with negative controls. Those animals similarly treated with TDI showed a lesser but still statistically significant ($p < 0.01$) increase in EPO activity compared with the negative controls.

The method using this refined protocol has clearly demonstrated respiratory sensitization, identified as pulmonary eosinophil infiltration, following exposure to PA and TDI without the need for challenge exposure to a protein conjugate. Furthermore, identical treatment of guinea pigs with the contact sensitizer DNCB produced no significant increase in EPO activity.

Histopathological examination of lung samples was undertaken to compare the morphological appearance of the lung tissue and, specifically, the cellularity of any inflammatory changes with the results of the EPO assay. One of the main perceived advantages of the EPO assay over histopathology for the determination of respiratory sensitization is that quantitative values can easily be obtained. This cannot be done by histopathology unless elaborate special staining techniques combined with image analysis or laborious cell-counting procedures are used. Thus, the histopathology results obtained are qualitative, or at best semiquantitative, in nature.

The PA- and TDI-repeated challenge animals that showed elevated EPO activities also showed statistically significant increases in the incidence and severity of eosinophil infiltration in the lung tissue as determined histopathologically. Histopathology also identified increased incidence and severity of eosinophils in the single-challenge TDI group but to a lesser degree than in the repeated-challenge group.

The histopathological determination of eosinophils correlated well with the results of the EPO assay, confirming the predictive nature of the assay. In particular, the more substantial elevation in eosinophil numbers, suggested by the EPO assay, for the PA-treated animals compared with the TDI group was confirmed by the histopathology results. An increased severity of eosinophil infiltration was described in the PA repeated challenge group compared with the corresponding TDI animals. The only discrepancy was in the single-exposure TDI group, which showed a significant eosinophilia by histopathology but not in the EPO assay, although a small, but not statistically significant, increase had been suggested by the EPO assay. This discrepancy, most likely results from the semiquantitative nature of the histopathology findings and the fact that histopathology necessarily focuses in on a small section of lung tissue (5 μm in thickness); when the condition may not occur uniformly throughout the tissue.

The results of this study show that the EPO assay can be used to provide a rapid and quantitative determination of pulmonary eosinophil infiltration caused by chemical respiratory sensitization. The protocol has potential for use as a screening test for chemical respiratory sensitizers and the quantitative nature of the assay suggests that it could be useful in identifying the potency of sensitizing chemicals.

12.7 INHALATION CONSIDERATIONS

As discussed previously, it is important for a predictive method for the determination of respiratory sensitization to include an inhalation phase. Clearly, it is inhalation of allergens that results in elicitation of asthmatic responses in sensitized individuals so a potential allergen must be capable of reaching the respiratory tract. The most useful methods are, therefore, likely to be those that at least allow challenge exposures to be undertaken by inhalation. It is not necessarily essential for initial sensitization (induction) to be conducted in this way. Respiratory sensitization in humans probably occurs in the majority of cases by solely inhalation exposure but considerable evidence indicates that it can occur through other routes of exposure as well. Studies in guinea pigs have demonstrated respiratory sensitization following dermal exposure and subsequent inhalation challenge (Karol et al., 1981). Furthermore, it is possible that other routes of exposure, in particular, the dermal route, could be of major importance in inducing sensitization, which subsequently results in occupational asthma. Workers are often exposed to chemicals by contact with the skin. Nevertheless, for respiratory hypersensitivity (asthma) to ensue, subsequent inhalation exposure is essential.

REFERENCES

Abraham, W.M., Sielczak, M.W., Waner, A., Perruchoud, A.P., Blinder, L., Stevenson, J.S., Ahmed, A., and Yerger, L.D., Cellular markers of inflammation in the airways of allergic sheep with and without allergen induced late responses, *Am. Rev. Respir. Dis.,* 138, 1565–1571, 1988.

Beasley, R., Roche, W.R., Robetrs, J.A., and Holgate, S.T., Cellular events in the bronchi in mild asthma and after bronchial provocation, *Am Rev. Respir. Dis.,* 139, 806–817,1989.

Bernstein, D.I., Patterson, R.M. and Zeiss, C.R., Clinical and immunological evaluation of trimellitic anhydride and phthalic anhydride exposed workers using a questionnaire and comparative analysis of enzyme-linked immunosorbent and radioimmunoassay studies, *J. Allergy Clin. Immunol.,* **69,** 311–318, 1982.

Blackwell, M.P., Demonstration of respiratory sensitisation in guinea pigs by a protein and a low molecular weight sensitiser using a biochemical assay of pulmonary eosinophilia, *Inhalation Toxicol.,* 10, 1023–1036. 1998.

Blackwell, M.P., Investigation of a biochemical marker of pulmonary eosinophil influx as a predictive assay for low molecular weight respiratory sensitisers, Ph.D. Thesis, University of Derby, U.K., 1999,

Botham, P.A., Hext, P.M., Rattray, N.J., Walsh, S.T., and Woodcock, D.R., Sensitisation of guinea pigs by inhalation exposure to low molecular weight chemials, *Toxicol. Lett.,* 41, 159–173, 1988.

Botham, P.A., Rattray, N.J., Woodcock, D.R., Walsh, S.T., and Hext, P. The induction of respiratory allergy in guinea pigs following i.d. injection of trimellitic anhydride. A comparison of response to 2,4-dinitrochlorobenzene, *Toxicol. Lett.,* 47, 25–39, 1989.

Bozeman, P.M., Learn, D.B., and Thomas, E.L., Assay of the human leukocyte enzymes myeloperoxidase and eosinophil peroxidase, *J. Immunol. Methods,* 126, 125–133, 1990.

Bradding, P., Feather, I.J., Howarth, P.H., Mueller, R., Roberts, J.A., and Britten, K., Interleukin-4 is localised to and released by human mast cells, *J. Exp. Med.,* 176, 1381–1386, 1992.

Bradding, P., Roberts, J.A., Britten, K.M., Montefort, S., Djukanovic, R., Mueller, R., Heusser, C.H., Howarth, P.H., and Holgate, S.T., Interleukin-4, -5 and –6 and tumour necrosis factor alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines, *Am. J. Respir. Cell Mol. Biol.,* 10, 471–480, 1994.

Briatico-Vangosa, G., Braun, C.L.J., Cookman, G., Hofmann, T., Kimber, I., Loveless, S.E., Morrow, T., Pauluhn, J., Scrensen, T., and Niessen, H.J., Respiratory allergy: Hazard identification and risk assessment, *Fundam. Appl. Toxicol.,* 23, 145–158, 1994.

Burstein, H.J., Tepper, R.I., Leder, P., and Abbas, A.K., Humoral immune functios in IL-4 transgenic mice, *J. Immunol.,* 147, 2950–2956, 1991.

Cartier, A., Chan, H., Malo, J.-L., Pineau, L., Tse, K.S., and Chan-Yeung, M., Occupational asthma caused by eastern white cedar (Thuja occidentalis) with demonstration that plicatic acid is present in this wood dust and is the causal agent, *J. Allergy Clin. Immunol.,* 77, 639–645, 1986.

Chand, N., Harrison, J.E., Rooney, S., Pillar, J., Jackubicki, R., Nolan, K., Diamantis, W., and Sofia, R.D., Anti-IL-5 monoclonal antibody inhibits allergic late phase bronchial eosinophilia in guinea-pigs: a therapeutic approach, *Eur. J. Pharmacol.,* 211, 121–124, 1992.

Cher, D.J. and Mossman, T.R., Two types of murine helper T cell clone. II. Delayed type hypersensitivity is mediated by Th1 clones, *J. Immunol.,* 138, 3688, 1987.

Coffman, R.L., Seymour, B.W.P., Hudak, S., Jackson, J., and Rennick, D., Antibody to interleukin-5 inhibits helminth-induced eosinophilia in mice, *Science,* 245, 308–311, 1989.

Coombs, R.R.A. and Gell, P.G.H., Classification of allergic reactions responsible for clinical hypersensitivity and disease, In *Clinical Aspects of Immunology,* 3rd ed., Gell. P.G.H., Coombs, R.R.A., and Lachmann, P.J., Eds., Blackwell Scientific, Oxford, U.K., 1975.

Corrigan, C.J. and Kay, A.B., T cells and eosinophilia in the pathogenesis of asthma, *Immunol. Today,* 13, 501–507, 1992.

Croft, M., Carter, L., Swain, S.L., and Dutton, R.W., Generation of polarised antigen specific CD-8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoying type 2 versus type 1 cytokine profiles, *J. Exp. Med.,* 180, 1715–1728, 1994.

Cruse, J.M. and Lewis, R.E., *Illustrated Dictionary of Immunology,* CRC Press, Boca Raton, FL, 1995.

Dearman, R.J., Basketter, D.A., Coleman, J.W., and Kimber, I., The cellular and molecular basis for divergent allergic responses to chemicals, *Chem.-Biol. Interact.,* 84, 1–10, 1992a.

Dearman, R.J., Basketter, D.A., and Kimber, I., Differential cytokine production following chronic exposure of mice to chemical respiratory and contact allergens, *Immunology,* 86, 545–550, 1995.

Dearman, R.J., Basketter, D.A., and Kimber, I., Characterisation of chemical allergens as a function of divergent cytokine secretion profiles in mice, *Toxicol. Appl. Pharmacol.,* 138, 308–316, 1996.

Dearman, R.J., Filby, A., Humphreys, I.R., and Kimber, I., Interleukins 5 and 13 characterize immune responses to respiratory sensitizing acid anhydrides, *J. Appl. Toxicol.,* 22, 317–325, 2002.

Dearman, R.J., Hegarty, J.M., and Kimber, I. Inhalation of mice to trimellitic anhydride induces both IgG and IgE anti-hapten antibody, *Int. Arch. Allergy Appl. Immunol.,* 95, 70–76, 1991.

Dearman, R.J. and Kimber, I. Differential stimulation of immune function by respiratory and contact chemical allergens, *Immunology,* 72, 563–570, 1991.

Dearman, R.J. and Kimber, I. Divergent immune responses to respiratory and contact chemical allergens: antibody elicited by phthalic anhydride and oxazolone, *Clin. Exp. Allergy,* 22, 241–250, 1992.

Dearman, R.J., Mitchell, J.A., Basketter, D.A., and Kimber, I. Differential ability of occupational chemical contact and respiratory allergens to cause immediate and delayed dermal hypersensitivity reactions in mice, *Int. Arch. Allergy Appl. Immunol.,* 97, 315–321, 1992b.

Dearman, R.J., Ramdin, L.S.P., Basketter, D.A., and Kimber, I., Inducible interleukin-4-secreting cells provoked in mice during chemical sensitisation, *Immunology,* 81, 551–557, 1994.

Dearman, R.J., Spence, L.M., and Kimber, I., Characterization of murine immune responses to allergenic diisocyanates, *Toxicol. Appl. Pharmacol.,* 112, 190–197, 1992c.

De Monchy, J.G.R., Kauffman, H.F., Venge, P., Koeter, G.H., Jansen, H.M., Sluiter, H.J., and De Vries, K. Bronchoalveolar lavage eosinophilia during allergen induced late asthmatic reactions, *Am Rev. Respir. Dis.,* 131, 373–376, 1985.Djukanovic, R., Wilson, J.W., Britten, K.M., Wilson, S.J., Walls, A.F., Roche, W.R., Howarth, P.H., and Holgate, S.T., Quantitation of mast cells and eosinophils in the bronchial mucosa of symptomatic atopic asthmatics and healthy control subjects using immunohistochemistry, *Am Rev. Respir. Dis.,* 142, 863–871, 1990.

Docker, A., Wattie, J.M., Topping, M.D., Luczynska, C.M., Newman-Taylor, A.J., Pickering, C.A., Thomas, P., and Gompertz, D., Clincal and immunological investigations of respiratory disease in workers using reactive dyes, *Br. J. Ind. Med.,* 44, 534–541, 1987.

European Commission (EC), Commission Directive No. 2001/59/EC, *Off. J. Eur. Communities,* L225, 44, 2001.

European Economic Community (EEC), Council Directive No. 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to classification, packaging and labelling of dangerous substances, *Off. J. Eur. Communities,* 196, 16.8, 1967.

EEC, Council Directive No. 79/831/EEC amending for the sixth time Directive No. 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to classification, packaging and labelling of dangerous substances, *Off. J. Eur. Communities,* L259, 15.10, 1979.

EEC, Council Regulation (EEC) No 793/93, *Off. J. Eur. Communities,* L84, 1–75, 1993.

EEC, Risk Assessment of Existing Substances, Technical guidance document, XI/919/94, European Commission DGXI, Brussells, 1994.

Ellis, A.G., The pathological anatomy of bronchial asthma, *Am. J. Med. Sci.,* 136, 407, 1908.

Elwood. W., Lotvall, J.O., Barnes, P.J., and Chung, K.F., Characterization of allergen-induced bronchial hyperresponsiveness and airway inflammation in actively sensitised brown-Norway rats, *J. Allergy Clin. Immunol.,* 88, 951–960, 1991.

EPA, *TSCA New Chemicals Program (NCP) Chemical Categories,* Office of Pollution Prevention and Toxics, U.S. EPA, Washington, DC, subject to review.

European Commission (EC), Annex to Commission Directive 96/54/EC, *Off. J. Eur. Communities,* L248, 227–228, 1996.

European Commission (EC), http://europa.eu.int/comm/environment/chemicals/whitepaper.htm, 2001.

Evans, P., Chemical respiratory allergy: a regulatory viewpoint, in *Toxicology of Chemical Respiratory Hypersensitivity,* Kimber, I., and Dearman, R.J., Eds., Taylor & Francis, London, 1997.

Finkelman, F.D., Katona, I.M., Urban, J.F., Jr., Holmes, J., Ohara, J., Tung, A.S., Sample, J.G., and Paul, W.E., IL-4 is required to generate and sustain in vivo IgE responses, *J. Immunol.,* 141, 2335–2341, 1988a.

Finkelman, R.D., Katona, I.M., Mossman, T.R., and Coffman, R.L., IFN-γ regulates the isotypes of Ig secreted during *in-vivo* humoral immune responses, *J. Immunol.,* 140, 1022–1027, 1988b.

Fong, T.A.T. and Mossman, T.R., The role of IFN-γ in delayed-type hypersensitivity mediated by Th1 clones, *J. Immunol.,* 143, 2887, 1989.

Foster, A. and Chan, C.C., Peptide leukotriene involvement in pulmonary eosinophil migration upon antigen challenge in the actively sensitised guinea pig, *Int. Arch. Allergy Appl. Immunol.,* 96, 179–284, 1991.

Frew, A.J., Teran, L.M., Madden, J.M., Trefilieff, A., St. Pierre, J., Semper, A., Carroll, M.P., and Holgate, S.T., Cellular changes 24 hours after endobronchial allergen challenge in asthma, *Int. Arch. Allergy Appl. Immunol.,* 107, 376–377, 1995.

Frigas, E. and Gleich, G.J., The eosinophil and the pathophysiology of asthma, *J. Allergy Clin. Immunol.,* 77, 527–537, 1986.

Gleich, G.J., The eosinophil and bronchial asthma: current understanding, *J. Allergy Clin. Immunol.,* 85, 422–436, 1990.

Gleich, G.J. and Adolphson, C.R., The eosinophilic leococyte: structure and function, *Adv. Immunol.,* 39, 177–253, 1986.

Graham, C., Rosenkranz, H.S., and Karol, M.H., Structure-activity model of chemicals that cause human respiratory sensitisation, *Regul. Toxicol. Pharmacol.,* 26, 296–306, 1997.

Gulbenkian. A.R., Egan, R.W., Fernandez, X., Jones, H., Kreutner, W., Kung, T., Payvandi, F., Sullivan, L., Zurcher, J.A., and Watnik, A.S., Interleukin-5 modulates eosinophil accumulation in allergic guinea pig lung, *Am. Rev. Respir. Dis.,* 146, 263–265, 1992.

Gundel, R.J., Gerritsen, M.E., and Wegner, C.D., Repeated antigen inhalation results in a prolonged airway eosinophilia and airway hyperresponsiveness in primates, *J. Appl. Physiol.,* 68, 779–786, 1990.

Gundel, R.H., Wegner, C.D., Torcellini, C.A., Clarke, C.C., Haynes, N., Rothlein, R., Smith, C.W., and Letts, L.G., ELAM-1 mediates antigen induced acute airway inflammation and late phase obstruction in monkeys, *J. Clin. Invest.,* 88, 1407–1411, 1991.

Hay, D.W.P., Torphy, T.J., and Undem, B.J., Cysteinyl leukotrienes in asthma: old mediators up to new tricks, *Trends Pharmacol. Sci.,* 16, 304–309, 1995.

Hilton, J., Dearman, R.J., Basketter, D.A., and Kimber, I., Serological responses induced in mice by immunogenic proteins and by protein respiratory allergens, *Toxicol. Lett.,* 73, 43–53, 1994.

Hilton, J., Dearman, R.J., Basketter, D.A., and Kimber, I., Identification of chemical respiratory allergens: dose-response relationships in the mouse IgE test, *Toxicol. Methods,* 5, 51–60, 1995.

Hilton, J., Dearman, R.J., Boylett, M.S., Fielding, I., Basketter, D.A., and Kimber, I., The mouse IgE test for identification of potential chemical respiratory allergens. Considerations of stability and controls, *J. Appl. Toxicol.,* 16, 165–170, 1996.

Horn, B.R., Robin, E.D., Theodore, J., and Van Kessel, A., Total eosinophil counts in the management of bronchial asthma, *N. Engl. J. Med.,* 292, 1152–1155, 1975.

Howe, W., Venables, K.M., Topping, M.D., Dally, M.B., Hawkins, R., Law, J.S., and Newman-Taylor, A.J., Tetrachlorophthalic anhydride asthma: Evidence for Specific IgE Antibody, *J. Allergy Clin. Immunol.,* 75, 5–11, 1983.

HSE, *Asthmagen? Critical Assessments of the Evidence for Agents Implicated in Occupational Asthma,* HSE Books, Sudbury, Suffolk U.K., 1997.

Ishida, K., Kelly, L.J., Thompson, R.J., Beattie, L.L., and Schelenberg, R.R., Repeated antigen challenge induces airway hyperresponsiveness with tissue eosinophilia in guinea pigs, *J. Appl. Physiol.,* 67, 1133–1139, 1989.

Iwami, T., Nagai, H., Suda, H., Tsuruoka, N., and Koda, A., Effect of murine recombinant interleukin-5 on the cell population in the guinea-pig airways, *Br. J. Pharmacol.,* 105, 19, 1992.

Iwami, T., Nagai, H., Tsuruoka, N., and Koda, A., Effect of murine recombinant interleukin-5 on bronchial reactivity in guinea pigs, *Clin. Exp. Allergy,* 23, 32–38, 1993.

Iwamoto, I., Nakajima, H., Endo, H., and Yoshida, S., Interferon-γ regulates antigen induced eosinophil recruitment into the mouse airways by inhibiting the infiltartion of CD4+ T cells, *J. Exp. Med.,* 177, 573–576, 1993.

Jefferey, P.K., Wardlaw, A.J., Nelson, F.C., Collins, J.V., and Kay, A.B., Bronchial biopsies in asthma: an ultrastructural quantification study and correlation with hyperreactivity, *Am. Rev. Respir. Dis.,* 140, 1745–1753, 1989.

Kapsenburg, M.L., Wierenga, E.A., Bos, J.D., and Jansen, H.M., Functional subsets of allergen reactive CD4+ T cells, *Immunol. Today,* 12, 392–395, 1991.

Karol, M.H., Assays to evaluate pulmonary hypersensitivity in methods, in *Immunotoxicology,* Vol. II, Burleson, G.R., Dean, J.H., and Munson, A.E., Eds., Wiley-Liss Inc., New York, 1995.

Karol, M.H., Dixon, C., Brady, M., and Alarie, Y., Immunologic sensitization and pulmonary hypersensitivity by repeated inhalation of aromatic isocyanates, *Toxicol. Appl. Pharmacol.,* 53, 260–270, 1980.

Karol, M.H. and Thorne, P.S., Pulmonary hypersensitivity and hyperreactivity: implications for assessing allergic responses, in *Toxicology of the Lung,* Gardner, D.E., Crapo, J.D., and Massaro, E.J., Eds., Raven Press, New York, 1988.

Karol, M.H., Hauth, B.A., Riley, E.J., and Magreni, C.M., Dermal contact with toluene diisocyanate (TDI) produces respiratory tract hypersensitivity in guinea pigs, *Toxicol. Appl. Pharmacol.,* 58, 221–230, 1981.

Kemeny, D.M., Noble, A., Holmes, B.J., and Diaz-Sanchez, D., Immune regulation: a new role for the CD8+ T cell, *Immunol. Today,* 15, 107–110, 1994.

Kimber, I., Chemical-induced hypersensitivity, in *Experimental Immunotoxicology,* Smialowicz. R.J. and Holsapple, M.P., Eds., CRC Press, Boca Raton, FL, 1996.

Kimber, I. and Dearman, R.J., Immunobiology of chemical respiratory sensitization, in *Toxicology of Chemical Respiratory Hypersensitivity,* Kimber, I. and Dearman, R.J., Eds., Taylor & Francis, London, 1997.

Kuhn, R., Rajewsky, K., and Muller, W., Generation and analysis of interleukin-4 deficient mice, *Science,* 254, 707–710, 1991.

Lin, C.C., Lin, C.Y., Liaw, S.F., and Chen, A., Pulmonary function changes and immunomodulation of cytokine expression by zafirlukast after sensitisation and allergen challenge in brown Norway rats, *Clin. Exp. Allergy,* 32, 960–966, 2002.

Maccia, C.A., Bernstein, I.L., Emmett, E.A., and Brooks, S.M., In-vitro demonstration of specific IgE in phthalic anhydride sensitivity, *Am. Rev. Respir. Dis.,* 113, 701–704, 1976.

Meredith, S.K., Taylor, V.M., and McDonald, J.C., Occupational respiratory disease in the United Kingdom 1989: a report to the British Thoracic Society and Society of Occupational Medicine by the SWORD Project Group, *Br. J. Ind. Med.,* 48, 292–298, 1991.

MITI/MHW, Amendment to law concerning examination and regulation of manufacture, etc. of chemical substances 1973*,* Japanese Ministry of International Trade and Industry/Ministry of Health and Welfare, Japan, 1986.

Moller, D.R., Gallagher, J.S., Bernstein, D.I., Wilcox, T.G., Burroughs, H.E., and Bernstein, I.L., Detection of IgE-mediated respiratory sensitisation in workers exposed to hexahydrophthalic anhydride, *J. Allergy Clin. Immunol.,* 75, 663–672, 1985.

Montefort, S., Gratziou, C., Goulding, D., Polosa, R., Haskard, D.O., Howarth, P.H., Holgate, S.T., and Carroll, M.P., Bronchial biopsy evidence for leucocyte infiltration and upregulation of leukocyte-endothelial cell adhesion molecules 6 hours after local allergen challenge of sensitised asthmatic airways, *J. Clin. Invest.,* 93, 1411–1421, 1994.

Montefort, S., Herbert, C.A., Robinson, C., and Holgate, S.T., The bronchial epithelium as a target for inflammatory attack in asthma, *Clin. Exp. Allergy,* 22, 511–520, 1992a.

Montefort, S., Roberts, J.A., Beasley, R., Holgate, S.T., and Roche, W.R., The site of disruption of the bronchial epithelium in asthmatic and non-asthmatic subjects, *Thorax,* 47, 499–503, 1992b.

Mossman, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., and Coffman, R.L., Two types of murine helper T cell clone. 1. Definition according to profiles of lymphokine activities and secreted proteins, *J. Immunol.,* 136, 2348–2357, 1986.

Mossman, T.R. and Coffman, R.L., Heterogeneity of cytokine secretion patterns and functions of helper T cells, *Adv. Immunol.,* 46, 111–145. 1989.

Mossman, T.R. and Sad, S., The expanding universe of T-cell subsets: Th1, Th2 and more, *Immunol. Today,* 17, 138–146,1996.

Mossman, T.R., Schumacher, J.H., Street, N.F., Budd, R., O'Garra, A., Fong, T.A.T., Bond, M.W., Moore, K.W.M., Sher, A., and Fiorentino, D.F., Diversity of cytokine synthesis and function of mouse CD4+ T cells, *Immunol. Rev.,* 123, 209–229, 1991.

Murdoch, R.D., Pepys, J., and Hughes, E.G., IgE antibody responses to platinum group metals: a large-scale refinery survey, *Br. J. Ind. Med.,* 43, 37–43, 1986.

O'Brien, I.M., Harries, M.G., Burge, P.S., and Pepys, J., Toluene diisocyanate-induced asthma. 1. Reactions to TDI, MDI, HDI and histamine, *Clin. Allergy,* 9, 1–6, 1979.

O'Byrne, P.M., Dolovich, J., and Hargeave, F.E., Late asthmatic responses, *Am Rev. Respir. Dis.,* 136, 740–751, 1987.

Okayama, Y., Petit-Frere, C., Kassel, O., Semper, A., Quint, D., Tunon de Lara, M.J., Bradding, P., Holgate, S.T., and Church, M.K., IgE-dependent expression of mRNA for IL-4 and IL-5 in human lung mast cells, *J. Immunol.,* 155, 1796–1808, 1995.

Pauluhn, J. and Eben, A., Validation of a non-invasive technique to assess immediate or delayed onset airway hypersensitivity in guinea pigs, *J. Appl. Toxicol.,* 11, 423–431, 1991.

Pauluhn, J. and Mohr, U., Assessment of respiratory hypersensitivity in guinea pigs sensitized to toluene diisocyanate: a comparison of sensitization protocols, *Inhalation Toxicol.,* 10, 131–154, 1998.

Quirce, S., Cuevas, M., Olaguibel, J.M., and Tabar, A.I., Occupational asthma and immunological responses induced by inhaled carmine among employees at a factory making natural dyes, *J. Allergy Clin. Immunol.,* 93, 44–52, 1994.

Rattray, N.J., Botham, P.A., Hext, P.M., Woodcock, D.R., Fielding, I., Dearman, R.J., and Kimber, I., Induction of respiratory hypersensitivity to MDI in guinea-pigs: influence of route of exposure, *Toxicology,* 88, 15–30, 1994.

Roche, W.R., Beasley, R., Williams, J.H., and Holgate, S.T., Subepithelial fibrosis in the bronchi of asthmatics, *Lancet,* i, 520–524, 1989.

Romagnani, S., Human TH1 and TH2 subsets: doubt no more, *Immunol. Today,* 12, 256–257, 1991.

Romagnani, S., Induction of TH1 and TH2 responses: a key role for the 'natural' immune response, *Immunol. Today,* 13, 379–381, 1992.

Ross, D.J., Sallie, B.A., and McDonald, J.C., SWORD '94: Surveillance of work related and occupational respiratory disease in the UK, *Occup. Med.,* 45, 175–178, 1995.

Sad, S., Marcotte, R., and Mossman, T.R., Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines, *Immunity,* 2, 271–279, 1995.

Sampson, A.P. and Holgate, S.T., Allergic asthma: an interaction between inflammation and repair, in *Toxicology of Chemical Respiratory Hypersensitivity,* Kimber, I. and Dearman, R.J., Eds., Taylor and Francis, London, 1997.

Sarlo, K., Animal models for assessing chemicals as airway allergens, in *Criteria for Classification of Skin And Airway Sensitising Substances in the Work and General Environments,* Flyvholm, MA., Ed., WHO Regional Office for Europe, Denmark, 1997.

Sarlo, K. and Clark, E.D., A tier approach for evaluating the respiratory allergenicity of low molecular weight chemicals, *Fundam. Appl. Toxicol.,* 18, 107–114, 1992.

Sarlo, K. and Karol, M.H., Guinea pig predictive tests for respiratory allergy, in *Immunotoxicology and Immunopharmacology,* 2nd ed., Dean, J.H., Luster, M.I., Munson, A.E., and Kimber, I., Eds., Raven Press, New York, 1994.

Smith, C.A. and Rennick, D.M., Characterisation of a murine lymphokine distinct from interlekin 3 (IL-3) possessing a T-cell growth factor activity and a mast cell growth factor activity that synergizes with IL-3, *Proc. Natl. Acad. Sci. USA,* 83, 1857–1861, 1986.

Tagari, P., Black, C., Marshall, S., and Ford-Hutchinson, A.W., A rapid biochemical method for measuring antigen induced pulmonary eosinophil margination in allergic guinea pigs, *J. Immunol. Methods,* 163, 49–58, 1993.

Tepper, R.I., Levinson, D.A., Stanger, B.Z., Campos-Torrez, J., Abbas, A.K., and Leder, P., IL-4 induces allergic-like inflammatory disease and alters T cell development in transgenic mice, *Cell,* 62, 457–467, 1990.

Thompson-Snipes, l., Dhar, V., Bond, M.W., Mosmann, T.R., Moore, K.W., and Rennick, D.M., Interleukin-10: a novel stimulatory factor for mast cells and their progenitors, *J. Exp. Med.,* 173, 507–510, 1991.

Topping, M.D., Venables, K.M., Luczynska, C.M., Howe, W., and Newman-Taylor, A.J., Specificity of the human IgE response to inhaled acid anhydrides, *J. Allergy Clin. Immunol.,* 77, 834–842, 1986.

Vandenplas, O., Cartier, A., Lesage, J., Cloutier, Y., Perrault, G., Grammer, L.C., Shaughnessy, M.A., and Malo, J.L., Prepolymers of hexamethylene diisocyanate as a cause of occupational asthma, *J. Allergy Clin. Immunol.,* 91, 850–861, 1993.

Vonderheide, R.H. and Springer, T.A., Lymphocyte adhesion through the very late antigen 4: evidence for a novel binding site in the alternatively spliced domain of VCAM-1 and an additional $\alpha 4$ integrin counter receptor on stimulated endothelium, *J. Exp. Med.,* 175, 1433–1442, 1992.

Wass, U. and Berlin, A., An in vitro method for predicting sensitizing properties of inhaled chemicals, *Scand. J. Work. Environ. Health,* 16, 208–214, 1990.

Weiss, S.J., Test, S.T., Eckman, C.M., Roos, D., and Regiani, S., Brominating oxidants generated by human eosinophils, *Science,* 234, 200–203, 1986.

Yokota, T., Coffman, R.L., Hagiwara, H., Rennick, D.M., Takebe, Y., Yokota, K., Gemmell, L., Schrader, B., Yang, G., and Meyerson, P., Isolation and characterisation of lymphokine cDNA clones encoding mouse and human IgA-enhancing and eosinophil-colony stimulating factor activities. Relationship to interleukin-5, *Proc. Natl. Acad. Sci. USA,* 84, 7388–7392, 1987.

Zeiss, C.R., Kanellakes, T.M., Bellone, J.D., Levitz, D., Pruzansky, J.J., and Patterson, R., Immunoglobulin E-mediated asthma and hypersensitivity pneumonitis with precipitating anti-hapten antibodies due to diphenylmethane diisocyanate (MDI) exposure, *J. Allergy Clin. Immunol.,* 65, 346–352, 1980.

13 Peripheral Chemosensory Irritation with Particular Reference to Respiratory Tract Exposure

Bryan Ballantyne

CONTENTS

13.1 NATURE OF THE PERIPHERAL SENSORY IRRITANT EFFECT

The word irritancy is used somewhat loosely in some toxicological texts, and it may not be specified in acute situations whether the word is being applied to a pathological or pharmacological process. In a pathological process (primary irritation), irritancy should be used to describe inflammatory effects and, in the pharmacological process, it is used to describe an interaction of the causative molecule with peripheral sensory nerve receptors (Ballantyne, 1983).

Peripheral sensory irritation (PSI) is a pharmacological effect in which xenobiotics interact with sensory nerve receptors in the skin or mucosae to produce a local sensation (discomfort, itching, burning sensation, or pain) together with related local and some systemic (autonomic) reflexes. The effects subside after removal of the stimulus and do not result in any long-term adverse sequelae. Hence, the major characteristics of a PSI event are that the materials act locally (in skin or mucosae) by stimulating sensory nerve receptors and producing local sensations with locally mediated and some systemic reflexes (Ballantyne, 1999). Many substances causing a pharmacological PSI effect will also produce, usually at a higher applied concentration, an inflammatory response. Thus, a PSI effect may result in a protective biological warning of exposure to potentially harmful materials. Originally, PSI effects were attributed to a common chemical sense independent of touch, temperature, and pain (Parker, 1912). However, it was subsequently demonstrated that PSI effects are mediated by several types of receptors, most of which also respond to noxious, thermal, and/or mechanical stimuli (Green, 2000). Thus, the idea of common chemical sense was replaced by the concept that PSI effects are mediated principally by chemically sensitive neural elements of pain and temperature; i.e., a process of peripheral chemosensory irritation (PCI). The word "chemesthesis" was introduced to stress that chemosensory irritation is a multimodal sense (Green et al., 1990).

The principal sites at which PCI effects are experienced are the eye, respiratory tract (nasal mucosa, oronasal pharynx, larynx, trachea, and bronchi), and skin. On the eye there is (depending on the concentration) local itching, discomfort, or pain, with excess lacrimation and blepharospasm as local reflexes. These effects may produce variable degrees of visual incapacitation and may also be accompanied by transient increases in intraocular pressure (Ballantyne, 1999). In the respiratory tract, and discussed in more detail later, discomfort or pain occurs in the nasal mucosa, nasal and oral pharynx, throat, larynx, and chest, together with the local reflexes of coughing, sneezing, and increased respiratory tract secretions. An increase or decrease in breathing rate and decreased tidal volume accompany these symptoms. An itching or burning sensation with local erythema usually occurs on the skin. By all routes, systemic reflexes include transient increased systolic and diastolic blood pressure with bradycardia. Incidental contamination of the oral cavity (from liquid splashes or from airborne material) may result in stinging sensation on the tongue and palate with increased salivation.

A characteristic feature produced by many PCI molecules is the development of a progressive decrease in the sensory irritant response with sequential applications of the material, a phenomenon usually referred to as tachyphylaxis (or tolerance). Also, cross-tachyphylaxis between different PCI molecules may occur with some, but by no means all, materials. When cross-tachyphylaxis develops it is possible that this may be by a similar mode of action of the responsible materials, with

stimulation of the same peripheral sensory receptor (Foster and Ramage, 1981). For example, Chang and Barrow (1986) used a depression-of-respiratory-rate model in the rat to study cross-tolerance between formaldehyde and chlorine. They showed that formaldehyde pretreatment induced a significant cross-tolerance to the PCI effects of chlorine, which was demonstrated by a shift to the right of the concentration–response curves. Babuik et al. (1985) showed that preexposure of rats to formaldehyde vapor caused cross-tolerance with acetaldehyde, as evidenced by the RD_{50} being increased three to five times, and similarly with acrolein, with which the RD_{50} increased five times. However, PCI cross-tolerance was not seen with propionaldehyde, butyraldehyde, crotonaldehyde, cyclohexanecarboxaldehyde, cyclohex-3-ene-1-carboxaldehyde, and benzaldehyde, confirming that cross-tolerance is not a generalized phenomenon. Tolerance and cross-tolerance are clearly of practical significance, because repeated exposure to a specific PCI material or preexposure to another may cause a reduced sensory warning on subsequent exposures and, hence, a correspondingly lesser degree of protection. Although the mechanism of production of tolerance is not clearly understood, it has been suggested that in some cases chemically induced histopathological changes in the nasal epithelium may cause tolerance and cross-tolerance. A detailed review of tolerance has been written by Bos et al. (1992).

13.2 PSI MOLECULE–SENSORY RECEPTOR INTERACTIONS

Many investigators consider that PCI molecules interact nonspecifically with sensory nerve receptors, and as noted above the expression "common chemical sense" was originally used to differentiate PSI function from other chemically induced sensations, such as taste or smell (Keele, 1962). Molecules that induce PCI have widely differing chemical structures and, in general, no identifiable specific morphological receptors for PCI exist. However, in skin PCI molecules show some selectivity in that they excite polymodal nociceptive receptors and warm thermoreceptors (Foster and Ramage, 1981), but the sensitivity of these receptors is divorced from their mechano- and thermoreceptive functions (Green and Tregear, 1964). The most distinct PCI effects result from stimulation of corneal and nasal mucosal sensory receptors, and in these tissues it is believed that a direct interaction occurs between PCI molecules and trigeminal nerve chemoreceptors that reside on C fibers and possibly Aδ fibers (Nielsen, 1991; Nielsen et al., 1996). The potent PSI material capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) is capable of binding with receptors on both C and Aδ fibers and desensitizing them to other PCI materials. This desensitization effect may be sustained (Bevan and Szolesanyi, 1990; Holzer, 1991; Thurauf et al., 1991). Thus, pretreatment with capsaicin can be used as a method to recognize PCI effects generated from capsaicin-sensitive nerves, and it

TABLE 13.1 The Influence of Molecular Weight on the Sensory Irritant Potential of a Homologous Series of *N*-(3′-hydroxy-4′-methoxyphenyl)-2-chloroamides Shown by the Guinea Pig Blepharospasm Test[a]

Chloroalkylamide[b]	Number of Side Chain C Atoms	EC_{50} (95% CL)[c] (*M*)
2-Chlorobutyramide	4	$5.37\,(4.27-6.75) \times 10^{-5}$
2-Chlorovaleramide	5	$1.20\,(1.05-1.66) \times 10^{-5}$
2-Chlorohexamide	6	$7.08\,(5.45-9.20) \times 10^{-6}$
2-Chloroheptamide	7	$2.83\,(2.18-3.68) \times 10^{-6}$
2-Chlorooctamide	8	$2.03\,(1.57-2.63) \times 10^{-6}$
2-Chlorononamide	9	$9.50\,(7.15-12.6) \times 10^{-6}$

[a] Data from Ballantyne (1999).

[b] Attached to 3′-hydroxy-4′-methoxyphenyl nucleus (Figure 13.1).

[c] Effective concentration to cause blepharospasm in 50% of the population studied (with 95% confidence limits).

TABLE 13.2 Depression of Respiratory Rate by 50% Produced by Vapor of Various Monoamines in Male Ssc:CF-1 or Swiss OF_1 Mice by Nasal Breathing (nRD_{50}) and Breathing through a Tracheal Cannula (tRD_{50})[a]

Amine	Formula	Molecular Weight	nRD_{50} (ppm)	tRD_{50} (ppm)	t/n[b]	log P[c]
Primary saturated aliphatic monoamines						
n-Propyl-	$CH_3CH_2CH_2NH_2$	59.12	115	500	4.35	0.58
isopropyl-	$(CH_3)_2CHNH_2$	59.12	157	489	3.11	0.43
n-Butyl-	$CH_3CH_2CH_2CH_2NH_2$	73.13	84	226	2.69	1.13
isobutyl-	$(CH_3)_2CHCH_2NH_2$	73.13	91	406	4.46	0.92
n-Pentyl-	$C_5H_{11}NH_2$	87.23	64	119	1.86	1.68
n-Hexyl-	$CH_3(CH_2)_5NH_2$	101.25	42	93	2.21	2.31
n-Heptyl-	$CH_3(CH_2)_6NH_2$	115.28	25	62	2.48	3.00
n-Octyl-	$CH_3(CH_2)_7NH_2$	129.31	17	35	2.06	3.30
tert-Octyl-	$(CH_3)_3CCH_2C(CH_3)_2NH_2$	129.31	80	96	1.20	2.19
Secondary saturated aliphatic monoamines						
Diethyl-	$(C_2H_5)_2NH$	73.13	184	549	2.98	0.58
Di-*n*-propyl-	$(CH_3CH_2CH_2)_2NH$	101.19	92	222	2.41	1.69
Di-isopropyl-	$[(CH_3)_2CH]_2NH$	101.19	161	102	0.63	1.09
Di-*n*-butyl-	$(CH_3CH_2CH_2CH_2)_2NH$	129.31	173	106	0.61	2.83
Di-isobutyl-	$[(CH_3)_2CHCH_2]_2NH$	129.31	300	289	0.96	2.67
Tertiary saturated aliphatic monoamine						
Triethyl-	$(C_2H_5)_3N$	101.19	186	691	3.72	1.45
Primary unsaturated aliphatic monoamine						
Allyl-	$CH_2{=}CHCH_2NH_2$	57.10	9	56	6.27	0.15
Secondary unsaturated aliphatic monoamine						
Diallyl-	$(CH_2{=}CHCH_2)_2NH$	97.16	4	157	39.25	1.08
Primary alicyclic monoamine						
Cyclohexl-	$C_6H_{11}NH_2$	99.18	27	78	2.87	1.54

[a] Data from Gagnaire et al. (1989, 1993) and Nielsen and Yamagiwa (1989).

[b] Ratio of tRD_{50}/nRD_{50}.

[c] Log P_{ow} (*n*-octanol-water partition coefficient).

is used as a pharmacological tool to investigate sensory irritant effects. For example, Hansen and Nielsen (1994), who studied the sensory irritant effects of *n*-propanol and ethylbenzene in mice after pretreatment with capsaicin, found that the intranasal preapplication of capsaicin decreased the sensory irritant response to these materials. They also found that pretreatment with indomethacin had no effect, indicating a direct receptor interaction rather than an indirect effect from tissue damage. Other aspects of desensitization have lead to suggestions concerning differential receptor binding. Thus, Hansen et al. (1992), using a reflexly induced decrease in breathing rate in mice as an index of PCI, found that methyl ethyl ketone at low vapor exposure concentrations caused a desensitization of the response, but little desensitization was seen at higher concentrations. In contrast, *n*-propanol desensitized the receptor at all concentrations. However, preexposure of mice to *n*-propanol did not influence the response to methyl ethyl ketone. The authors concluded that the results indicated that the two ketones bind to different receptors sites, which have different properties.

TABLE 13.3 Guinea Pig Blepharospasm Test on a Series of *N*-Phenyl-2-Chloroamides[a]

N-Phenyl Derivative	Chloroalkyl Group (X) Attached to *N*-Phenylamide[b]	EC_{50} (95% CL) ($\times 10^{-5}$ *M*)[c]
2-Chloroacetamide	$-CH_2Cl$	40.6 (30.5–54.5)
2-Chloropropionamide	$-CH(Cl)CH_3$	8.70 (6.61–11.43)
2-Chorobutyramide	$-CH(Cl)CH_2CH_3$	4.30 (3.52–5.37)
2-Chlorovaleramide	$-CH(Cl)(CH_2)_2CH_3$	3.54 (2.72–4.60)
2-Chlorohexanamide	$-CH(Cl)(CH_2)_3CH_3$	2.87 (2.18–3.77)
2-Chloroheptanamide	$-CH(Cl)(CH_2)_4CH_3$	2.68 (2.05–3.50)
2-Chloro-octanamide	$-CH(Cl)(CH_2)_5CH_3$	1.15 (0.87–1.50)
2-Chlorononamide	$-CH(Cl)(CH_2)_6CH_3$	1.07 (0.82–1.40)
2-Chlorodecanamide	$-CH(Cl)(CH_2)_7CH_3$	1.23 (0.92–1.65)

[a] Over the range of molecular weights tested the PCI effects (as EC_{50} values) were similar.
[b] Ø-NHCO-X.
[c] Effective concentration 50% (95% confidence limits).
Source: Data according to Ballantyne (1999).

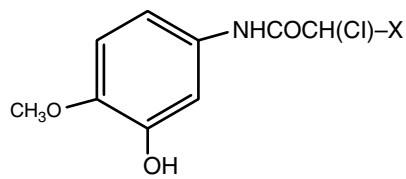


FIGURE 13.1 Nucleus for a series of *N*-(3′-hydroxy-4′-methoxyphenyl) chloroamides tested for determination of the EC_{50} by the guinea pig blepharospasm procedure (Table 13.1); X = aliphatic attachment.

Molecules with a variety of chemical structures are capable of causing PCI effects (Nielsen, 1991). For example, molecules having C=C and carbonyl groupings, in particular, in the presence of a halogen, have sensory irritant properties (Dixon and Needham, 1946). With aldehydes, the degree of irritation increases with α,β-unsaturation (Moncrief, 1944). With a known PCI material, introduction of certain groupings into that molecule may alter the sensory irritant potential, the magnitude of which depends on the specific nature of the grouping introduced. For example, based on RD_{50} measurements, compared with the sensory irritant styrene the chloro-derivative 3-chlorostyrene is 2.6 times more potent and β-nitrostyrene is 364 times more potent (Alarie, 1973). Some homologous series of chemicals may show a clear relationship between molecular weight (MW) and PCI potential. Thus, for a series of C3–C7 *n*-alkylamines Nielsen and Vingaard (1988) noted that PCI potential increased, as reflected by decreasing values for the nRD_{50}, as the chain length increased. Also, Table 13.1 indicates that for a series of *N*-(3′-hydroxy-4′-methoxyphenyl)-2-chloroamides (Figure 13.1) the PCI potential as determined by a guinea pig blepharospasm test increased (became more potent) as the C chain length increased. Less clear relationships have been shown for other series; for example, differences exist within the monoalkylamine series by RD_{50} measurements (Table 13.2). The primary saturated aliphatic monoamines show a decrease in RD_{50}

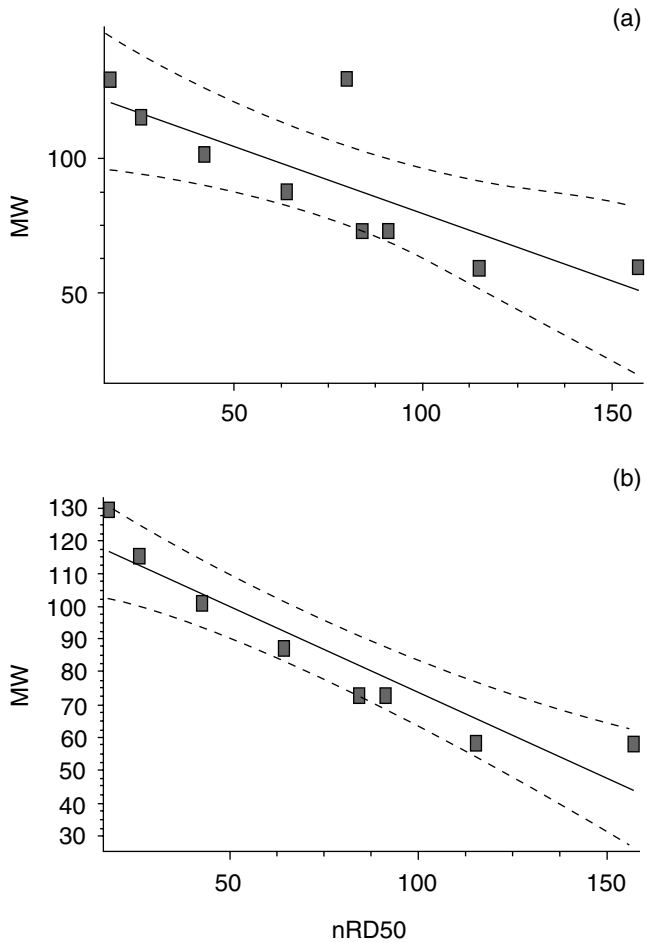


FIGURE 13.2 Linear regression analysis for assessment of the correlation between nRD_{50} in mice and molecular weight (MW) of the primary saturated aliphatic monoamines listed in Table 13.2. (a) Analysis for all nine primary amines shown in Table 13.2; $r = -0.79$; $p = 0.01$. (b) Analysis for all primary monoamines except *tert*-octylamine; $r = -0.9874$; $p = 0.0006$.

value (i.e., increased PCI potency) by nasal exposure (nRD_{50}) as the molecular weight increases (Figure 13.2). Thus, for this series, whose nRD_{50} values ranged from 17 to 157 ppm, analysis by linear regression shows a significant ($p = 0.01$) correlation between nRD_{50} and molecular weight (Figure 13.2(a); $r = -0.79$). If *tert*-octylamine is omitted from the analysis, because this compound does not fall into the generally decreasing nRD_{50} series (Table 13.2), then the association with MW is stronger (Figure 13.2(b); $r = -0.9874$, $p = 0.0006$). In contrast, the secondary saturated aliphatic monoamines do not show a statistical relationship between nRD_{50} and MW ($r = 0.4262$, $p = 0.47$). The unsaturated aliphatic amines, allylamine, and di-allylamine, were the most potent PCI materials in the series, with respective values of 9 and 4 ppm. This agreed with measured nRD_{50} values for other allyl compounds, including allyl acetate (2.9 ppm), allyl glycidyl ether (5.7 ppm), allyl alcohol (3.9 ppm), and allyl ether (5 ppm) (Gagnaire et al., 1987; Nielsen et al., 1984). However, with many homologous series MW has little influence on sensory irritant potential, as shown in Table 13.3 for guinea pig blepharospasm studies with a series of *N*-phenyl-2-chloroamides.

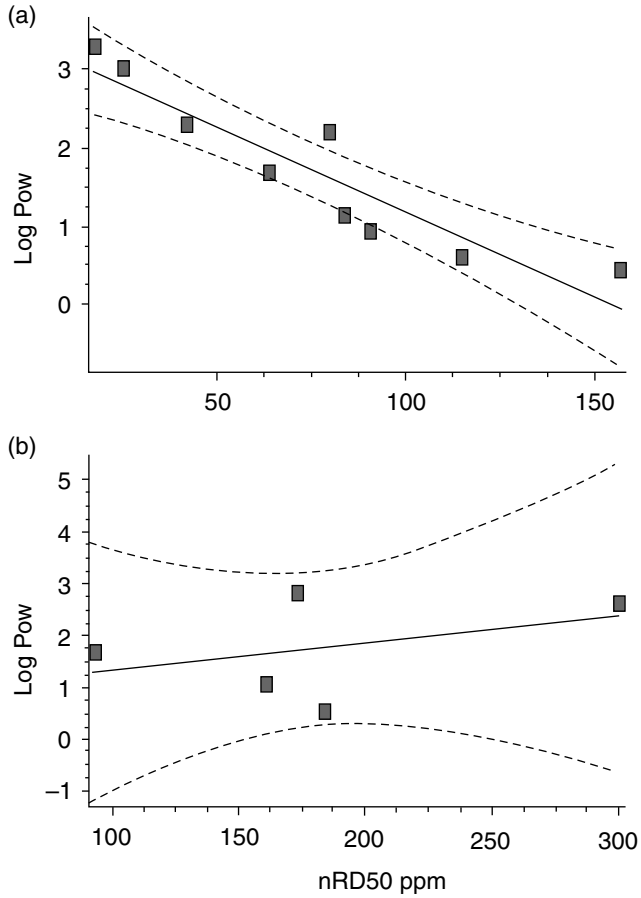


FIGURE 13.3 Linear regression analysis for assessment of the correlation between nRD_{50} in mice and log P_{ow} (octanol-water partition coefficient) of the saturated aliphatic monoamines shown in Table 13.2. (a) Analysis for all nine primary saturated aliphatic amines listed in Table 13.2 shows a strong correlation; $r = –0.9524$; $p = 0.0003$. (b) Analysis for the five secondary saturated aliphatic monoamines listed in Table 13.2 shows a poor correlation; $r = 0.3945$; $p = 0.51$.

Experimental studies have suggested that PCI molecules interact with membrane-associated binding sites and that binding affinity is a major factor in determining the potency of the PCI response (Green et al., 1979). In certain cases these effects may be associated with –SH or $–NH_2$ groups of membrane proteins or enzymes, or the amide nitrogen of a peptide bond, or both (Dixon and Needham, 1946; Silver et al., 1967; Alarie, 1973; Schauenstein et al., 1977; Douglas, 1981). However, different mechanisms probably operate for different chemical groupings. For example, the PCI potential of saturated aliphatic aldehydes decreases with their hydration constant, which may determine their degree of cross-linking with receptor proteins. In contrast, unsaturated aliphatic aldehydes such as acrolein and crotonaldehyde do not hydrate to any degree, but they undergo addition reactions with –SH, $–NH_2$, and other groups (Schauenstein et al., 1977; Steinhagen and Barrow, 1984). For higher molecular weight, less reactive aldehydes, the sensory irritant effects may be due to a physiological mechanism involving thermodynamic and solubility properties of the molecule in a lipid bilayer containing receptor proteins (Luo et al., 1983; Nielsen

and Alarie, 1982). Gagnaire et al. (1993) have demonstrated that RD_{50} values for aliphatic amines are related to their octanol-water partition coefficients, with a linear relationship between lipophilicity and sensory irritation. For the series of monoamines shown in Table 13.2, the primary saturated aliphatic compounds show a strong association between PCI potential (as nRD_{50}) and log P_{ow} (Figure 13.3(a); $r = -0.9524$, $p = 0.0003$) indicating a linear relationship between lipophilicity and PCI potency, but this does not extend to the secondary aliphatic amines (Figure 13.3(b); $r = 0.3945$, $p = 0.51$). Cometto-Muniz and Cain (1995) studied a homologous series of aliphatic alcohols, ketones, and alkylbenzenes and found that thresholds for eye irritation decreased with increasing carbon chain length, implicating lipophilicity in the irritant potency. Dudek et al. (1992) compared the Swiss–Webster mouse RD_{50} value for toluene (4,900 ppm) with those for various halogenated derivatives of toluene, which were found to be significantly more irritant (range, 4.3–27 ppm). They believed that their findings were compatible with the interaction of these PCI molecules with a receptor protein in a lipid bilayer. The trends in RD_{50} values were related to the development of a partial positive charge on the toluene α-carbon-halogen bond by the positioning of ring chlorine and also the bond dissociation energies of the α-carbon-halogen bond for the halogenated isomers of benzyl halide. Quantitative structure–activity relationship (QSAR) considerations have suggested the importance of hydrogen bonding in relation to the activation of sensory irritant receptors by nonreactive volatile organic molecules (Abraham et al., 1990; Nielsen, 1991). The effect of hydrogen bonding on upper respiratory tract irritation, assessed in detail by QSAR, demonstrated that the process involved nonreactive irritants as hydrogen bond acids; i.e., the receptor site is a hydrogen bond base. However, the range of nonreactive irritants was not large, with most hydrogen bond acid compounds being simple alcohols. To further investigate this area, Nielsen et al. (1996) studied the following three compounds with differing hydrogen bond acidities: methyl hexafluoroisopropyl ether, trifluoroethanol, and hexafluoropropan-2-ol. The respective RD_{50} values determined in CF-1 mice were ≥160,000, 110,400–23,300, and 165 ppm. QSAR showed that methyl hexafluoroisopropyl ether and trifluoroethanol behaved as predicted as nonreactive sensory irritants, whereas hexafluoropropan-2-ol was much more potent than predicted, which could result from a coupled reaction involving both strong hydrogen bonding and weak Brønsted activity. Kasanen et al. (1998) studied stereospecificity in detail.

13.3 PHYSIOLOGICAL SIGNIFICANCE OF PERIPHERAL CHEMOSENSORY IRRITATION

The significance and importance of peripheral chemosensory irritant effects for normal biological circumstances are as outlined below.

13.3.1 Biological Warning

The local sensation induced by PCI materials gives a warning of the presence of such materials in the immediate environment. They are thus likely, if of sufficient potency, to cause the affected individual(s) to seek an uncontaminated area. Because many materials causing a PCI effect also produce tissue injury (inflammation) at higher exposure concentrations, a determination of the exposure conditions leading to a PCI response can be an important factor in determining, or qualifying, what are suitable safe occupational or environmental exposure limits (Ballantyne, 1983).

Although the biological importance of a positive PCI response is clear, it is also obvious that the absence of such a response with a material capable of causing tissue injury can result in a potentially hazardous situation. Such an absence of a warning on contact can be conducive to local injury. Examples of materials that may injure because of the absence of sensory warning on contact include dimethyl sulfate and methyl bromide (Grant and Schuman, 1993). In these circumstances it may be necessary, or advantageous, to add a material with PCI properties; e.g., addition of chloropicrin to the fumigant ethyl bromide. For materials with a PCI effect, eye irritation thresholds are usually well above odor thresholds. For example, Cometto-Muñiz and Cain (1995), who studied a homologous

series of aliphatic alcohols, ketones, and alkylbenzenes, found that the ratio of eye irritation threshold to odor threshold varied from 15 with 2-propanone to 2,695 for propyl benzene. This indicates that with a known PCI substance the odor may increase the warning margin of exposure.

13.3.2 Biological Protective Mechanisms

The local reflexes induced from exposure to a PCI material are of importance as early protective mechanisms that limit further exposure. For example, lacrimation removes material from the surface of the eye, and blepharospasm restricts further access of material to the surface of the eye. With the respiratory tract, decreased breathing rate, decreased tidal volume, and cough all restrict further inhalation of irritant material.

13.3.3 Harassment and Incapacitation

Both the sensory effects (discomfort and pain) and the local reflex effects can be distracting and harassing, and thus produce variable degrees of incapacitation. For example, blepharospasm and excess lachrimation result in an impairment of vision. This may be detrimental to efficient working and also may predispose the affected individual to accidents. Therefore, PCI is an important consideration in relation to safe working conditions.

13.3.4 PCI and Pulmonary Defense Mechanisms

The effect of PCI materials on the pulmonary defense mechanisms is variable. For example, sulfur dioxide and formaldehyde at low concentrations may stimulate phagocytosis (Katz and Laskin, 1976), whereas other PCI materials may inhibit the phagocytic capacity of pulmonary macrophages. A dose-related inhibition of the normal phagocytic response of the pulmonary alveolar macrophage has been shown with cigarette smoke (Green and Carolin, 1967) and marijuana smoke (Cutting et al., 1974). In a study on the effect of dibenz[b.f]-1,4-oxazepine (CR) and ten of its derivatives on the phagocytosis of rabbit pulmonary alveolar macrophages toward *Staphylococcus epidermis,* it was found that CR was the most potent inhibitor, and the 1- and 2-chloro derivatives were the least potent (Hogg et al., 1983). Whereas inhibition of phagocytosis was dose dependent, the degree of inhibition was not correlated with either the nature or the position of the substituent groups on the CR molecule. Also, no discernable relationship existed between the inhibition of phagocytosis and the sensory irritancy of the compounds tested.

13.4 FACTORS INFLUENCING THE PCI RESPONSE

Numerous endogenous and exogenous factors may influence the concentration required to induce a PCI response, its subjective severity, and its latency and duration. Some of the more important of these are discussed below.

13.4.1 Concentration of PCI

With progressively increasing concentrations, no PCI effects are produced until a certain concentration (the threshold) is attained. At suprathreshold concentrations, the exposure concentration is a determinant of the following.

a. The proportion of the exposed population that respond: the higher the concentration, the larger the proportion of the population that is affected. On a linear basis, in general, there is a typical biological sigmoid curve (with proportionate values; Figure 13.4) or a Gaussian curve (with absolute data).
b. The latency to onset of PSI effects: the higher the concentration, the shorter the time to onset of effects (Figure 13.5).

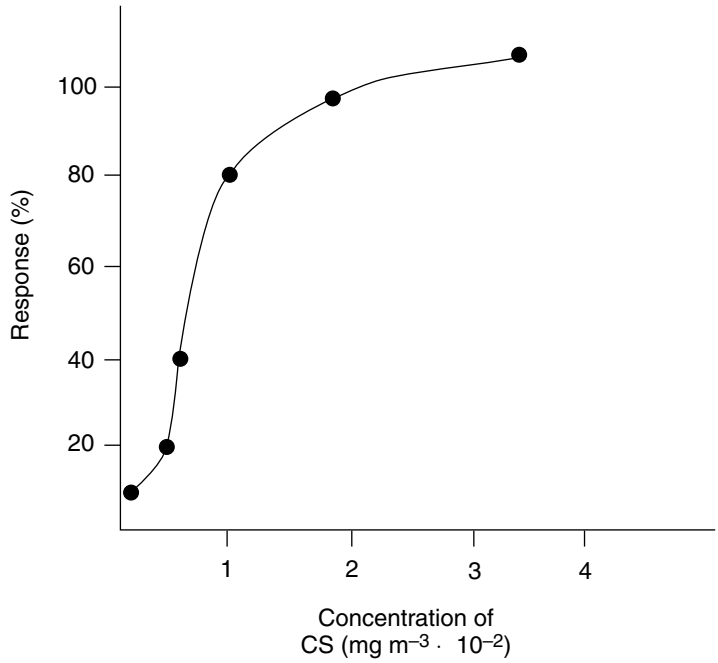


FIGURE 13.4 Sigmoid concentration–response curve of the threshold irritant response for sensation induced in the human eye by an aerosol of 2-chlorobenzylidene malononitrile (CS). The percent response is the proportion of the population responding for each concentration tested. By probit analysis, the TC_{50} (with 95% confidence limits) is 4.0 (2.3–6.6) × 10^{-3} mg/m^3.

c. The duration of the sensory irritant effect, in particular, after a brief (pulsed) exposure. The higher the concentration, in general, the longer is the persistence of the effect (Figure 13.5).
d. The subjective experience of the response. For example, the higher the concentration of PCI applied to the eye the more marked is the induced blepharospasm (intermittent to sustained) and the more severe is the induced pain.

13.4.2 Particle Size

For PCI materials in particulate form, size may be an important determinant of both the severity and site of the response. For example, small particles of respirable dimensions rapidly produce both ocular and respiratory PCI effects, whereas larger particles produce predominantly ocular irritation with prolonged recovery. For example, Owens and Punte (1963) showed this by using particles of *o*-chlorobenzylidene malononitrile having respective sizes of 1- and 60-μm MMAD.

13.4.3 Motivation and Distraction

Both increased motivation and distractions can raise the threshold for the induction of PCI effects and enhance tolerance for suprathreshold sensory irritancy. For example, in the workplace the concentration at which sensory discomfort becomes apparent with a particular material may be significantly higher than that causing threshold effects with the same material tested under carefully controlled laboratory conditions. It thus follows that the quantitative values obtained for sensory irritant effects in experimental situations probably represent the most sensitive measurements for the effects and thus give the most reliable and safest estimates on which to base occupational exposure guidelines.

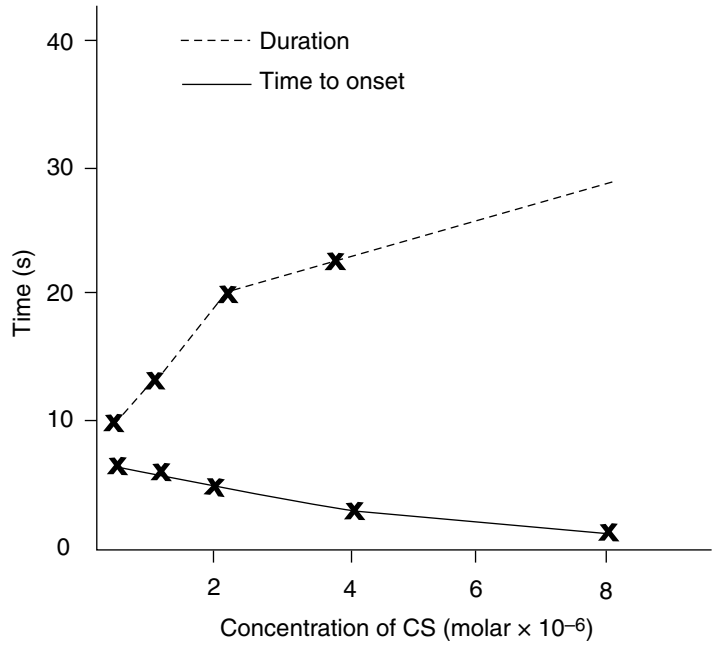


FIGURE 13.5 Influence of the concentration of a solution of the PCI, 2-chlorobenzylidene malononitrile (CS), on the latency to onset (solid line) and duration (interrupted line) of human eye discomfort. As the concentration of CS applied to the eye increases, latency decreases and the duration of the induced effect is prolonged. (Data according to Ballantyne and Swanston, 1973a.)

13.4.4 Tolerance

Individuals may develop tolerance to a PCI material if exposure is gradual and at low concentrations; the individual adapts by a decrease in the apparent feeling of discomfort and harassing effects (Beswick et al., 1972). The development of tolerance is clearly of importance in the workplace, because an adaptation to a warning of potentially harmful chemicals may lead to a decrease in safe working conditions, in particular, if carefully derived occupational exposure limits are not strictly adhered to.

13.4.5 Personality

There are significant personal and psychological factors in tolerance to pain, and a similar variability exists between individuals with respect to the discomfort produced by PCI materials. Thus, under a given set of circumstances some individuals will tolerate the discomfort more readily than others. This also is a factor to be considered when assessing the variability to sensory irritants in the workplace. Variation in the perceived irritation from a chemical can result from sensory factors that limit the quantity or quality of information available to process and cognitive factors that influence how the information is evaluated. Assessing the degree to which differences in cognitive states can produce differences in the ratings of irritancy appears to be critical when evaluating the perceived sensory and health impact on humans following exposure to chemicals in occupational and residential environments (Dalton et al., 1997).

13.4.6 Personal Habits and State of Health

Smoking, medication, and certain diseases may affect susceptibility to PCI materials. For example, in a study of worker sensitivity to sodium borate dust, Woskie et al. (1998) found that those who

smoked, used nasal sprays, or had allergies or colds were more sensitive to sensory irritant effects, and Dunn et al. (1982) showed that smokers were less sensitive to nasal CO_2 irritation.

13.4.7 Temperature and Humidity

It has been found that increases in environmental temperature and humidity may decrease endurance to peripheral sensory irritation (Punte et al., 1963).

13.4.8 Vehicle

With sensory irritants in solution the vehicle used to dissolve or suspend the active PCI substance may modify its irritant potential. Surface-active vehicles, for example, can facilitate penetration and enhance the PCI response, as shown by a lowering of the concentration necessary to induce both threshold and incapacitating effects.

13.5 QUANTITATION AND INTERPRETATION OF PERIPHERAL SENSORY IRRITANT OBSERVATIONS

As noted above, a clear, positive relationship usually exists between the exposure concentration for a PCI material and the proportion of the exposed population responding, and this is sigmoid or Gaussian in form (Figure 13.4). This indicates that although most of the exposed population respond over a well-defined region about the median, a small proportion (at the left-hand side of the curve) is hyperreactive to sensory irritant stimulation and a small proportion (to the right) is hyporeactive. For ease of presentation and calculations the concentration–response data are usually converted to a linear configuration by log-probit plot. It is conventional to calculate a 50% response level, with 95% confidence limits (CL), to allow statistical comparison of the relative potency of the PCI response under defined conditions. This should also include a determination of the slope on the concentration–response regression line to obtain the best possible estimate of comparative sensory irritant potency. A variety of values, discussed below, are calculated to allow the sensory irritant response to be quantified.

For effects that can be objectively evaluated it is common to refer to the effective concentration (EC); i.e., the exposure concentration that is effective in producing the specific effect under consideration. When sufficient data exist it is common to calculate the sensory irritant potency as the EC_{50}; i.e., the concentration, calculated from the exposure concentration–response data, which results in a specific irritant response in 50% of the exposed population under the particular conditions of the observations. Examples of effects that may be quantified and expressed as an EC_{50} include depression of breathing rate in animals, blepharospasm in experimental animals and human volunteers, and subjectively assessed discomfort (e.g., pain). Some effects are referred to by specific expressions; e.g., a 50% depression in breathing rate is usually referred to as the RD_{50}. For subjective evaluations in humans, such as the degree of discomfort, it is also possible to determine and cite 50% response levels; e.g., the threshold concentration 50% (TC_{50}) and, above-threshold concentrations, if the degree of discomfort is severe, the incapacitating (intolerable) concentration 50% (IC_{50}) can be obtained. In some situations it may be useful to calculate different levels of incapacitation; e.g., IC_{90}.

As with the proportion of the population responding to various exposure concentrations of a PCI challenge, variability also occurs between individuals in the latency to effect and in the duration of effect. Therefore a complete description of a PCI response requires information on the exposure concentration, proportionate response, latency, and duration of response. Figure 13.6 (left-hand graph) shows the relationship between the exposure concentration and exposure time to produce different degrees of incapacitation in a particular group of human subjects. For any given exposure concentration, the proportion of the population responding increases with exposure time, reflecting variable latency within the group. Also, and a further indication of variability, within limits a given degree of incapacitation can be produced by various reciprocally related exposure times and exposure concentrations. The reciprocal relationship between exposure time and concentration to

produce a given degree of incapacitation also has implications for the exposure dosage (Ct) received. The right-hand graph in Figure 13.6 shows that for any given level of incapacitation the exposure dosages are greater for the high concentration–short exposure duration conditions than for the lower concentration–more prolonged conditions. Thus, although increasing the exposure concentration shortens latency to response, it also results in a greater exposure dosage to produce a given equipotent incapacitating effect.

Ideally, data derived from the use of animal models should indicate the range of concentrations over which PCI effects may be expected to occur in humans. However, and as discussed later, most animal models are less sensitive than the human to many PCI materials. In general, animal models will detect those materials having a potential to produce PSI effects in humans but normally do not rank different materials in their order of potency for the human. Also, they do not accurately predict the concentration range over which effects may be anticipated in humans.

In examining the relative potency of different PCI materials it is important not only to compare the EC_{50} and 95% CL, but also to take into account the slope of the regression line. For example, if two different PCI materials have significantly different EC_{50} values and the slopes of the regression lines on the dose–response lines are essentially parallel to each other then it may be concluded that the materials are significantly different with respect to PCI potency over a wide exposure concentration range (Figure 13.7). In contrast, even with materials of closely comparable EC_{50} values, if

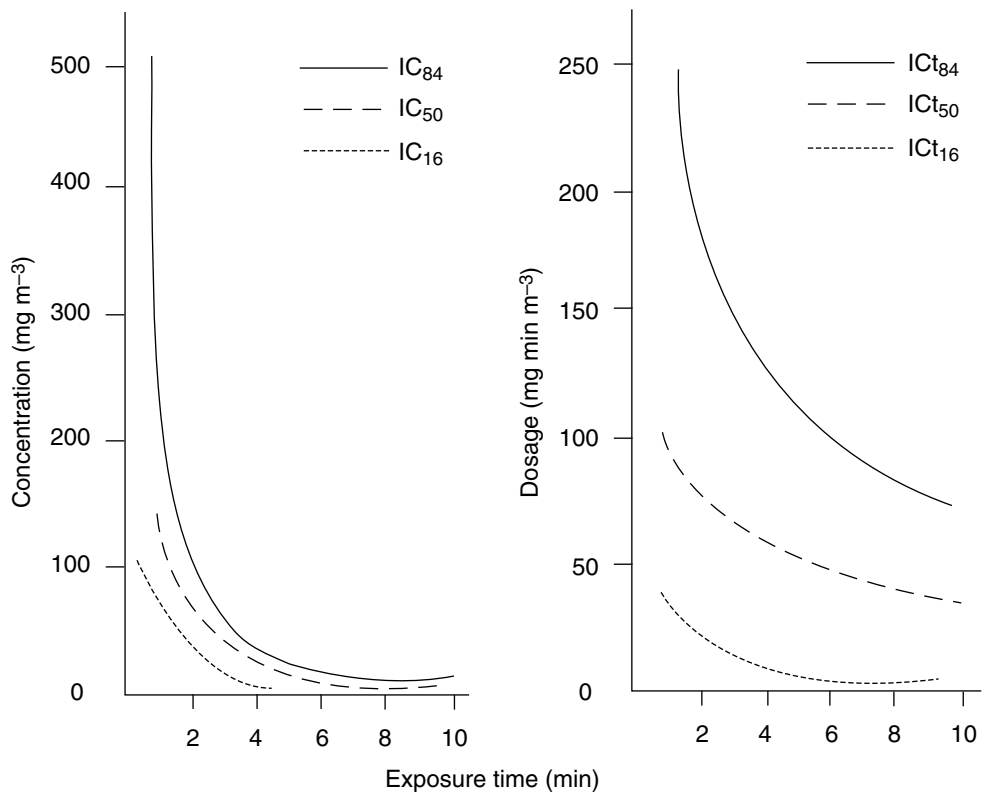


FIGURE 13.6 Graphs showing the relationship between the atmospheric concentration of the PCI ω-chloroacetophenone (CN) and the exposure time (left-hand graph) and the exposure dosage (Ct) of CN and time (right-hand graph) required to produce defined levels of incapacitation. The incapacitating concentrations (IC) and incapacitating doses (ICt) are plotted as a function of the time required to cause intolerable effects in 16, 50, and 84% of the population studied. (Data according to McNamara et al., 1968.)

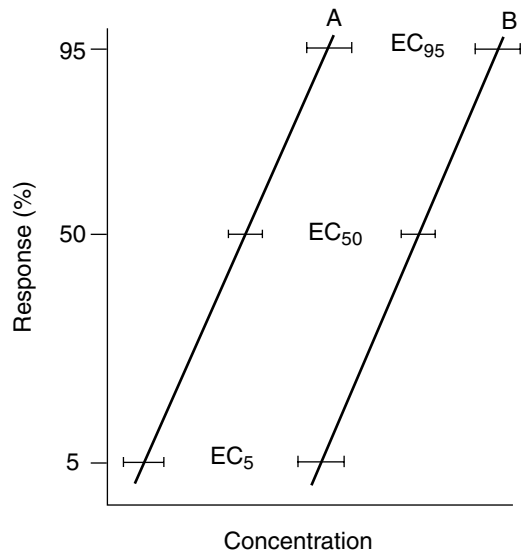


FIGURE 13.7 Comparison of two PCI materials (A and B) having significantly different EC_{50} values but similar slopes on the concentration–response regression lines. Because of the parallel nature of the two regressions lines, the EC_5 and EC_{95} values are also significantly different for materials A and B. Thus, A and B are significantly different from each other with respect to PCI potency over a wide concentration range.

the slopes are different this may have important practical relevance with respect to proportionate population response. This is illustrated in Figure 13.8 for two materials having identical EC_{50} values but differing slopes of the regression lines. Because of the differing slopes, the values of the EC for the two materials differ significantly from each other at both high-response (e.g., EC_{95}) and low-response (e.g., EC_5) levels. It follows that for the material having the steeper slope, once the threshold concentration is exceeded then only a small incremental increase in exposure concentration is required to cause an effect in the majority of the population. Thus, in such workplace situations it may be necessary to have a wider margin of safety below the relatively narrow concentration range required to convert a threshold to an incapacitating effect. In contrast, with the material having the shallower slope the concentration range necessary to convert a small response rate in the population to a large response rate will be much greater. However, in these circumstances it may be necessary to consider the measures necessary to protect the small proportion (EC_5) that is hypersensitive.

When differences in slopes exist there are two major implications with respect to comparative evaluations. First, the relative potency of two materials will not be the same at different levels of response. Second, it may not be statistically valid to calculate a potency ratio. However, in such circumstances it may be of value to calculate the comparative potencies (CP) at different levels of irritancy. This is defined as follows (Ballantyne, 1977):

$$\mathrm{CP}x = [A] \text{ causing } (x) \text{ response}/[B] \text{ causing } (x) \text{ response}$$

where $[A]$ and $[B]$ are concentrations of PCI materials A and B, and (x) is a specific response rate.

It is often useful to compare the threshold and incapacitating concentrations for individual materials, e.g., IC_{50}/TC_{50}. This effectiveness ratio can be used to indicate if the threshold value gives an adequate

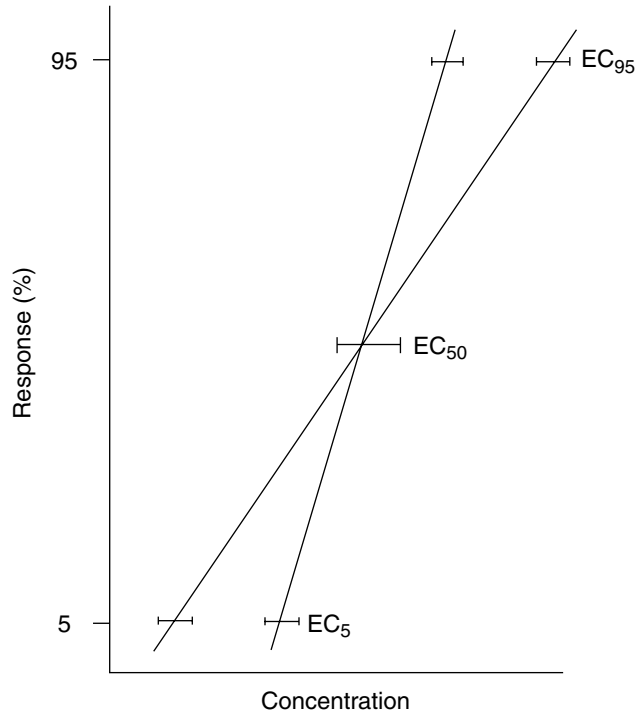


FIGURE 13.8 Comparison of two PCI materials having the same EC_{50} values but differing slopes on the concentration–response regression lines. This results in the EC_5 and EC_{95} values being significantly different from each other. This has implications for hyperreactive individuals in the group with the shallow slope and safety margins for the group with the steeper slope (see text).

warning of potentially incapacitating exposure; the greater the value of the ratio, the more effective is the warning of the threshold effect. The value can also be used to indicate how many times the threshold concentration has to be increased to result in an incapacitating effect. Table 13.4 gives an example of values for the comparative potencies by respiratory exposure to aerosols of *o*-chlorobenzylidene malononitrile (CS) and dibenz[b.f]-1,4-oxazepine (CR), and the effectiveness ratios for the individual materials. It can be seen that as the concentration increases the comparative potency of CR is greater than that for CS; thus the aerosol concentration of CS required to attain a TC_{50} is twice that for CR, for an IC_{50} is 5.1 times, and for an IC_{75} response is 9.1 times. Also, there is a wide margin between the TC_{50} and IC_{50} for CS and CR, indicating a significant PCI warning effect at the TC_{50}. However, the increase in concentration required to convert threshold to incapacitating effects is greater with CS ($\times$900) than with CR ($\times$350), indicating the latter to have a greater PCI potency. A similar situation applies with the IC_{75}, but the proportionate increases required are even greater with CS.

13.6 METHODS FOR ASSESSING SENSORY IRRITANT POTENTIAL

Non-animal methods for determining the potential for substances to produce PCI effects have included QSAR considerations and chemical models. For example, Abraham et al. (1996, 1998) developed a QSAR equation for nasal pungency in subjects with anosmia caused by volatile organic compounds. The equation described sensory potency in terms of interaction via electron pairs, dipolarity/polarizability, hydrogen bond acidity and basicity, and hydrophobicity. Based on correlations obtained between the results of the potency of nonreactive airborne chemicals as PCI materials

Table 13.4 Comparative Potencies (CP) and Effectiveness Ratios (ER) for Aerosols of *o*-Chlorobenzylidene Malononitrile (CS) and Dibenz[b.f]-1,4-Oxazepine (CR) by Respiratory Exposure of Male Human Volunteer Subjects[a]

Material	Degree of Irritancy (mg m^{-3})[b]			ER	
	TC_{50}	IC_{50}[c]	IC_{75}[d]	IC_{50}/TC_{50}	IC_{75}/TC_{50}
CS	4.0×10^{-3}	3.6	10.0	900	2,500
CR	2.0×10^{-3}	0.7	1.1	350	550
CP	2.0	5.1	9.1		

[a] Data according to after Ballantyne (1977).
[b] TC_{50} = threshold concentration 50% for sensation (1-min exposure).
[c] IC_{50} = incapacitating concentration 50% (1-min exposure).
[d] IC_{75} = incapacitating concentration 75% (1-min exposure).

(RD_{50} measurements) and several of their physicochemical properties, including vapor pressure and Ostwald gas-liquid partition coefficients, an alternative for bioassays was presented with physicochemical variables such as those to estimate RD_{50} values for nonreactive volatile organic compounds (Alarie et al., 1995). Biological approaches include the use of *in vitro* and *in vivo* biological models, and the controlled exposure of human volunteer subjects. Some of these, with their relative advantages and disadvantages, are considered below.

13.6.1 Chemical Models

Some correlations have been made between PCI potential and certain physicochemical characteristics, although this has been only for a limited series of materials, and comparisons between irritant and nonirritant materials are sparse. In an effort to predict what materials are likely to have PCI properties, various artificial membranes have been devised to simulate biological membranes. These have included monolayers and bilayers of lecithin, with monitoring of phase transitions to measure interaction with a PCI. Approaches used to investigate transitions have included differential scanning calorimetry, electron spin resonance, and X-ray diffraction. In general, the use of such chemical membrane simulants has not been a reliable predictor for PCI effects.

13.6.2 Animal Biological Models

Several biological models, both *in vivo* and *in vitro* and of varying degrees of sophistication and sensitivity, have been used to qualitatively or quantitatively assess the PCI potential of various classes of chemicals. These include the following.

13.6.2.1 Isolated Intestinal Segment

This is a nonspecific method based on the use of segments of small intestine, which are suspended in an incubating bath in a manner that allows the contraction of the segment to be measured mechanically. The PCI substance being tested is added to the bath at varying concentrations, and a record is kept of those concentrations causing contraction of the intestinal segment.

13.6.2.2 Frog Flexor Reflex (FFR)

This approach, also nonspecific, involves a determination of the minimum concentration of irritant that causes withdrawal of the hind limb of decerebrate frogs from test solutions in an incubating bath. The method involves the sequential immersion of the hind-limb preparation in increasing

concentrations of PCI in solution. After each immersion, the limb is washed with saline, and after a period of 5 min the limb is immersed in the next solution. The time (T) between immersion of the hind limb and its reflex withdrawal from the solution is recorded. The log-molar concentration of PCI is plotted against T and the curve extrapolated to infinite time by the use of a template of a standard curve to permit the minimum irritant concentration (T_m) to be determined. The mean minimum irritant concentration from six frog preparations ($\check{T}_m$) is used for comparison between different materials. The method is essentially *in vitro,* simple, economic, and easy to conduct, and some have used it as a reliable screening test for detection of materials having a PCI potential (Ballantyne, 1999; Feniak, 1966). However, with some materials the FFR test shows poor reproducibility and tachyphylaxis may develop. Although the FFR test is relatively insensitive compared with other methods, such as the *in vivo* guinea pig blepharospasm and mouse depression of respiration (RD_{50}) tests (Table 13.5), the procedure correlates well ($r = 0.99$) with the guinea pig blepharospasm test (Table 13.6), but is less well correlated with the mouse RD_{50} ($r = 0.76$) (Table 13.7).

13.6.2.3 Neurophysiological Preparations

Several peripheral nerves known to contain afferent nerves carrying PCI-induced action potentials from skin or mucosal surfaces have been used to detect materials having sensory irritant properties. Measurement of afferent nerve activity has been used with the following neurophysiological preparations.

a. Ciliary nerve recording following corneal stimulation. This is frequently undertaken using an excised cat eye mounted in a warm chamber, with the cornea exposed, and the attached long ciliary nerve laid over recording electrodes (Green and Tregear, 1964). Test sensory discharge can be induced by light tactile stimuli or cooling the surface. The respective thresholds are 0.1–1.0 g and 5–20ºC (Green and Tregear, 1964).
b. Nasopalatine, ethmoidal and sphenoidal nerve recording following nasal mucosal stimulation. Such studies have been carried out with success in the rat (Cooper, 1970; Kulle and Cooper, 1975). For example, Tsubone and Kawahi (1991) used the ethmoidal nerve to record afferent impulses during exposure of the rat to various irritant gases. They found that formaldehyde and acrolein stimulated nasal mucosal sensory endings at about 1.0 ppm, with acetaldehyde producing a significantly lesser degree of stimulation. The concentrations producing a 50% increase in nerve activity were 1.8 ppm for formaldehyde, 1.2 ppm for acrolein, and 908 ppm for acetaldehyde.

TABLE 13.5 Comparison of the Relative Sensitivity of the Frog Flexor Reflex (FFR), Guinea Pig Blepharospasm (GPB), and Mouse Respiratory Rate Depression (RRD) Tests for the Assessment of PCI[a]

Compound[b]	FFR $\check{T}_m$ (M)[c]	GPB EC_{50} (M)[d]	RRD RD_{50} (M)[e]
CS	9.78×10^{-6}	2.16×10^{-5}	6.00×10^{-8}
CR	1.10×10^{-4}	9.00×10^{-5}	3.40×10^{-7}
HMPC	2.14×10^{-6}	7.70×10^{-7}	3.82×10^{-8}

[a] Data according to Ballantyne (1999).
[b] CS = *o*-chlorobenzylidene malononitrile; CR = dibenz[b.f]-1,4-oxazepine; HMPC = *N*-(4′-hydroxy-3′-methoxyphenyl)-2-chloro-octanamide.
[c] $\check{T}_m$ = mean minimum irritant concentration.
[d] EC_{50} = effective concentration 50%.
[e] RD_{50} = depression of respiration 50%.

TABLE 13.6 Comparative PSI Potential for Various Chemicals Measured by the Frog Flexor Reflex (FFR) and Guinea Pig Blepharospasm (GPB) Tests[a]

Compound	FFR $\check{T}_m$ (*M*)[b]	GPB EC_{50} (*M*)[c]
N-(4′-Hydroxy-3′-methoxyphenyl) 2-chlorodecanamide	1.64×10^{-6}	3.58×10^{-7}
N-(3′-Hydroxy-4′-methoxyphenyl) 2-chloroheptanamide	1.89×10^{-6}	2.83×10^{-6}
N-(3′-Hydroxy-4′-methoxyphenyl) 2-chlorononanamide	1.97×10^{-6}	9.50×10^{-7}
N-Nonanoylvanillylamide	2.00×10^{-6}	3.30×10^{-7}
N-(4′-Hydroxy-3′-methoxyphenyl) 2-chlorononamide	2.10×10^{-6}	3.17×10^{-7}
N-(4′-Hydroxy-3′-methoxyphenyl) 2-chloro-octanamide	2.14×10^{-6}	7.69×10^{-7}
N-(3′-Hydroxy-4′-methoxyphenyl) 2-chloro-octanamide	3.32×10^{-6}	2.03×10^{-6}
N-(4′-Hydroxy-3′-methoxyphenyl) 2-chloroheptanamide	5.23×10^{-6}	1.90×10^{-6}
o-Chlorobenzylidene malononitrile	9.78×10^{-6}	2.16×10^{-5}
Dibenz[b.f]-1,4-oxazepine	2.09×10^{-5}	3.48×10^{-5}
4-Methyldibenzoxazepine	2.20×10^{-6}	9.30×10^{-5}
3-Methyldibenzoxaxepine	4.16×10^{-5}	5.40×10^{-5}
β,β-Diacetyl-3-chlorostyrene	9.43×10^{-5}	1.30×10^{-4}
ω-Chloroacetophenone	1.0×10^{-4}	9.00×10^{-5}
Phenanthrine	1.77×10^{-3}	4.11×10^{-3}

[a] Data according to Ballantyne (1999).
[b] $\check{T}_m$ = mean minimum irritant concentration.
[c] EC_{50} = effective concentration 50%; Correlation coefficient (r) = 0.99 $p < 0.001$.

TABLE 13.7 Comparative PSI Potential for Various Chemical Classes Measured by the Frog Flexor Reflex (FFR) and Mouse Respiratory Rate Depression (RRD) Tests[a]

Compound	FFR $\check{T}_m$ (*M*)$_b$	RRD RD_{50} (*M*)[c]
o-Chlorobenzylidene malononitrile	9.78×10^{-6}	6.00×10^{-8}
Dibenz[b.f]-1,4-oxazepine	2.09×10^{-6}	2.30×10^{-7}
ω-Chloroacetophenone	1.10×10^{-4}	3.40×10^{-7}
N-(4′-hydroxy-3′-methoxyphenyl) 2-chloro-octanamide	2.14×10^{-6}	3.82×10^{-8}
N-(4′-hydroxy-3′-methoxyphenyl) 2-chlorononamide	2.10×10^{-4}	1.43×10^{-7}
N-(4′-hydroxy-3′-methoxyphenyl) 2-chlorodecanamide	1.64×10^{-6}	2.16×10^{-7}

[a] Data according to Ballantyne (1999).
[b] $\check{T}_m$ = mean minimum irritant concentration.
[c] RD_{50} = respiratory rate depression 50%.

c. Laryngeal nerve recording has been conducted after PCI stimulation of the laryngeal mucosa. It is usual to conduct the study in anesthetized and tracheotomized rats, with action potentials recorded from the recurrent laryngeal nerve. Unlike the corneal (ciliary nerve) preparation, the laryngeal model is more complex and slowly adapting (Dirnhuber et al., 1965).
d. For cutaneous application of a PSI material, a convenient model is by topical application of a PCI to the hind limb, with recording of afferent action potentials from the saphenous nerve (Foster and Ramage, 1981).

13.6.2.4 Blepharospasm Test

The induction of blepharospasm in conscious animals in response to topical application of a PCI to the cornea, or from exposure to atmospherically dispersed material, is a very frequently used approach to assess the PCI potential on the cornea. It is a simple and reliable *in vivo* method that can also be performed in most species, including human subjects. For solutions, the test involves applying increasing concentrations to the surface of the cornea and noting the proportion of the test population that develop blepharospasm following the initial blink reflex. In this way, the response rate can be calculated and an EC_{50} for blepharospasm calculated. If the test substance is in solution, or has to be diluted, it is important that an inert solvent be used. For materials dispersed in the atmosphere similar observations are employed to sequential increasing concentrations of the test substance.

There is a species variation in the sensitivity of the blepharospasm test for the same substances applied to the eye (Table 13.8). In general, the guinea pig is the most suitable test species because of cost, size, and sensitivity to PCI materials. It is reproducible, free from tachyphylaxis, and differentiates PCI molecules having closely chemically similar structures. However, it does not correlate well with the depression of breathing rate test in the mouse ($r = 0.68$; Table 13.9).

In addition to detecting chemicals having PCI potential, the guinea pig blepharospasm (GPB) test has found application in various drug applications. For example, it has been successfully used as a screen for the development of comfortable ophthalmic drug preparations. Bar-Ilan (1997) compared results from a GPB test with the number of human subjects who noted pain or discomfort on applying various concentrations (2.5–17.5%) of sulfacetamide. The dose–response curves for the guinea pigs and humans were essentially identical, with a threshold at 5% sulfacetamide and a linear increase up to a maximum at 12.5–15.0%. The good relationship between the GPB test and the human nociceptive response with sulfacetamide indicated the usefulness of the procedure for predicting the degree of ocular discomfort in humans.

13.6.2.5 Depression of Breathing Rate

Inhalation of PCI materials by laboratory animals results in a decrease in breathing rate, which is reflexly induced by stimulation of trigeminal sensory receptors in the nasal mucosa. The decrease in breathing rate is caused by a pause in the expiratory phase of the breathing cycle (expiratory bradypnea), resulting in a characteristic notching pattern (Figure 13.9). The approach basically involves the head-only exposure of animals to various concentrations of test material and recording of the breathing rate by means of a plethysmograph. By using a minimum of four animals, the mean proportionate decrease in breathing rate for a given concentration of PCI is calculated. Up to a limiting value, there is usually a relation between the increase in concentration (C) of the test substance inhaled and the resultant decrease (R) in breathing rate (Figure 13.10). Because C and C/R are linearly related,

TABLE 13.8 Comparison of the Blepharospasm-Inducing Effects in Various Species of Solutions (in Polyethylene Glycol 300) of 2-Chlorobenzylidene Malononitrile (CS) and Dibenz[b.f]-1, 4-Oxazepine (CR) Applied Topically to the Cornea[a]

Material	Blepharospasm as EC_{50} with 95% CL (M)[b]		
	Guinea Pig	Rabbit	Human
CS	$2.2\ (1.9–2.4) \times 10^{-5}$	$5.9\ (3.8–10.0) \times 10^{-5}$	$3.2\ (2.1–6.1) \times 10^{-6}$
CR	$3.5\ (2.8–4.3) \times 10^{-5}$	$7.9\ (5.1–12.5) \times 10^{-5}$	$8.6\ (6.8–12.5) \times 10^{-7}$

[a] Data according to Ballantyne and Swanston (1973a, 1973b).

[b] Results as effective concentration (EC) 50% with 95% confidence limits.

TABLE 13.9 Comparison of the PSI Potential for Various Classes of Compounds as Assessed by the Guinea Pig Blepharospasm (GPB) and Mouse Depression of Respiratory Rate (DRR) Tests[a]

Compound	DRR RD_{50} (*M*)[b]	GPB EC_{50} (*M*)[c]
N-Undec-10-enoyl-4-hydroxy-3-methoxy benzylamine	2.04×10^{-8}	1.55×10^{-7}
cis-N-(4-*cyclo*-Hexylmethyl)-*cyclo*-hexylacetamide	2.95×10^{-8}	8.83×10^{-7}
N-(4′-Hydroxy-3′-methoxyphenyl) 2-chloroheptanamide	3.82×10^{-8}	7.69×10^{-7}
2-Chloro-3,4-dimethoxy-ω-nitrostyrene	7.10×10^{-8}	9.26×10^{-6}
N-(4′-Hydroxy-3′-methoxyphenyl) 2-chlorononamide	1.43×10^{-7}	3.17×10^{-7}
N-(4′-Hydroxy-3′-methoxyphenyl) chlorodecanamide	2.16×10^{-7}	3.58×10^{-7}
Dibenz[b.f]-1,4-oxazepine	2.30×10^{-7}	3.48×10^{-5}
N-Phenyl-2-chloro-octanamide	3.08×10^{-7}	1.15×10^{-5}
ω-Chloroacetophenone	3.40×10^{-7}	9.00×10^{-5}

[a] Data after Ballantyne (1999).

[b] Depression of respiratory rate 50%.

[c] Effective concentration 50%. Correlation coefficient (r) = 0.68; p = 0.06.

a least-squares regression between the two can be undertaken and the concentration causing a 50% depression in the breathing rate (RD_{50}) can be calculated. To ensure that the most sensitive response is obtained, preliminary studies are needed to determine that the exposure is long enough to obtain the maximum decrease in breathing rate at the exposure concentrations. This may vary with different chemicals. For example, with glutaraldehyde vapor a plateau for decrease in breathing rate was obtained within 5–10 min (Figure 13.11), and with methyl isocyanate within 10–15 min (Ferguson et al., 1986). If the exposure time is not sufficiently long (i.e., a plateau is not reached), then a misleadingly high value may be obtained; e.g., with 1,6-hexamethylene di-isocyanate the 10-min RD_{50} is 0.96 ppm and the 120-min RD_{50} is 0.22 ppm; for toluene di-isocyanate the 10-min RD_{50} is 0.81 ppm and the 120-min value is 0.25 ppm (Sangha et al., 1981).

Species variations in sensitivity may occur, and the mouse is the most frequently used test animal. It has also been shown that stain variations may occur, and a factor of 10 has been demonstrated between the least sensitive and the most sensitive strains of mice (Alarie et al., 1980). Swiss–Webster mice are used most frequently, although no significant differences in sensitivity have been shown between Swiss–Webster and $B6C3F_1$ mice (Steinhagen and Barrow, 1984). The rat is not regarded as being an appropriate species by some investigators (Babiuk et al., 1985). To permit head-only exposure of the test species, and to simultaneously record the breathing movements, it is necessary to use a whole-body plethysmograph with a neck restraint made of rubber. Studies involving multiple exposures have suggested that this method could result in pituitary gland lesions (Kaempfe and Dudek, 1994). Subsequent detailed studies with ND-4 Swiss–Webster mice given single sham and test material (dust) exposures did show pituitary lesions with both the sham (7 of 7) and test material (52 of 80) groups (Werley et al., 1996). Histology revealed pituitary gland hemorrhages. The authors concluded that the lesion is produced by increased pressure in the blood supply to the pituitary gland but believe that the lesion (as an artifact of methodology) should have little effect on the usefulness of the procedure as a screening test. Details of the RD_{50} investigation have been published elsewhere (Ballantyne et al., 1977), and comprehensive lists of measured RD_{50} values have been published (Alarie, 1981a, 1981b; Bos et al., 1992; Schaper, 1993).

For any substance, there is a difference in the concentration causing a typical *in vivo* PCI response by stimulation of receptors in the nasal mucosa and the concentration that is required to stimulate pulmonary receptors. In most cases, and in the intact conscious animal, the nasal trigeminal afferent

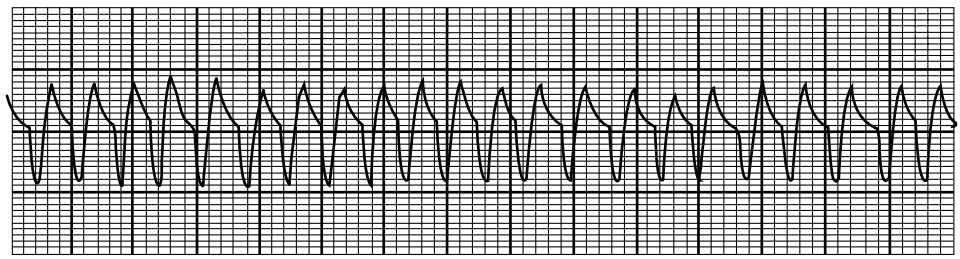


FIGURE 13.9 The effect of glutaraldehyde vapor on the breathing cycle of the mouse. The respiratory cycle shows a prolongation of the expiratory phase which produces a characteristic notching.

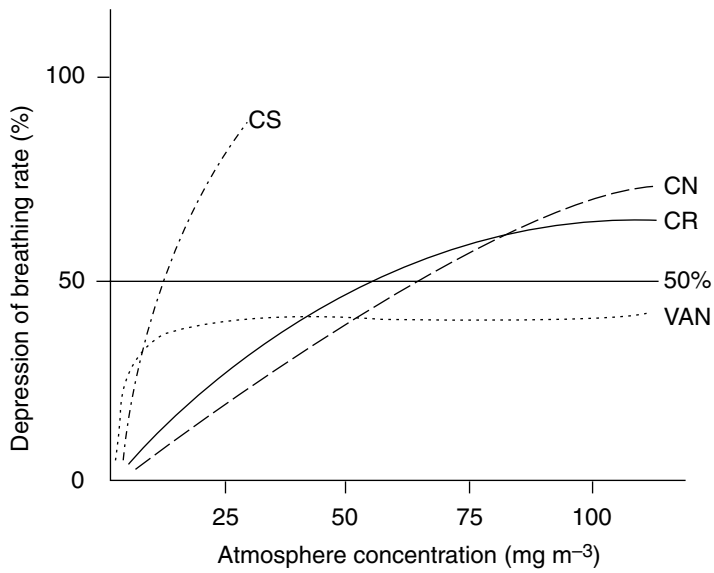


FIGURE 13.10 Relationship between depression of breathing rate in the conscious mouse and the airborne concentration of several PCI materials; CS = 2-chlorobenzylidene malononitrile; CN = ω-chloroacetophenone; CR = dibenz[b.f]-1,4-oxazepine; VAN = *N*-nonanoylvanillylamide.

response will predominate. It follows that a determination of the difference between the concentrations causing nasal and pulmonary irritation could be used as an index of the margin of warning for potential lung injury. This has been studied by using intact mice to determine the PCI response from trigeminal stimulation and comparing the outcome with that resulting from exposure of tracheal-cannulated mice to measure the pulmonary response (Alarie, 1981c). The mouse, unlike other species, develops a decrease in breathing rate in response to pulmonary receptor stimulation due to the development of a pause between the end of expiration and the start of the subsequent inspiration. The duration of this pause increases with exposure concentration. The decrease in breathing rate and calculation of the tRD_{50} are performed as for the nasal RD_{50}. The ratio of the RD_{50} values in the tracheal cannulated and intact mouse (tRD_{50}/RD_{50}) can be used as the warning margin produced by the intact PCI response. The closer the ratio is to unity, the closer is the concentration to cause pulmonary irritation to that causing nasal trigeminal stimulation. Examples are shown in Table 13.2 for several monoamines. It can be seen that only one primary saturated aliphatic monoamine, *tert*-octylamine, is close

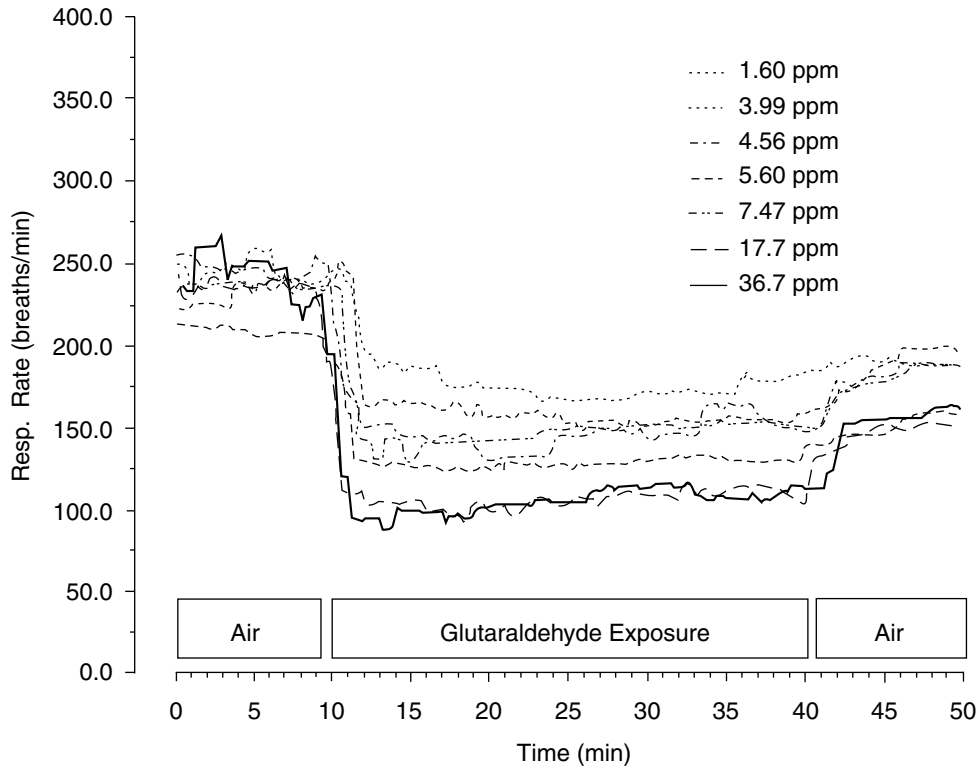


FIGURE 13.11 Mean time–response curves for breathing rate changes in male ND4 Swiss–Webster mice exposed for 10 min to air, followed by a 30-min exposure to various concentrations of glutaraldehyde vapor, and then again exposed to air.

to the nRD_{50} and tRD_{50} values, with a nRD_{50}/tRD_{50} (t/n) ratio of 1.2. However, three secondary saturated aliphatic monoamines have tRD_{50}/nRD_{50} ratios less than 1.0; namely, di-isopropylamine (0.63), di-*n*-butylamine (0.61), and di-isobutylamine (0.96), indicating that occupational exposure guidelines for these materials need to be strictly controlled. Methods that may be used for a determination of concentrations of irritant-causing structural and/or functional lung injury for a direct comparison with the intact (nasal) RD_{50} value include ultrastructural changes, alterations in lung function (including resistance) (Matijak-Schaper et al., 1983), detection of pulmonary edema by a sensitive method based on the intravenous injection ^{51}Cr-ethylenediamine tetraacetate followed by lung lavage (Valentini et al., 1983), and measurement of protein and lactate dehydrogenase in lung lavage fluid.

13.6.3 Studies in Human Volunteer Subjects

The animal studies briefly discussed above, in general, allow the detection of substances that can produce PCI effects. They do not, however, allow for the assessment of subjective effects such as discomfort or pain that are experienced in human subjects. In view of this, and because the human may be more sensitive to some PCI materials than is estimated in the animal models, it may be desirable to conduct PCI tests in volunteer human subjects. These studies not only allow an assessment of subjective responses but, when appropriately planned, allow a quantitation of the PCI response at threshold and suprathreshold levels and permit a determination of the group variability and the influence of environmental conditions. Additionally, the physiological and potential medical effects of exposure can be monitored simultaneously; e.g., systemic reflexes (such as blood pressure, heart rate). These tests should, of course, be only conducted in subjects who have been fully informed of the nature

and conduct of the tests, are aware of any possible discomfort and risks, have been informed that they may withdraw from the study at any time, and not be attracted by possible overcompensation if the volunteers are to be paid. The toxicology and pharmacology of the test substance should be fully investigated, the potential adverse effects should be fully understood, and any possible side effects should carry the minimum of risks for the subjects. The protocol should be reviewed and approved by an appropriate independent Institutional Review Board (Ballantyne, 2005).

13.6.3.1 Cutaneous Exposure

Various concentrations of material can be applied to the skin, and the subjective degree of discomfort assessed. Measurement of chemosensory irritation of the skin has been discussed in detail by Green (2000) and Green and Bluth (1995). It should be remembered that there are anatomical regional variations in the sensitivity of the skin to PCI materials (Ballantyne et al., 1973).

Because the epidermis presents a barrier to the access of PCI materials to subepidermal sensory endings, a method was devised to overcome this and obtain a comparative evaluation of different substances. This is the blister base technique and involves the production of a blister on the forearm by covering the area of the skin with tape having perforations at regular intervals, and to which is applied a paste of 0.2% cantharidin in kaolin. The tape and paste are removed after 6 h and the blisters allowed to develop overnight. Currently, this technique seems to have been used little or not at all.

13.6.3.2 Eye Irritation

Human ocular chemoreceptors are located mainly in the central corneal area (Coe and Douglas, 1984). As with animal *in vivo* models, in the human it is possible to determine the concentration of PCI that causes blepharospasm. However, in addition in the human it is possible to determine the degree of local discomfort or pain produced at various concentrations and to calculate concentrations that are thresholds for sensation and also incapacitating (e.g., TC_{50} and IC_{50}). PCI effects of solutions on the eye are relatively easy to determine by carefully applying measured small volumes of such solutions to the surface of the cornea (Ballantyne and Swanston, 1973a, 1973b). For vapors and gases, tests may be conducted in a chamber with the subject wearing oronasal respiratory protective equipment or in a chamber with specially constructed eye ports (Bender et al., 1983). Also, specially designed goggles are available for localized exposures of the eye to prevent simultaneously exposing the respiratory tract (Kjaegaard, 1992; Hempel-Jørgensen et al., 1996, 1999). Some examples of EC_{50} values for blepharospasm are shown for humans and compared with animal results in Table 13.8; values for subjectively assessed discomfort (TC_{50}) in humans are shown in Table 13.10. These values show that, for the species studied, the blepharospasm-inducing effects of the test materials were greater in the human; i.e., the human is more sensitive. Also, sensory discomfort was a more sensitive indication of exposure to a PCI than was blepharospasm.

An interesting and potentially valuable technique described by Kjaergaard et al. (1990) for measuring what they described as sensory irritation of the eye was the photographic measurement of changes in conjunctival redness before and after exposure to an airborne irritant. It is a sensitive and reproducible approach. Using a series of volatile organic compounds, Hempel-Jorgensen et al. (1998) found that the degree of conjunctival hyperemia measured in human volunteer subjects was positively related to the exposure concentration. This noninvasive approach may be a useful approach for studies on the irritating potential of environmental pollutants. It has been used, for example, to study exposure to formaldehyde, *n*-decane, tobacco dust, and birch pollen (Bach et al., 1988; Kjaergaard and Pederson, 1989; Kjaergaard et al., 1989, 1990).

13.6.3.3 Respiratory Tract Exposure

For exposure to airborne PCI materials it is usual to determine the concentration causing discomfort within the respiratory tract. Depending on the reason for conducting the study this may be nasal

TABLE 13.10 Threshold Concentrations for Solutions of *o*-Chlorobenzylidene Malononitrile (CS) and Dibenz[b.f]-1,4-Oxazepine (CR) to Produce Sensations in the Human Eye[a]

Material	TC_{50} (*M*)[b]
CS	$7.3\ (4.2–11.2) \times 10^{-7}$
CR	$4.9\ (3.8–6.5) \times 10^{-7}$

[a] Data after Ballantyne and Swanston (1973a, 1973b).
[b] Threshold concentration 50%, with 95% confidence limits.

irritation, cough, or chest discomfort. Nasal irritation may be experienced principally as uncomfortable sensations and stinging (nasal pungency). Exposures may be conducted in various ways, most frequently by using whole-body chambers of sufficient size to house the volunteers comfortably with any required monitoring equipment. In some cases, and when available, the use of a wind tunnel may offer a more convenient approach to control and alter the concentration of test material. To avoid complications of interpretation due to ocular irritation, either comfortable airtight goggles may be worn, or the test material can be delivered by a mask. The latter approach, however, may have psychological disadvantages with some subjects. Comparative measurement of nasal pungency in those with normal olfaction and anosmics suggests that the presence of intact olfaction may decrease the threshold for nasal pungency (Cometto-Muñiz and Cain, 1990). They also found that eye irritation thresholds were very close to nasal pungency thresholds measured in anosmics (Cometto-Muñiz and Cain, 1995), and suggested that eye irritation data may serve as a basis for assessing potency for induction of nasal pungency; i.e., nasal trigeminal threshold. Eye irritation thresholds generally do not differ significantly between normosmics and anosmics of similar age, gender, and smoking status (Cometto-Muñiz and Cain, 1998). In this respect it is of interest to note that human pungency thresholds correlate well with some RD_{50} values in animal studies (Cometto-Muñiz and Cain, 1994).

Techniques to evaluate and differentially compare both olfactory and chemosensory irritant thresholds have been described. One of these depends on the observation that if a volatile compound is sniffed into one nostril, and simultaneously air is sniffed into the contralateral nostril, then only if the material is a PCI (stimulating the nasal trigeminal nerve receptors) can it be readily identified which nostril is being stimulated; olfaction by itself does not permit this spatial discrimination (Dalton et al., 2000; Roscher et al., 1996; Wysocki et al., 1992). Nasal localization thresholds in normosmics, in general, are approximately equivalent to nasal pungency thresholds in anosmics, and the threshold for nasal localization thus offers a suitable way to measure nasal irritation in normosmic subjects (Cometto-Muñiz and Cain, 1998). This lateralization, or monorhinal stimulation, technique was used by Wysocki et al. (1997) to study the odor and sensory irritant threshold for acetone. In acetone-exposed workers (36,699 ppm) they found the lateralization thresholds were elevated compared with a control group not occupationally exposed (15,758 ppm), indicating that acetone is a weak sensory irritant and that adaptation is an important factor in the response. Olfactory adaptation was also noted (855 compared with 41 ppm).

13.6.3.4 Total Body Exposure

With unprotected workers in the occupational environment, PCI materials in the atmosphere may contact skin, eye, and respiratory tract and thus produce sensory irritant responses in one or all of these sites. Hence, for such situations it is most meaningful for studies to be conducted by whole-body

exposures, simulating the workplace. These studies can indicate if any particular tissue is more susceptible to the PCI potential of an airborne material and thus would likely be the determinant for sensitivity as a warning effect. Appropriately planned, these studies can give significant amounts of information. For example, Lunquist et al. (1992) investigated the effects on human volunteer subjects of diethylamine vapor that was increased in atmospheric concentration from 0 to 12 ppm over a 1-h period (average concentration was 10 ppm). A moderate to strong olfactory response with distinct subjective nasal and ocular irritation was noted. Some, but not all, subjects showed an adaptation. Nasal irritation appeared to be a slightly more sensitive index, and a significant correlation existed between nasal and ocular irritation ($r = 0.87$; $p < 0.001$). To study acute physiological changes in the nasal mucosa, they exposed adult male volunteers to 25 ppm diethylamine vapor in a chamber for 15 min. Changes in nasal volume and nasal resistance were measured by acoustic rhinometry and rhinomanometry. No reaction occurred for either acute nasal volume or resistance, indicating that these procedures are not useful measures for the detection PCI effects at lower (near threshold) concentrations.

13.7 CHEMOSENSATION BY THE RESPIRATORY TRACT ROUTE

Human subjects inhaling PCI materials will experience discomfort or pain in the nasal mucosa, nasopharynx, throat, larynx, and chest, with local reflexes of coughing, sneezing, rhinorrhea, increased respiratory tract secretions, changes in breathing rate, and decreased tidal volume. The clinical presentation varies somewhat based on the chemistry of the PCI material, its solubility, and the principal site of receptor activation (Table 13.11). A feature characteristic of respiratory tract chemosensory responses is that they simulate the effects of afferent cholinergic fiber stimulation in the nasal mucosa, leading to reductions in the breathing rate and tidal volume (Cauna et al., 1969; James and Daly, 1969; Ulrich et al., 1972). Various sensory receptors are present in the lower respiratory tract (Crofton and Douglas, 1981; Widdicombe, 1981). With respect to sensitivity to inhaled chemoirritant materials, the most important appear to be J receptors and airways irritant receptors (Kristiansen et al., 1986, 1988; Nielsen and Vinngaard, 1988). Stimulation of J receptors results in apnea, rapid shallow breathing, and systemic hypertension with bradycardia. Stimulation of airways irritant receptors causes hyperpnea and bronchoconstriction (Douglas, 1981). The overall effect of stimulation of lower respiratory tract receptors is, in most species, an increase in breathing rate and tidal volume (Table 13.11). However, with most PCI materials, effects are produced at lower concentrations by trigeminal nerve stimulation than occurs with lower respiratory tract receptor stimulation. Therefore with a biologically effective PCI challenge by respiratory route exposure, the trigeminal reflex will usually predominate. These respiratory regional differences in chemoreceptor

TABLE 13.11 Effects Produced by Peripheral Chemosensory Irritant Materials on the Respiratory Tract Irritant Receptors

Location	Effect
Nasal mucosa	Decreased breathing rate
	Decreased tidal volume
Lung	Increased breathing rat[a]
	Decreased tidal volume
	Bronchospasm

[a] A few species have decreased breathing rate (e.g., mouse).

sensitivity form the basis for the (tRD_{50}), described earlier, concerned with assessing the margin of warning for respiratory tract injury provided by the PCI response.

13.8 PRACTICAL APPLICATIONS AND IMPLICATIONS OF PERIPHERAL CHEMOSENSORY IRRITATION

The biological effects of PCI are of applied relevance in the following situations.

13.8.1 Occupational Exposure and Protection

As noted above, both the local sensory and reflex effects resulting from the PSI response may be detrimental to safe and efficient working conditions and in many cases may be a basis for assigning airborne occupational exposure limits, which are discussed in detail below.

13.8.2 Harassing Agents

The use of PCI materials for warning purposes with other materials not giving a warning on contact has been mentioned previously. More frequent is the use of certain PCI materials for use in peacekeeping operations (riot control agents). By the very nature of their intended usage, riot control agents produce marked PSI effects, which result in hindering the conduct of unlawful activities and causing malefactors to leave the contaminated area. These aspects of PSI have been discussed in detail elsewhere (Ballantyne, 1977; Salem, et al., 2005).

13.8.3 Combustion Products

Many materials of widely differing chemistry are produced during the processes of combustion, the nature of which depends on the circumstances of the fire. Some of these may produce PCI effects; e.g., hydrogen chloride, sulfur dioxide, isocyanates. The resultant harassing or incapacitating effects may impede escape from a fire. This may be additive to other factors that also hinder escape from a fire; e.g., obstacles, physical injury, hypoxia, and absorbed products that produce a disturbance of consciousness, such as carbon monoxide, hydrogen cyanide, and volatile organic solvents (Ballantyne, 1981; Norris and Ballantyne, 1999). Sensory irritation caused by thermal decomposition products has been investigated by RD_{50} measurements (Barrow et al., 1978).

13.8.4 Environmental Considerations

Many airborne chemicals in the environment can evoke chemosensory irritant effects. Perceived irritation, particularly in the indoor environment, is a common symptom and resembles the effects experienced in the "sick building syndrome" (SBS) or in multiple chemical sensitivity (MCS) (Koren et al., 1992; Otto et al., 1992; Dalton et al., 1997; Anderson and Anderson, 1999; Sparks, 1999). There are several syndromes, including SBS and MCS, that are classified together by some as idiopathic environmental intolerance (IEI) (Sparks, 1999) and that are characterized by irritation of the face (burning sensation), sore eyes and throat, difficulty with breathing (including asthma), often (particularly with MCS) neurological problems including confusion, fatigue, difficulty with concentration, and poor memory. The considerable overlap of symptoms related to peripheral sensory irritation, pulmonary irritation, and airflow limitation and to the various types of IEI has lead to proposals that common air pollutant mixtures (occupational, domestic, and environmental) can cause these various syndromes. The term IEI has been preferred by organizations such as the World Health Organization and the International Labour Organization because "sensitivity" in a description implies an immunological response, the relation between environmental exposure and symptomatology is as yet totally unproven, and the underlying pathophysiological mechanisms and criteria for diagnosis yet are to be fully defined. However, it is established that air samples taken from domestic sites associated with repeated human complaints of poor air quality have caused sensory irritation, pulmonary irritation,

and airflow limitation in mice (Anderson and Anderson, 1997a, 1997b, 1998, 1999). Also, behavioral changes have been detected by functional observation battery studies in mice after exposure to product emissions or air from sites where individuals have made complaints. Findings of these types suggest that many symptoms of SBS and MCS could be the result of exposure to airborne irritant chemicals such as those liberated by common commercial products and found in polluted air. For example, Kjaergaard et al. (1989) found that *n*-decane caused a concentration-dependent irritation of mucosae and increased the sensation of odor intensity. They showed that even at small concentrations, *n*-decane caused symptoms similar to those of SBS. Where many relatively nonreactive chemicals are present in the atmosphere at concentrations below their respective PCI thresholds, their combined effect could induce irritation in mucosae (Cometto-Muñiz et al., 1997). Anderson and Anderson (1999) have noted that repeated exposures (two or three 1-h exposures over 24 h) of mice to certain airborne mixtures of chemicals (e.g., emissions of a solid air freshener) yielded the same degree of sensory irritation, whereas repeat exposure to other mixtures (e.g., emissions of some fabric softeners, disposable diapers, and vinyl mattress covers) resulted in increasing sensory irritant responses (2- to 4-fold). Thus, in the latter cases the intensity of each exposure was constant but the magnitude of the sensory irritant response was increased, leading the authors to conclude that the sensitivity of the mice to the chemical mixtures had changed. The response is specific to certain mixtures of chemicals.

The individuals who complain of the symptoms of IEI are a heterogeneous group, and more than one mechanism may be operative in different cases (NIEHS, 1997). The suggested pathogenesis has included exposure to an airborne mixture of materials having PCI properties, as discussed above, and to preexisting or concurrent psychiatric illness, sensitivity to odors, or combinations of these factors. However, the role of exposure to airborne materials having PCI properties, notably in mixtures, offers a possible major etiologic avenue, for which there is some laboratory support, and that deserves further exploration. IEI has increasingly impacted the overall burden of chronic disability, and deserves further future medical and scientific investigation and evaluation.

13.9 PERIPHERAL CHEMOSENSORY IRRITATION AS A FACTOR IN DETERMINING OCCUPATIONAL (WORKPLACE) EXPOSURE GUIDELINES

13.9.1 General Considerations on Occupational Exposure Guidelines

Occupational exposure guidelines (OEGs) are usually recommended on the basis of providing protection from potential adverse effects of overexposure for a working lifetime of exposure. Depending on the nature of the major toxic process determining the quantitative value of the guideline, they may be set as permitted average exposure values or may be qualified to further restrict exposure potential. Thus, the threshold limit values (TLVs) recommended by the American Conference of Governmental Industrial Hygienists (ACGIH, 2005) may be set as a time-weighted average exposure values to be permitted over an 8-h work day (time-weighted average over 8 h for a 40-h workweek; TWA_8). Additional considerations may lead to a qualification that:

1. The recommended guideline is a set value that may not be exceeded during any part of the working period, a Ceiling (C) value, or
2. The TWA is qualified (supplemented) by an additional concentration, to which it is believed that workers can be exposed for a short period without developing irritation, narcotic effects, or chronic or irreversible tissue damage, and providing that the daily TWA_8 is not exceeded. This qualification is the short-term exposure limit (STEL) and is usually set for a 15-min period that should not be occurring for more than four times a working day, with a period of at least 60 min between successive STEL exposures.

TLVs are based on credible information derived from *in vitro* and *in vivo* toxicology laboratory investigations, controlled human studies, in-use occupational medical studies and experience, and epidemiological studies. The values are usually recommended on the basis of the most sensitive index of adverse effects that may occur in exposed workers. These may sometimes be toxic effects causing an impairment of health of variable concern and resulting from either acute or repeated exposure; examples include carcinogenic effects, nononcogenic chronic health effects, neurological effects, reproductive effects, developmental toxicity, immune-mediated effects including asthma, and other organ-specific or enzyme-specific pathology. In other cases the effects may have a physiological or pharmacological basis, such as PCI or narcosis, both of which are detrimental to efficient working conditions and may result in accidents due to impairment of vision or locomotion or from distraction. In yet other cases, a qualified TLV may be based on the potential for differing adverse effects from the same material. In coming to conclusions about the most appropriate value for a TLV, in addition to the particular chemical-related adverse effect(s), it is necessary to take into account how other workplace or personal factors may influence the expression of the adverse effect and how a hypersusceptible proportion of the potentially exposed worker population would react; from these considerations the TLV is appropriately modified. These additional considerations will include, for example, the possible influence of age, sex, smoking habits, genetic factors, and general state of health. For many adverse effects resulting from repeated exposure, the TWA-TLV provides the most satisfactory and convenient method for controlling workplace exposure. These values should take account of both the inhaled dose of material and, where relevant and when data are available, the possible percutaneous absorption of the chemical that in some cases may make a significant contribution to the systemic toxicity from airborne gases, vapors, or mists. Information on the possible relevance and quantitative contribution from percutaneous absorption can be derived from repeated cutaneous contact and percutaneous toxicokinetic studies in laboratory animals, or from exposed volunteer human subjects by wipe sampling and measurement of parent material or metabolite in sequential blood samples (Leung and Paustenbach, 1994; McDougal et al., 1990). Excursions permitted on the TWA_8 depend on a number of factors, including the nature of the workplace, the duration of the excursion periods, and whether higher concentrations produce acute toxicity. TWA excursions are allowed above the TLV if they are compensated for by equivalent excursions below the TLV-TWA during the workday. The magnitude of the excursions is not usually definable with any degree of precision based on toxicological data. However, Leidel et al. (1975) reviewed numerous industrial hygiene surveys conducted by the National Institute of Occupational Safety and Health (NIOSH) and found that short-term exposure measurements were, in general, log normally distributed with geometric standard deviations generally in the range of 1.5 to 2.0. If short-term exposure values in a given situation have a geometric standard deviation of 2.0, then 5% of all values will exceed 3.13 times the geometric mean. This forms the basis for the excursions permissible on TLV-TWA values that do not have a qualifying STEL (ACGIH, 2003); i.e., excursions may exceed three times the TLV-TWA for no more than a total of 30 min during a workday and under no circumstances should they exceed five times the TWA, provided that the TLV-TWA is not exceeded. The STEL supplements the TWA where acute effects from a material whose more serious effects are primarily of a long-term or chronic nature are recognized; a STEL is usually only assigned where toxic effects have been established from high-concentration, short-term exposures. The Ceiling (C) value is appropriate for acute effects (principally PCI), usually occurring at low concentrations, which have a demonstrable threshold that is below the exposure concentration (NOAEL) which may result in cumulative or long-term toxicity. A variety of other, mainly environmental, exposure guidelines exist which are designed to protect individuals who may be incidentally exposed from workplace or transport releases.

Other countries have different approaches and terminologies for their own national exposure limits for the workplace. In the United Kingdom, for example, under the Control of Substances Harmful to Health (COSHH) Regulations, the Health and Safety Executive (on advice from an independent Advisory Committee on Toxic Substances, ACTS) set occupational exposure limits (OELs). COSHH defines two types of OELs—the occupational exposure standard (OES) and the

maximum exposure limit (MEL). OESs are set for materials for which it is possible to identify a concentration at which there are no significant risks to health, and MELs are set for materials that have serious implications for health (notably carcinogens and asthmagens), and for which an OES cannot be assigned (Topping, 2001).

13.9.2 Chemosensory Irritation in the Assignment of Occupational Exposure Guidelines

Because PCI materials produce distracting, harassing, and/or incapacitating effects, which are not conducive to efficient working conditions and may predispose to accidents, this is the basis for assigning OEGs to many materials producing chemosensory irritation. This is particularly valuable when PCI effects occur at concentrations lower than those resulting in toxicity by acute or repeated exposure, because sensory irritant effects then give a warning of potential overexposure. About 40% of ACGIH TLVs are set on the basis of chemosensory irritation. Because a threshold exists for a PCI response and the slope on the exposure concentration–response data varies between different materials, a TWA_8 is, by itself, inappropriate for workplace protection. Therefore either a *C* value is recommended for a material having a potent PCI effect as the main biological effect, or if a TWA_8 is recommended based on other toxicity this can be qualified by a STEL.

For a limited number of substances, quantitative information is available on the chemosensory irritant potential in humans, which allows a reasonable estimate for threshold and incapacitating effects and permits a reliable OEG to be assigned. Where data are available attention should be paid to the threshold and incapacitating concentrations, slopes of the exposure concentration-response relationship, and effectiveness ratios. Also, with both human and animal-derived data, consideration should be given as to how the following may affect the OEG: development of tolerance, environmental conditions, nature of the workplace, type of occupation, and the physical characteristics and pattern of the workplace exposure.

In the absence of reliable human data, or else to support and confirm the human data, PCI studies in animals have been widely used. Most frequently employed have been nRD_{50} studies in mice. Those who recommend the value of nRD_{50} studies have supported their case by comparing nRD_{50} values with TLVs for known irritants that have been assigned by ACGIH or other recognized agencies (Barrow et al., 1977; Alarie et al., 1980; Kane et al., 1980; de Ceaurriz et al., 1981; Alarie, 1984; Steinhagen and Barrow, 1984; Nielsen and Yamagiwa, 1989; Schaper, 1993). Predictive conversion methods that have been proposed include the following:

1. The TLV should be between 0.01 nRD_{50}, where it is predicted that there will either be no sensory irritation or it will be threshold, and 0.1 nRD_{50}, which is likely to be an uncomfortable but tolerable concentration (Barrow et al., 1977; de Ceaurriz et al., 1981). At the nRD_{50} the predicted response in humans is intolerable or incapacitating.
2. Based on a good correlation between the then available TLVs, 0.03 nRD_{50} was recommended for establishing a TLV based on chemosensory irritation (Alarie, 1981b; Schaper, 1993).
3. Because at an airborne concentration of 0.1 nRD_{50} it is anticipated that humans will experience slight discomfort, this should be the highest exposure concentration, and form the basis of a STEL to qualify a TWA_8-TLV defined on the basis of 0.03 nRD_{50} (Gagnaire et al., 1994).
4. Where data are available from tracheal-cannulated animals, and the tRD_{50} is close to, or less than the nRD_{50}, because of the prediction of pulmonary irritation, somewhat greater caution is recommended with the TLV set at 0.01 tRD_{50} (Weyel et al., 1982; Weyel and Schaffer, 1985; Nielsen, 1991).

The need for a safety margin between the measured nRD_{50} and the recommended OEG is supported by the observation that respiratory tract histopathological inflammatory lesions may be induced at

the nRD_{50} concentration, For example, Buckley et al. (1984) found that with ten sensory irritants of widely differing mouse nRD_{50} values, short-term repeated exposures at the RD_{50} caused inflammatory lesions in the respiratory tract. The materials studied (with nRD_{50} values) were 2,4-toluene diisocyanate (0.4 ppm), acrolein (1.7 ppm), formaldehyde (3.1 ppm), chloropicrin (8.0 ppm), sulfur dioxide (117 ppm), ammonia (303 ppm), hydrogen chloride (309 ppm), dimethylamine (511 ppm) and epichlorohydrin (687 ppm). After exposure of mice for 6 h a day for 5 days, all irritants produced lesions in the nasal mucosa with an anterior-posterior severity gradient. The lesions ranged from slight epithelial hypertrophy or hyperplasia to epithelial erosions, ulceration with variable subepithelial inflammatory cell infiltration. Only chlorine, chloropicrin, and epichlorohydrin caused lesions in the lower respiratory tract.

Some illustrative examples of the use of measured RD_{50} values in mice to derive OEGs are as follows. Rydzynski and Jedrychowski (1944) established an nRD_{50} of 5.9 (1.3–13.0; 95% CL; slope, 1.37) mg m^{-3} for cyanuric chloride, indicating the material to be a potent PCI, and suggested an OEG of 0.17 mg m^{-3} for the substance (0.03 nRD_{50}). The respiratory PCI potential of chlorine and of nitrogen trichloride were studied by mouse plethysmography by Gagnaire et al. (1994), who found respective nRD_{50} values of 3.5 and 2.5 ppm. Based on 0.03 and 0.1 of the nRD_{50} they proposed TWA_8 (and STEL) values of 0.1 (0.5) ppm for chlorine and 0.1 (0.3) ppm for nitrogen trichloride. Werley et al. (1995) determined the nRD_{50} for glutaraldehyde vapor in ND4 Swiss–Webster mice was 13.86 ppm (95% CL, 9.86–23.58 ppm), which is in the range of nRD_{50} values measured for other aliphatic aldehydes (Bos et al., 1992). With a correlation factor of 0.03 nRD_{50}, this suggests an OEG of 0.42 ppm, which is close to the threshold irritancy concentration of 0.3 ppm found for exposed human volunteer subjects (Ballantyne and Jordan, 2001). The current ACGIH TLV for glutaraldehyde of 0.05 ppm is a *C* value; although in part based on sensory irritation, the value is mainly recommended because of a suggested potential (yet not totally proven) for respiratory sensitization by glutaraldehyde (ACGIH, 2005). Steinhagen et al. (1982) found the nRD_{50} value for dimethylamine vapor to be 573 ppm in male Fischer 344 rats and 511 ppm in male Swiss–Webster mice. Use of the nRD_{50} value from the mouse, and the range of 0.01–0.1 RD_{50} as a guideline interval in which the OEG should be set, suggested an OEG between 5 and 51 ppm; the current TLV for dimethylamine is 5 ppm with a STEL of 15 ppm (ACGIH, 2003). The next higher alkylamine, diethylamine, has the following mouse RD_{50} values (Nielsen and Yamagiwa, 1989). A nRD_{50} value of 550 mg m^{-3} [(184 ppm); i.e., approximately one-third that of dimethylamine] and a tRD_{50} of 1650 mg m^{-3}. These would suggest TLV values of 16.5 mg m^{-3} (0.03 RD_{50}) and 5.5 mg m^{-3} (0.01 tRD_{50}), respectively. It is relevant to note that a 1-h exposure of human volunteer subjects to an average concentration of 30 mg m^{-3} of diethylamine vapor caused distinct nasal and eye sensory irritation. The current ACGIH TWA_8 for diethylamine TWA is 5 ppm (15 mg m^{-3}) with a STEL of 15 ppm (45 mg m^{-3}) (ACGIH, 2005). Di-isopropylamine is a secondary saturated monoamine whose tRD_{50} (102 ppm) is lower than the nRD_{50} (161 ppm) with a t/n ratio of 0.63, indicating a potential for lung injury at concentrations below those giving a typical nasal PCI warning (Table 13.2). Therefore an OEG of 0.01 tRD_{50} would be most appropriate; i.e., 1 ppm. However, the current ACGIH TLV is set at a TWA_8 of 5 ppm (ACGIH, 2005), a value that is more appropriate for one derived from the nRD_{50} (x 0.03 = 4.83 ppm).

RD_{50} values for aerosols can be readily measured in mice. For example, Ballantyne et al. (1994) exposed mice, nose-only, for 30 min to triethylene glycol aerosols of MMAD of 2.45–3.10 μm. Maximum decreases in respiratory rate occurred at 15–25 minutes of the start of exposure and were sustained. The nRD_{50} was calculated to be 5.14 mg l^{-1}, from which it is suggested that an appropriate OEG ($0.0RD_{50}$) would be 150 mg m^{-3}.

A comparison of isocyanates provides an instructive example of the use of sensory irritation data for establishing OEGs and the additional influence of pulmonary irritation on establishing the values. James et al. (1987) determined the nasal nRD_{50} for methyl isocyanate was 2.9 ppm in mice, and Ferguson et al. (1986) obtained a value for the nRD_{50} of 1.3 ppm and for the tRD_{50} of 1.9 ppm. These RD_{50} values indicate that methyl isocyanate is a potent peripheral chemosensory irritant. With respect to nasal sensory irritancy (nRD_{50}), methyl isocyanate is not as potent as (60-min values)

the aliphatic hexyl di-isocyanate and the following aromatic mono- and di-isocyanates: phenyl isocyanate (0.9 ppm), *p*-toluene isocyanate (0.84 ppm), and toluene di-isocyanate (0.39 ppm). It is, however, more potent than hexyl isocyanate (9.2 ppm) (Sangha et al., 1981). Although the nRD_{50} for methyl isocyanate (1.3 ppm) is numerically larger than that for toluene di-isocyanate (0.39 ppm), the main difference between these two isocyanates is that there was no evidence for pulmonary irritation with the toluene compound (even in cannulated mice inhaling $5 \times RD_{50}$) (Sangha and Alarie, 1979), but the methyl compound had a tRD_{50} of 1.9 ppm; thus the ratio tRD_{50}/nasal RD_{50} was 1.46, indicating that methyl isocyanate is a potent pulmonary as well as sensory irritant. In comparison with other potent PCI irritant materials, such as acrolein (nRD_{50} 1.7 ppm) and formaldehyde (nRD_{50} 3.7 ppm), methyl isocyanate is much more hazardous by inhalation as indicated by the respective tRD_{50}/nRD_{50} ratios of 142, 31, and 1.46. The current ACGIH (2005) TLV values for the isocyanates agree with the measured nRD_{50} values as follows:

Methyl isocyanate: 0.03 nRD_{50} = 0.04 ppm; 0.01 tRD_{50} = 0.02 ppm; TLV = 0.02 ppm
1,6-Hexamethylene di-isocyanate: 0.03 nR_{50} = 0.0066; TLV = 0.005 ppm (also a respiratory sensitizer).
Toluene-1.4-di-isocyanate: 0.03 nRD_{50} = 0.0075 ppm; TLV = 0.005 ppm (also a respiratory sensitizer).

Several investigators have expressed reservations on the suitability of the RD_{50} test as a basis for assigning OEGs. For example, Bos et al. (1992) have questioned the suitability of this approach based on considerations of interlaboratory variations, the finding that toxicity (inflammation) may be seen at or below RD_{50} concentrations and that the rationale for deriving an OEG from RD_{50} data is based on empirical findings. As noted above, although effects may be observed at the nRD_{50} concentration, using a correction factor of up to 0.1 nRD_{50} allows, at least in part, for slight effects at the RD_{50}. Bos et al. (1992) recommended the following procedures with respect to use of the sensory irritation test:

a. In view of interspecies variations, two species (rat and mouse) should be used, with the results from the most sensitive species being chosen.
b. Time–response and log concentration–response curves should be obtained.
c. Exposure should be continued until a plateau is obtained.
d. There should be verification that no pulmonary irritation is occurring.
e. For every material tested, it should be verified whether the observed response is due to irritation or toxicity and that toxicity is not occurring below the concentration causing sensory irritation.

The latter point is important, and it cannot always be assumed that by preventing irritation the occurrence of other manifestations of systemic irritancy can be prevented. For example, the mouse nRD_{50} values for 1,2-dichlorobenzene and hexachloro-1,3-butadiene vapor were found to be 181 and 211 ppm, respectively. The median active level of exposure (MAL) for 1,2-dichlorobenzene to produce a 50% decrease in hepatic glucose-6-phosphate dehydrogenase (an index of hepatotoxicity) was 598 ppm, and the MAL for hexachloro-1,3-butadiene to cause 50% renal tubular injury (based on alkaline phosphatase histochemistry) was 7.2 ppm. Thus, the MAL for 1,2-dichlorobenzene hepatotoxicity is about 3.3 times that of the nRD_{50}, and hence this material can be treated as a PCI from the OEG viewpoint. However, the RD_{50} for hexachloro-1,3-butadiene vapor is 29.3 times higher than the MAL for nephrotoxicity, and the latter should receive greater consideration in the assignment of an OEG (de Ceaurriz et al., 1988).

OEGs are usually based on irritancy and toxicology information collected at ambient temperature. However, in some workplace situations there may be an elevated environmental temperature; e.g., in tropical conditions. Also, certain industrial processes may not be totally investigated. For example, thermoplastic resins are heated to liquefy them for processes such as blow, extrusion,

or injection molding. This can result in the liberation of residual monomers, additives, or thermal decomposition products, to which workers may be exposed. Schaper et al (1994) conducted a study on the respiratory responses to the thermal decomposition products of thermoplastics. They measured nRD_{50} values at processing temperatures between 200°C and 300°C for four resins; polyacrylonitrile-polybutadiene-polysterene, polypropylene-polyethylene copolymer, polypropylene homopolymer, and plasticized poly(vinyl chloride). The respective nRD_{50} values were 21.10, 3.51, 2.60, and 11.51 mg m^{-3}. Based on these findings the authors recommended exposure limits of 0.63, 0.11, 0.08, and 0.35 mg m^{-3} (0.03 nRD_{50}). Although such studies yield valuable practical information relevant to certain specific workplace activities, Muller and Black (1995) cautioned against the routine use of extreme generation conditions. They investigated the PCI potential of various samples of indoor materials (including carpets, ceiling tiles, and wall coverings) that were ventilated at either 23°C or 70°C. Using mouse plethysmographic procedures (nRD_{50} measurements) they found irritation at 70°C but not at 23°C. They concluded that increasing the environmental temperature increased the concentration of chemical emissions, which account for the sensory irritant effects. They considered that ambient generation conditions were more appropriate for assessing emissions in relation to such indoor air considerations.

In the workplace or general environment exposure to several different irritant materials can be simultaneous. Using mixtures of volatile organic compounds, Hempel-Jørgensen et al. (1999) produced ocular irritation by interactive additive effects. Although, in the context of the use of nRD_{50} measurements to assign OEGs, PCI materials probably act on the same trigeminal chemoreceptors, several considerations suggest that a predictive approach by addition of concentrations may not always be reliable. For example, competition for the same chemoreceptor may result in a decrease in breathing rate that is less then predicted on the basis of additive considerations. Thus, Cassee et al. (1996) found that sensory irritation in rats resulting from exposure to mixtures of aldehydes (formaldehyde, acrolein, and acetaldehyde) was more pronounced than that caused by each aldehyde separately, but less than that of the sum of the individual irritant potencies. Competitive agonism has been demonstrated for acrolein and formaldehyde (Kane and Alarie, 1978) and for cumene and *n*-propanol (Nielsen et al., 1988). Also, different materials may display different desensitization patterns. Cassee et al. (1996) measured the decrease in breathing rate in rats for formaldehyde, acetaldehyde, and acrolein and for mixtures of these substances. They found that sensory irritation was more pronounced for mixtures than for each compound tested separately, but less than for the sum for individual components. They concluded that the decrease in breathing rate as a result of exposure to mixtures of PCI materials could be predicted with a model for competitive antagonism. Threshold responses for odor, nasal pungency (in anosmics), and eye irritation were measured by Cometto-Moñiz et al. (1997) for single chemicals (1-propanol, 1-hexanol, ethyl acetate, heptyl acetate, 2-pentanone, 2-heptanone, toluene, ethyl benzene, and propyl benzene) and for various component mixtures of them. They found various degrees of stimulus agonism for the three sensations when testing mixtures. As the number of components and the lipophilicity of the components in the mixtures increased, so did the degree of agonism. Synergistic agonism characterized the eye irritation response for the most complex and most lipophilic mixtures. Korpi et al. (1999) reported that microbial volatile organic compounds may have some synergistic effects for sensory irritation and also that if a particular component of a mixture is much more potent than the other components of a mixture it may dominate the PCI effect. In addition to the use of such information for determining OEGs for the workplace, it may also be of value for investigating situations such as the "sick building" syndrome, and the authors suggest that the combined action of many relatively nonreactive chemicals at concentrations below their respective PCI thresholds could induce irritation in mucosae.

REFERENCES

Abraham, M. H., Andonian-Haftvan, J., Cometto-Muñiz, J. E. and Cain, W. S., An analysis of nasal irritation thresholds using a new solvation equation, *Fundam. Appl. Toxicol*., 31, 71–76, 1996.

Abraham, M. H., Kumarsingh, R., Cometto-Muñiz, J. E., Cain, W. S., Rosés, M., Bosch, E., and Diaz, L. D., The determination of solvation descriptors for terpenes, and the prediction of nasal pungency threshold, *J. Chem. Soc. Perkins Trans. I*, 2, 2405–2411, 1998.

Abraham, M. H., Whiting, G. S., Alarie, Y., Morris, J. J., Taylor, P. J., Doherty, R. M., Taft, R. W., and Nielsen, G. D., Hydrogen bonding. 12. A new QSAR for upper respiratory tract irritation by airborne chemicals in mice, *Quant. Struct.-Act. Relat*., 9, 6–10, 1990.

Alarie, Y., Sensory irritation by airborne chemicals, *Crit. Rev. Toxicol.,* 2, 299–363, 1973.

Alarie, Y., Bioassay for evaluating the potency of airborne irritants and predicting acceptable levels of exposure in man, *Food Cosmet. Toxicol*., 19, 623–626, 1981a.

Alarie, Y., Dose-response analysis in animal studies: prediction of human responses, *Environ. Health Perspect*., 42, 9113, 1981b.

Alarie, Y., Toxicological evaluation of airborne chemical irritation and allergens using respiratory reflex reactions, in *Proceedings of the International Toxicology and Technology Symposium*, Leong, B. K. J., Ed., Ann Arbor Science Publications, Ann Arbor, MI, pp. 207–231, 1981c.

Alarie, Y., Establishing threshold limit values for airborne sensory irritants from an animal model and the mechanisms of action of sensory irritants, in *Advances in Modern Environmental Toxicology,* Vol. VIII. *Occupational and Industrial Hygiene: Concepts and Methods,* Esmen, N. E. and Mehlman, M. A., Eds., Princeton Scientific Publishing, Princeton, NJ, pp. 153–164, 1984.

Alarie, Y., Kane, L., and Barrow, C., Sensory irritation: the use of an animal model to establish acceptable exposure to airborne chemical irritants, in *Toxicology: Principles and Practice*, Vol. 1, Reeves, A. L., Ed., Wiley, New York, pp. 48–92, 1980.

Alarie, Y., Kane, L., and Barrow, C., Sensory irritation: the use of an animal model to establish acceptable exposure to airborne chemical irritants, in *Toxicology; Principles and Practice*, Vol. 1, Reeves, A. L., Ed., Wiley, New York, pp. 48–92, 1980.

Alarie, Y., Nielsen, G. D., Andonian-Haftvan, J., and Abraham, M. H., Physicochemical properties of nonreactive volatile organic chemicals to estimate RD50: alternatives to animal studies, *Toxicol. Appl. Pharmacol.*, *134,* 92–99, 1995.

American Conference of Governmental Industrial Hygienists (ACGIH), *TLVs® and BEIs®. Thresold Limit Values. Biological Exposure Indices.* ACGIH, Cincinnati, OH, 2005.

Anderson, R. C. and Anderson, J. H., Toxic effects of air freshener emissions, *Arch. Environ. Health*, 52, 433–441, 1997a.

Anderson, R. C. and Anderson, J. H., Airflow limitation in mice caused by emissions of fabric softeners, *The Lancet Conference on Asthma and the Environment*, Tours, France, October 9–10, 1977(Abstr.), The Lancet, London, 1997b.

Anderson, R. C. and Anderson, J. H., Acute toxic effects of fragrance products, *Arch. Environ. Health*, 53, 138–146, 1998.

Anderson, R. C. and Anderson, J. H., Sensory irritation and multiple chemical sensitivity, *Toxicol. Ind. Health*, 15, 339–345, 1999.

Babuik, C., Steinhagen, W. H., and Barrow, C. S., Sensory irritation response to inhaled aldehydes after formaldehyde pre-treatment, *Toxicol. Appl. Pharmacol.*, 79, 143–149, 1985.

Bach, B., Molhave, L., and Pederson, O. F., *Human Reactions to Formaldehyde Vapor*, Institute of Environmental and Occupational Medicine, Århus, Denmark, 1988.

Ballantyne, B. Biomedical and health aspects of the use of chemicals in civil disturbances, in *Medical Annual 1977,* Scott, R. B., and Frazer, J., Eds., Wright, Bristol, pp. 7–41, 1977.

Ballantyne, B., Toxic effects of inhaled materials, in *Respiratory Protection. Principles and Applications*, Ballantyne, B. and Schwabe, P. J., Eds., Chapman & Hall, London, pp. 93–134, 1981.

Ballantyne, B., Peripheral sensory irritation as a factor in defining workplace exposure guidelines, in *Occupational Health in the Chemical Industry*, Orford, R. R., Cowell, J. W., Jamieson, G. G., and Love, E. J., Eds., MEDICAM Calgary '83 Association, Canada, p. 119–149, 1983.

Ballantyne, B., Peripheral sensory irritation: basics and applications, in *General and Applied Toxicology,* Vol. 2, 2nd ed., Ballantyne, B., Marrs, T. C., and Syversen, T., Eds., Macmillan Reference Ltd., London, pp. 611–629, 1999.

Ballantyne, B. and Salem, H., Forensic aspects of riot control agents, in *Riot Control Agents: Issues in Toxicology, Safety and Health*, Olajos, E. J., and Stopford, W., Eds., CRC Press, Boca Raton, FL, 231–258, 2005.

Ballantyne, B., The occupational toxicologist: professionalism, morality, and ethical standards in the context of legal and non-litigation issues, *J. Appl. Toxicol.*, in press, 2005.

Ballantyne, B., Gall, D., and Robson, D.C., Effects on man of drenching with dilute solutions of o-chlorobenzylidene malononitrile (CS) and dibenzoxazepine (CR), *Med. Sci. Law*, 16, 159–170, 1973.

Ballantyne, B., Gazard, M. F., and Swanston, D. W., Irritancy testing by respiratory exposure, in *Current Approaches in Toxicology*, Ballantyne, B., Ed., Wright, Bristol, pp. 129–138, 1977.

Ballantyne, B. and Jordan, S. L. P., Toxicological, medical, and industrial hygiene of glutaraldehyde with particular reference to its biocidal use in cold sterilization procedures, *J. Appl. Toxicol.*, 21, 131–151, 2001.

Ballantyne, B. and Swanston, D. W., The irritant potential of solutions of orthochlorobenzylidene malononitrile (CS), *Acta Pharmacol. Toxicol.*, 32, 266–277, 1973a.

Ballantyne, B. and Swanston, D. W., The irritant potential of dilute solutions of dibenzoxazepine (CR) on the eye and tongue, *Acta Pharmacol. Toxicol.*, 35, 412–423, 1973b.

Ballantyne, B., Werley, M. S., Snellings, W. M., and Burleigh-Flayer, H. D., Sensory irritation study of triethylene glycol aersol in ND4 Swiss-Webster mice, *Toxicologist*, 14, 310, 1994.

Bar-Ilan, A., The guinea pig blepharospasm test: a comparison with human responses, *J. Ocul. Pharmacol. Ther.*, 13, 207–212, 1997.

Barrow, C. S., Alarie, Y., and Stock, M. F., Sensory irritation and incapacitation evoked by thermal decomposition products of polymers and comparison with known sensory irritants, *Arch. Environ. Health*, 4, 79–88, 1978.

Barrow, C. S., Alarie, Y., Warrick, J., and Stock, M., Comparison of the sensory irritant response in mice to chlorine and hydrogen chloride, *Arch. Environ. Health*, 3, 68–76, 1977.

Bender, J. R., Mullin, L. S., Graepel, G. J., and Wilson, W. E., Eye irritation response of humans to formaldehyde, *Am. Ind. Hyg. Assoc. J.*, 44, 463–465, 1983..

Beswick, F. W., Holland, P., and Kemp, K. H., Acute effects of exposure to orthochlorobenzylidine malononitrile (CS) and the development of tolerance, *Br. J. Ind. Med.*, 29, 298–306, 1972.

Bevan, S. and Szolesanyi, J., Sensory neuron-specific actions of capsaicin: mechanisms and applications, *Trends Pharmacol. Sci.*, 11, 330–333, 1990.

Bos, P. M. J., Zwart, A., Reuzel, P. G. J., and Bragi, P. C., Evaluation of the sensory irritation test for assessment of occupational health risk, *Crit. Rev. Toxicol.*, 21, 423–450, 1992.

Buckley, L. A., Jiang, X .Z., James, R. A., Morgan, K. T., and Barrow, C. S., Respiratory tract lesions induced by sensory irritants at the RD50 concentration, *Toxicol. Appl. Pharmacol.*, 74, 417–429, 1984.

Cassee, F. R., Arts, J. H. E., Groten, J. P., and Feron, V. J., Sensory irritation to mixtures of formaldehyde, acrolein, and acetaldehyde in rats, *Arch. Toxicol.*, 70, 329–337, 1996.

Cauna, N., Hinderer, K. H., and Wentges, R. T., Sensory receptor organs in human nasal respiratory mucosa, *Am. J. Anat.*, 14, 295–300, 1969.

Chang, J. C. F. and Barrow, C.S., Sensory irritation tolerance of F-344 rats exposed to chlorine or formaldehyde gas, *Toxicol. Appl. Pharmacol.*, 76, 319–327, 1986.

Coe, J. E. and Douglas, R. B., *In vivo* localization of human ocular chemoreceptors, *Cutan. Ocular, Toxicol.*, 3, 3–5, 1984.

Cometto-Muñiz, J. E. and Cain, W. S., Thresholds for odor and nasal pungency, *Physiol. Behav.*, 48, 719–717, 1990.

Cometto-Muñiz, J. E. and Cain, W. S., Sensory reactions of nasal pungency and odor to volatile organic compounds. The alkylbenzenes, *Am. Ind. Hyg. Assoc. J.*, 55, 811–817, 1994.

Cometto-Muñiz, J. E. and Cain, W. S., Relative sensitivity of the ocular trigeminal, nasal trigeminal and olfactory systems to airborne chemicals, *Chem. Senses*, 20, 191–198, 1995.

Cometto-Muñiz, J. E. and Cain, W. S., Trigeminal and olfactory sensitivity: comparison of modalities and methods of measurement, *Int. Arch. Occup. Environ. Health*, 71, 105–110, 1998.

Cometto-Muñiz, J. E., Cain, W. S., and Hudnell, H. K., Agonistic sensory effects of airborne chemicals in mixtures: odor, nasal pungency, and eye irritation, *Percept. Psychophys.*, 59, 665–674, 1997.

Cooper, G. P., Responses of single units in the rat ethmoidal nerve to cutaneous and olfactory stimuli, *Physiologist*, 13, 171, 1970.

Crofton, J. and Douglas, A., *Respiratory Diseases*, 3rd ed., Blackwell, Oxford, 1981.

Cutting, M., Goodenough, S., Simmons, G., Watson, A., LaGuarda, A., and Huber, G., *Clin. Res.*, 22, 501A, 1974.

Dalton, P. H., Dilks, D. D., and Banton, M. I., Evaluation of odor and sensory irritation thresholds for methyl isobutyl ketone in humans, *Am. Ind. Hyg. Assoc. J.*, 61, 340–350, 2000.

Dalton, P., Wysocki, C. J., Brody, M. J., and Lawley, H. J., The influence of cognitive bias on the perceived odor, irritation and health symptoms from chemical exposure, *Int. Arch. Occup. Environ. Health*, 69, 407–417, 1997.

de Ceaurriz, J. C., Miccilino, J. C., Bonnet. P., and Guenier, J. P., Sensory irritation caused by various industrial airborne chemicals, *Toxicol. Lett.*, 9, 137–143, 1981.

de Ceaurriz, J., Gagnaire, F., Ban, M., and Bonnet, P., Assessment of the relative hazard involved with airborne irritants with additional hepatotoxic or nephrotoxic properties in mice, *J. Appl. Toxicol.*, 8, 417–422, 1988.

Dirnhuber, P., Green, D. M., and Tregear, R. T., Excitation of sensory neurones in the cat larynx by ω-chloroacetophenone and *n*-nonanoylvanillamide, *J. Physiol.*, 178, 41–42, 1965.

Dixon, M. and Needham, D. M., Biochemical research on chemical warfare agents, *Nature*, 158, 432–438, 1946.

Douglas, R., Inhalation of irritant gases and aerosols, in *Respiratory Pharmacology*, Widdicombe, J., Ed., Pergamon Press, Oxford, pp. 297–333, 1981.

Dudek, B. R., Short, R. D., Brown, M. H., and Roloff, M. V., Structure-activity relationship of a series of sensory irritants, *J. Toxicol. Environ. Health*, 37, 511–518, 1992.

Dunn, J. D., Cometto-Muniz, J. E., and Cain, W. S., Nasal reflexes: reduced sensitivity to CO_2 irritation in cigarette smokers, *J. Appl. Toxicol.*, 2, 176–178, 1982.

Feniak, G., The common chemical sense of the frog, Suffield Technical Paper 310, Suffield, Experimental Station, Alberta, Canada, 1966.

Ferguson, J. S., Schaper, M., Stock, M. F., Weyel, D. A., and Alarie, Y., Sensory and pulmonary irritation with exposure to methyl isocyanate, *Toxicol. Appl. Pharmacol.*, 82, 329–335, 1986.

Foster, R. W. and Ramage, A. G., The action of some chemical irritants on somatosensory receptors of the cat, *J. Neuropharmacol.*, 20, 191–198, 1981.

Gagnaire, F., Zizzu, P., Bonnet, P., and De Ceaurriz, J., Nasal and pulmonary toxicity of allyl glycidyl ether in mice, *Toxicol. Lett.*, 39, 329–335, 1987.

Gagnaire, F., Azim, S., Bonnet, P., Hecht, G., and Hery, M., Comparison of the sensory irritation response in mice to chlorine and nitrogen trichloride, *J. Appl. Toxicol.*, 14, 405–409, 1994.

Gagnaire, F., Azim, S., Bonnet, P., Simon, P., Guenier, J. P., and de Ceaurriz, J., Nasal irritation and pulmonary toxicity of aliphatic amines in mice, *J. Appl. Toxicol.*, 9, 301–304, 1989.

Gagnaire, F., Azim, S., Simon, P., Cossee, B., Bonnet, P., and de Ceaurriz, J., Sensory and pulmonary irritation of aliphatic amines in mice: a structure-activity relationship study, *J. Appl. Toxicol.*, 13, 129–135, 1993.

Grant, W. M. and Schuman, J. S., *Toxicology of the Eye*, 4th ed., Charles C Thomas, Springfield, IL, 1995.

Green, B. G., Measurement of sensory irritation of the skin, *Am. J. Contact Derm.*, 11, 170–180, 2000.

Green, D. M., Balfour, D. J. K., and Muir, A., Effect of methyl substitution on the irritancy of dibenz[b.f]-1,4-oxazepine (CR), *Toxicology*, 12, 151–153, 1979.

Green, B. G. and Bluth, J., Measuring the chemosensory irritability of human skin, *J. Toxicol. Cutan. Ocul. Toxicol.*, 14, 23–48, 1995.

Green, G. M. and Carolin, D., *N. Engl. J. Med.*, 276, 421, 1967.

Green, B. G., Mason, J. R., and Kare, M. R., *Chemical Senses*, Vol. 2, *Irritation*, Dekker, New York, 1990.

Green, D. M. and Tregear, R. T., The action of sensory irritants on the cat's cornea, *J. Physiol.*, 175, 37–38, 1964.

Hansen, L. F., Knudsen, A., and Nielsen, G. D., Sensory irritation effects of methyl ethyl ketone and its receptor activation mechanism, *Pharmacol. Toxicol.*, 71, 201–208, 1992.

Hansen, L. F. and Nielsen, G. D., Sensory irritation effects of n-propanol and ethylbenzene after pretreatment with capsaicin or indomethacin, *Pharmacol. Toxicol.*, 75, 154–161, 1994.

Hempel-Jørgensen, A., Kjaergaard, S. K. and Mølhave, L., Measurement of human eye irritation using a CO_2 reference scale. In *Characterizing Sources of Indoor Air Pollution and Related Sink Effects*, B.A. Tichenor (Ed.).West-Conshohocken, PA. American Society for Testing and Materials. ATSM, STP 1287, pp. 350–366, 1996.

Hempel-Jørgensen, A., Kjaergaard, S. K., and Molhave, L., Cytological changes and conjunctival hyperemia in relation to sensory eye irritation, *Int. Arch. Occup. Environ. Health*, 71, 225–235, 1998.

Hempel-Jørgensen, A., Kjaegaard, S. K., and Mølhave, L., Sensory eye irritation in humans exposed to mixtures of volatile organic compounds, *Arch. Environ. Health*, 54, 416–424, 1999.

Hogg, S. I., Curtis, C. G., Russell, N. J., Upshall, D. G., and Powell, G. M., The effects of sensory irritants on phagocytosis by pulmonary macrophages, *Environ. Res.*, 30, 492–497, 1983.

Holzer, P., Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurones, *Pharmacol. Rev.*, 43, 143–163, 1991.

James, J. T., Buetnner, L. C., and Hsu, S. S., Sensory irritation of methyl isocyanate vapor, *J. Appl. Toxicol.*, 7, 147–148, 1987.

James, J. E. A. and Daly, M. de B., Nasal reflexes. *Proc. R. Soc. Med.*, 62, 1287–1293, 1969.

Kaempfe, T. A. and Dudek, B. R., Evaluation of 2 carpet samples for sensory irritation in mice with multiple exposure modification of ASTME981, *Toxicologist*, 14, 254, 1994.

Kane, L. E. and Alarie, Y., Evaluation of sensory irritation from acrolein-formaldehyde mixtures, *Am. Ind. Hyg. Assoc. J.*, 39, 270–274, 1978.

Kane, L. E., Dombroske, L., and Alarie, Y., Evaluation of sensory irritation from some common industrial solvents, *Am. Ind. Hyg. Assoc. J.*, 41, 451–455, 1980.

Kasanen, J.-P., Pasanca, A.-L., Pasanen, P., Liesivuori, J., Kosma, V.-M., and Alarie, Y., Stereospecificity of the sensory irritant receptor for non-reactive chemicals illustrated by pinene enantiomers, *Arch. Toxicol.*, 72, 514–523, 1998.

Katz, G. V. and Laskin, S., In *Air Pollution and the Lung. Proceedings of the 12th Annual OHOLO Biological Conference, Maalot, Israel, 1975*, Aharonson, E. F., Ben-David, A., and Klingberg, M. A., Eds., Wiley, New York, 1976.

Keele, C. A., The common chemical sense and its receptors, *Arch. Int. Pharmacodyn. Ther.*, 39, 547–557, 1962.

Kjaergaard, S. K., Sensitivity of the eyes to airborne irritant stimuli: the influence of individual characteristics, *Arch. Environ. Health*, 47, 45–50, 1992.

Kjaergaard, S. and Pederson, O. F., Dust exposure, eye redness, eye cytology and mucous membrane irritation in a tobacco industry, *Int. Arch. Occup. Environ. Health*, 61, 519–525, 1989.

Kjaergaard, S., Pederson, O. F., and Molhave, L., Human exposure to indoor pollutants: *n*-decane, *Environ. Int.*, 15, 473–482, 1989.

Kjaergaad, S., Pederson, O. F., Taudorfe, E., and Molhave, L., Assessment of changes in eye redness by a photographic method and the relation to sensory irritation, *Int. Arch. Environ. Health*, 62, 133–137, 1990.

Koren, H. S., Graham, D. E., and Devlin, R. B., Exposure of humans to a volatile organic mixture. III. Inflammatory response, *Arch. Environ. Health*, 47, 38–44, 1992.

Korpi, A., Kosma, V.-M., Kasanen, J.-P., Pasanen, A.-L., and Alarie, Y., Sensory irritating potency of some microbial volatile organic compounds (MVOCs) and a mixture of five MVOCs, *Arch. Environ. Health*, 54, 347–352, 1999.

Kristiansen, U., Hanse, G. D., Nielsen, G. D., and Holst, E., Sensory irritation and pulmonary irritation of cumene and n-propanol: mechanisms of receptor activation and desensitization, *Acta Pharmacol. Toxicol.*, 59, 60–72, 1986.

Kristiansen, U., Vinggaard, A. M., and Nielsen, G. D., The effects of n-butanol vapour on respiratory rate and tidal volume, *Arch. Toxicol.*, 61, 229–236, 1988.

Kulle, T. J. and Cooper, G. P., Effects of formaldehyde and ozone on the trigeminal nasal sensory system, *Arch. Environ. Health*, 30, 237–243, 1975.

Leidel, N. A., Busch, K. A., and Crouse, W. E., Exposure Measurement Action Level and Occupational Environmental Variability, Department of Health Education and Welfare (NIOSH) Publication 76–13; NTIS Publication PB-267–509. Dated December 1975. National Technical Information Service, Springfield, VA, 1975.

Leung, H.-W. and Paustenbach, D., J., Techniques for estimating the percutaneous absorption of chemicals due to occupational and environmental exposure, *Appl. Occup. Environ. Hyg.*, 9, 187–197, 1994.

Lundquist, G. R., Yamagiwa, M., Pederson, O. F., and Nielsen, G. D., Inhalation of diethylamine—acute nasal effects and subjective response, *Am. Ind. Hyg. Assoc. J.*, 53, 181–185, 1992.

Luo, J. E., Nielsen, G. D., and Alarie, Y., Formaldehyde: an exception in the series of saturated aldehydes, aliphatic alcohols and alkylbenzenes, *Toxicologist*, 3, 74, 1983.

Matijak-Schaper, M., Wong, K.-L., and Alarie, Y., A method to rapidly evaluate the acute pulmonary effects of aerosols in unanesthetized guinea pigs, *Toxicol. Appl. Pharmacol.*, 69, 451–460, 1983.

McDougal, J. N., Jepson, G. W., Clewell, H. J., III, Gargas, M. L., and Andersen, M. E., Dermal absorption of organic chemical vapors in rats and humans, *Fundam. Appl. Toxicol.*, 14, 299–308, 1990.

McNamara, B. P., Vocci, F. J., and Owens, E. J., The toxicology of CN, Edgewood Arsenal Technical Report Series, 4207, December 1968, Edgewood Arsenal, MD, 1968.

Moncrief, R. H., *The Chemical Senses*, Leonard Hill, London, 1944.

Muller, W. J. and Black, M. S., Sensory irritation in mice exposed to emissions from indoor products, *Am. Ind. Hyg. Assoc. J.*, 56, 794–803, 1995.

National Institute of Environmental Health Sciences (NIEHS), Experimental approaches to chemical sensitivity: a monograph based on papers presented at the Workshop on Approaches to Chemical Sensitivity, Princeton, New Jersey, September 20–22, 1995. *Environ. Health Perspect.*, 105, Supplement 2, 1997.

Nielsen, G. D., Mechanism of activation of the sensory irritant receptor by airborne chemicals, *Crit. Rev. Toxicol.,* 21, 183–208, 1991.

Nielsen, G. D., Abraham, M. H., Hansen, L., Haminer, L. F., Cooksey, C. J., Andonian-Haftvan, J. and Alarie, Y., Sensory irritation mechanisms investigated from model compounds: trifluoroethanol, hexafluoroisopropanol and methyl hexafluoroisopropyl ethyl. *Arch. Toxicol.*, *70,* 319–328, 1996.

Nielsen, G. D. and Alarie, Y., Sensory irritation, pulmonary irritation and respiratory stimulation by airborne benzene and alkylbenzenes. Prediction of safe industrial exposure levels and correlation with their thermodynamic properties, *Toxicol. Appl. Pharmacol.*, 65, 459–477, 1982.

Nielsen, G. D., Bakbo, J. C., and Holst, E., Sensory irritation and pulmonary irritation by airborne allyl acetate, allyl alcohol and allyl ether compared to acrolein, *Acta Pharmacol. Toxicol.*, 54, 292–298, 1984.

Nielsen, G. D., Krastiansen, U., Hansen, L., and Alarie, Y., Irritation of the upper airways from mixtures of cumene and *n*-propanol, *Arch. Toxicol.*, 62, 209–215, 1988.

Nielsen, G. D. and Vinggaard, A. M., Sensory irritation and pulmonary irritation of C3-C7 n-alkylamines: mechanisms of receptor activation, *Pharmacol. Toxicol.*, 63, 293–304, 1988.

Nielsen, G. D. and Yamagiwa, M., Structure-activity of airway irritative aliphatic amines. Receptor activation mechanisms and predicted industrial exposure limits, *Chem.-Biol. Interact.*, 71, 223–244, 1989.

Norris, J. C. and Ballantyne, B., Toxicology and implications of the products of combustion, in *General and Applied Toxicology*, 2nd ed., Ballantyne, B., Marrs, T.C., and Syversen, T., Eds. Macmillan Reference Ltd., London, pp. 1915–1933, 1999.

Otto, D. A., Hudnell, H. K., House, D. E., Molhave, L., and Counts, W., Exposure of humans to a volatile organic mixture. I. Behavioral assessment, *Arch. Environ. Health*, 47, 23–30, 1992.

Owens, E. J. and Punte, C. L., Human respiratory and ocular irritation studies using *ortho*-chlorobenzylidene malononitrile aerosol, *Am. Ind. Hyg. Assoc. J.*, 24, 22–264, 1963.

Parker, G. H., The relations of smell, taste, and the common chemical sense in vertebrates, *J. Acad. Nat. Sci. Phila.*, 15, 21–234, 1912.

Punte, C. L., Owens, E. J., and Gutentag, E. J., Exposure to *ortho*-chlorobenzylidene malononitrile, *Arch. Environ. Health*, 6, 366–374, 1963.

Roscher, S., Glaser, C., Hummel, T., and Kobal, G., An easy method for separating olfactory from trigeminal stimulations, *Chem. Senses*, 21, 492–493, 1996.

Ryydzynski, K. and Jedrychowski, R., Sensory irritating properties of cyanuric chloride as revealed with plethysmographic method, *Int. J. Occup. Med. Environ. Health*, 7, 149–154, 1994.

Salem, H., Ballantyne, B., and Katz, Sidney A., Inhalation toxicology of riot control agents, in Inhalation Toxicology, CRC Press, Boca Raton, FL, 497–514, 2005.

Sangha, G. K. and Alarie, Y., Sensory irritation by toluene diisocyanate in single and repeated exposure, *Toxicol. Appl. Pharmacol.*, 50, 533–547, 1979.

Sangha, G. K., Matijak, M., and Alarie, Y., Comparison of some mono- and diisocyanates as sensory irritants, *Toxicol. Appl. Pharmacol.*, 57, 241–246, 1981.

Schaper, M. M., Development of a database for sensory irritants and its use in establishing occupational exposure limits, *Am. Ind. Hyg. Assoc. J.*, 54, 488–544, 1993.

Schaper, M. M., Thompson, R. D., and Detwiler-Okabayashi, K. A. Respiratory responses of mice exposed thermal decomposition products from polymers heated at and above workplace processing temperatures, *Am. Ind. Hyg. Assoc. J.*, 55, 924–934, 1994.

Schauenstein, E., Esterbauer, M., and Zollne, H., *Aldehydes in Biological Systems*, Pion, London, 1977.

Silver, R. F., Kerr, A. K., Frandson, P. D., Kelley, S. J., and Holmes, H. L., Synthesis and chemical reactions of some conjugated heteroenoid compounds, *Can. J. Chem.*, 45, 1001–1006, 1967.

Sparks, P. J., Idiopathic environmental intolerances, in *General and Applied Toxicology*, 2nd ed., Ballantyne, B., Marrs, T. C., and Syversen, T., Eds., Macmillan Reference Ltd., London, pp. 1703–1720, 1999.

Steinhagen, W. H. and Barrow, C. S., Sensory irritation structure-activity study of inhaled aldehydes in B6C3F1 and Swiss-Webster mice, *Toxicol. Appl. Pharmacol.*, 72, 495–503, 1984.

Steinhagen, W. H., Swenberg, J. A., and Barrow, C. S., Acute inhalation toxicity and sensory irritation of dimethylamine, *Am. Ind. Hyg. Assoc. J.*, 43, 411–417, 1982.

Thurauf, N., Friedel, I., Hummel, C., and Kohol, G., The mucosal potential elicited by noxious chemical stimuli with CO_2 in rats: is it a peripheral nociceptive event? *Neurosci. Lett.*, 18, 297–300, 1991.

Topping, M., Occupational exposure limits for chemicals, *Occup. Environ. Med.*, 58, 138–144, 2001.

Tsubone, H. and Kawata, M., Stimulation of the trigeminal afferent nerve of the nose by formaldehyde, acrolein and acetaldehyde gas, *Inhalation Toxicol.*, 3, 211–222, 1991.

Ulrich, C. E., Haddock, M. P., and Alarie, Y., Airborne chemical irritants, *Arch. Environ. Health*, 24, 37–42, 1972.

Valentini, J. E., Wang, K.-L., and Alarie, Y., Single tracer technique to evaluate pulmonary edema and its application to detect the effect of hexamethylene diisocyanate trimer aerosol exposures, *Toxicol. Appl. Pharmacol.*, 69, 461–470, 1983.

Werley, M. S., Burleigh-Flayer, H. D., and Ballantyne, B., Respiratory peripheral sensory irritation and hypersensitivity studies with glutaraldehyde vapor, *Toxicol. Ind. Health*, 11, 489–501, 1995.

Werley, M. S., Burleigh-Flayer, H. D., Fowler, E. H., Rybka, M. L., and Adler, A. W., Development of pituitary lesions in ND4 Swiss Webster mice when estimating the sensory irritancy of airborne chemicals using ASTM method E981–84, *Am. Ind. Hyg. Assoc. J.*, 57, 712–716, 1966.

Weyel, D. A., Rodney, B. S., and Alarie, Y., Sensory irritation, pulmonary irritation and acute lethality of a polymeric isocyanate and sensory irritation of toluene-2,6-diisocyanate, *Toxicol. Appl. Pharmacol.*, 64, 423–430, 1982.

Weyel, D. A. and Schaffer, R. B., Pulmonary and sensory irritation of diphenylmethane-4,4′- and dicyclohexylmethane-4,4′-diisocyanate, *Toxicol. Appl. Pharmacol.*, 77, 427–433, 1985.

Widdicombe, J. G., Physiological response to inhaled material, in *Respiratory Protection: Principles and Applications*, Ballantyne, B., and Schwabe, P., Eds., Chapman & Hall, London, pp. 47–64, 1981.

Woskie, S. R., Eisen, E. E., Wegman, D. H., Hu, X., and Kriebel, D., Worker sensitivity and reactivity: indicators of worker susceptibility to nasal irritation, *Am. J. Ind. Med.*, 34, 614–627, 1998.

Wysocki, C. J., Dalton, P., Brody, M. J.. and Lawley, H. J. Acetone odor and irritation thresholds obtained from acetone-exposed factory workers and control (occupationally unexposed) subjects, *Amer. Ind. Hyg. Assoc. J.*, 58, 704–712, 1997.

Wysocki, C. J., Green, B. G., and Malia, T. P., Monorhinal stimulation as a method for differentiating between threshold for irritation and odor, *Chem. Senses*, 17, 722–723, 1992.

14 Role of Cytokines in Pulmonary Inflammation and Fibrosis Induced by Inhaled Mineral Particles

François Huaux

CONTENTS

14.1 INTRODUCTION

Pulmonary fibrosis is characterized by alveolar inflammation, mesenchymal cell proliferation, and increased production of collagen resulting in fibrous deposits, impairing the gas-exchange function of the lung. In numerous cases no causative agent or associated condition can be found and these are referred to as cryptogenic fibrosing alveolitis or idiopathic pulmonary fibrosis. In certain instances, lung fibrotic diseases may result from the pulmonary response to inhaled environmental agents, mineral or organic in nature. Interstitial pulmonary fibrosis resulting from workplace exposure to inorganic dusts (pneumoconiosis) persists throughout the world despite identification of the causes and preventive measures. Pneumoconioses are presently incurable and may be progressive even after dust exposure has ceased; therefore, early recognition and preventive intervention are important.

Cytokines are of central importance in the regulation of immunity, inflammation, tissue remodeling, and embryonic development. Uncontrolled or excessive cytokine production may, however, contribute to the pathophysiology of acute and chronic diseases. This is, in particular, the case in the development of interstitial lung disease (fibrosing alveolitis), which is triggered by numerous cytokines produced in excess by pulmonary cells, especially alveolar macrophages. Studies on the cytokine network implicated in the dust-induced alterations of lung structure and function are important for understanding the basic mechanisms of the disease. In addition, understanding the contribution of cytokines in the

responses to pneumotoxic agents and identification of key mediators in the pathogenic process could contribute to: (a) the identification of biomarkers for monitoring exposure or lung disease activity, (b) the development of therapeutic interventions for lung disease; and finally, (c) the development and validation of *in vitro* and *in vivo* approaches for assessing the toxicity of new materials.

14.2 PULMONARY RESPONSES TO INORGANIC DUST

Chronic inhalation of high concentrations of inorganic dust may result in a form of interstitial lung disease referred to as pneumoconiosis, which is a broad term used to describe the deposition of any inhaled dusts in the lung irrespective of the effects that they cause (excluding asthma and neoplasia). Like other interstitial lung disorders, malignant pneumoconioses are chronic inflammatory reactions and collagenous fibrotic disorders confined to the lower respiratory tract, essentially within the alveolar walls. Malignant pneumoconioses are characterized by: (a) the presence of exaggerated collagen deposition, (b) a permanent impairment of function or destruction of the alveolar architecture, (c) a permanent cicatricial state of the lung (Heppleston, 1994). The three major classes of inorganic dusts that can cause interstitial lung disease are crystalline silica (silicosis), asbestos (asbestosis), and coal mine dust (coal workers' pneumoconiosis, CWP) (Harvey and Crystal, 1997). These lung diseases can progress even after cessation of exposure (Steenland and Brown, 1995; Davis Smith 1996) and malignant pneumoconioses predispose to infections, in particular, tuberculosis, and to lung cancer.

Benign pneumoconioses consist essentially of a simple accumulation of inorganic material without significant impairment of the pulmonary function or of the alveolar architecture. Dusts of titanium, iron, tin, barium, or tungsten carbide are considered as "inert" material (noninflammatory and nonfibrogenic) and the pulmonary reactions to these dusts are classified as noncollagenous pneumoconioses (Driscoll et al., 1990b; Lasfargues et al., 1992; Parkes, 1998).

Pneumoconioses do not affect the general population but are confined to individuals chronically exposed to high concentrations of fibrogenic dusts in the occupational setting. According to epidemiological studies, the risk of interstitial lung disease induced by exposures to inorganic dusts relates largely to the cumulative exposure to hazardous dust (Davis Smith, 1996). Simple chronic silicosis has, however, been described after environmental exposures to silica in regions where soil silica content is high and dust storms are common (Franco and Massola 1992; Norboo et al., 1991). Moreover, nonoccupational exposure to aluminum silicates resulting in fibrogenic disease has also been reported in some arid regions of the world, including the southwestern United States (Osornio-Vargas et al., 1991).

In the United States, between 1979 and 1992, 4,882 death certificates listed silicosis as an underlying or contributing cause of death; 8,761 death certificates listed asbestosis during that period. Silicosis mortality rates calculated for various European countries ranged from 0.91 per 100,000 men in the United Kingdom to 7.36 per 100,000 men in Belgium during the period 1985 and 1986. General population mortality rates, however, are not necessarily illuminating for purposes of comparison because information about the number of people at risk of disease due to work is not fully available. Contrary to a common opinion, silicosis is not a disease of the past. Surveillance for silicosis in four States in the United States identified 447 new cases between 1988 and 1992 (American Thoracic Society Committee, 1997; Wagner, 1997). In France, approximately 200 cases of silicosis are recorded each year, and today, it is estimated that more than 250,000 persons are exposed to silica particles (Gout, 1996). The situation in developing countries appears much more preoccupant. From 1991 to 1995, China recorded more than 500,000 cases of silicosis, with approximately 6,000 new cases and more than 24,000 deaths occurring each year. In Colombia, the government estimates that 1.8 million workers are at risk of developing silicosis (World Health Organization [WHO], 2000).

Epidemiological studies have demonstrated wide variations in the response of individuals to similar exposures to inorganic dusts. The reasons for these variations are not clear and genetic background could probably play a role (Borm et al., 1992; Shih et al., 1993).

No effective treatment is available to reverse the course of malignant pneumoconiosis (Davis Smith, 1996). Prevention through elimination of hazardous exposure conditions is therefore of primary

importance. Some epidemiological and laboratory investigations have also suggested that early identification of disease followed by withdrawal from exposure may lead to more favorable long-term outcomes (Wagner, 1997). Chest radiography, high-resolution computed tomography (CT) scan, and bronchoalveolar lavage or biopsy, which are useful for the recognition of advanced pathologies (Begin et al., 1988, 1991, 1995; Kipen et al., 1987), do however not always allow an early detection of the lung disease (Wagner, 1997). Therefore, any biological investigation leading to the discovery of an early indicator of pneumoconiosis and to evaluate the incipient progression of the disease could be of great value in preventive medicine.

Several lines of evidence support the view that the pathogenesis of the interstitial disease induced by certain inorganic dusts involves normal biological processes albeit exaggerated. The fundamental concept, usually put forward to explain the pathogenesis of pneumoconiosis, is similar to that of all interstitial lung diseases and is relatively simple: a chronic inflammatory status (referred to as alveolitis), in which the inflammatory cells are activated and release toxic mediators, damages the pulmonary architecture and modulates the accumulation of mesenchymal cells and their connective tissue products, which forms the basis of the fibrotic scar (Crouch, 1990; Harvey and Crystal, 1997; Wolff and Crystal, 1997). What is not well understood is what maintains the alveolitis and why normal wound healing, which is normally localized in space and confined in time, overflows and promotes the establishment of lung fibrosis. The transition from normal defense to disease may simply be a matter of intensity: if the host defense system is overwhelmed by an exaggerated and chronic exposure to inorganic particles and if normal repair processes cannot keep up with the ensuing damages, the consequences are inevitable and lead to the disease. Another plausible hypothesis suggests that normal biological processes operating after insults caused by mineral dusts are amplified and/or uncontrolled only in susceptible individuals (Harvey and Crystal, 1997).

The hypothetical biological process leading to the extension of particles-induced lung inflammation and fibrosis can be summarized as follows.

14.2.1 Alveolitis

Inorganic particles that reach the alveolar epithelial surface are phagocytized mostly by alveolar macrophages. Evidence that alveolar macrophages are activated after phagocytosis comes from *in vitro* and *in vivo* studies that show morphological characteristics of activation (Takemura et al., 1989) and spontaneous release of a broad array of typical inflammatory mediators such as cytokines (Driscoll, 1995; Vanhee et al., 1995a) and arachidonic acid metabolites (eicosanoids) such as leukotrienes B4 (LT B4) (Englen et al., 1990; Ghio et al., 1992; Koren et al., 1992; Kuhn et al., 1993a; Demers and Kuhn, 1994). These mediators induce a pronounced recruitment of inflammatory cells both in the alveolar walls and on the alveolar epithelial surface. The resulting alveolitis is dominated by alveolar macrophages (Oghiso and Kubota, 1986; Rom et al., 1987), but also includes neutrophils (Rom, 1991), lymphocytes (Rom and Travis, 1992), and, to a much lesser extent, eosinophils (Robinson et al., 1986) and mast cells (Hamada et al., 2000). The major components of alveolitis accumulate by a recruitment of inflammatory cells from blood but also by an increased proliferation of inflammatory cells in the lung (Bitterman et al., 1984; Hoogsteden et al., 1989, 1993).

Although the process of injury may be initiated by the action of mineral particles themselves (Flaherty et al., 2002; Schins et al., 2002a), additional injuries seem to be associated with the influx of inflammatory cells. In particular, toxic oxygen derivatives and proteolytic enzymes released by inflammatory cells cause further cellular damage and disruption of the associated extracellular matrix (Weiss, 1989; Brown et al., 1992). In this context, several lines of evidence strongly suggest that exaggerated release of oxidants (e.g., superoxide anion, hydrogen peroxide, and nitric oxide) by alveolar macrophages, neutrophils, and probably other pulmonary cells plays a major role in the alteration of epithelial and endothelial cells that are particularly susceptible to these radicals (Blackford et al., 1994; Gossart et al., 1996; Mossman et al., 1990; Simeonova and Luster, 1995; Thomas et al., 1994; Vallyathan et al., 1992). The fragmentation of interstitial collagen fibers and

basement membrane components observed after exposure to fibrogenic dusts strongly suggests that proteases also play a role in the injury process (Kawanami et al., 1995). Matrix metalloproteinases (MMPs) and elastase are proteolytic enzymes specifically directed against extracellular matrix components. They are secreted by inflammatory cells such as activated alveolar macrophages and neutrophils and may consequently contribute to the alveolar damage observed during the development of alveolitis (D'Ortho et al., 1994; Ferry et al., 1997; Torii et al., 1997; Perez-Ramos et al., 1999). Collagenase overexpression in the lungs of transgenic mice causes a clear disruption of the alveolar walls and coalescence of the alveolar spaces without inflammation or fibrosis, providing evidence that extracellular matrix proteases could play a major role in the injury process (D'Armiento et al., 1992). The plasminogen-activating (PA) system plays a role in the injury process. For example, alveolar macrophages recovered from a sheep model of asbestosis release plasminogen activator that converts plasminogen into the potent protease plasmin and may play a role in injury to the parenchyma (Cantin et al., 1989). In a mouse model of silicosis, increased urokinase-type PA activity was measured in the lung early after treatment (Lardot et al., 1998b).

In the acute response to fibrogenic particles, apoptosis, and DNA damages are observed in macrophages and epithelial cells (Gozal et al., 2002; Kamp et al., 2002; Schins et al., 2002b; Panduri et al., 2003). Recent observations presented by Borges and coauthors demonstrated that apoptosis, induced early after administration of silica in mice, plays a central proinflammatory role by modulating the tumor necrosis factor α (TNF-α) production (Borges et al., 2001).

14.2.2 Fibrotic Reaction

The inflammatory phase is followed by a second or reparative phase in which polypeptide growth factors stimulate the recruitment and the proliferation of mesenchymal cells and regulate neovascularization and reepithelialization of injured tissues. Tissue repair is accomplished by extracellular matrix deposition (mainly collagens I and III, fibronectin, and proteoglycans) and tissue remodeling. During this phase, abnormal or uncontrolled reparative mechanisms may result in the development of fibrosis (Limper and Roman, 1992). Fibrosis occurs by local proliferation of mesenchymal cells and apparition of myofibroblasts with accompanying secretion of connective tissue proteins, resulting in a direct expansion of the alveolar interstitium. It is also now admitted that fibrosis could result from the organization of alveolar exudates (Lee and Kelly, 1993; Kawanami et al., 1995). Through defects in the basement membrane resulting from damage produced during alveolitis, mesenchymal cells and their connective tissue products also accumulate in the air spaces, and a subsequent reepithelialization, mainly by proliferative type II epithelial cells, incorporates these intraalveolar masses into the markedly thickened alveolar wall (Wolff and Crystal, 1997). The ability of the normal reparative mechanisms to maintain the original architecture of the lung seems to depend on many factors, including host inflammatory responses but also the nature and the persistence of injurious agents, the duration of exposure, and most notably, whether the injury has destroyed the continuity of the basement membrane, which is essential for the normal lung architecture (Burkhardt, 1989).

In addition to injury of the parenchyma during alveolitis, alveolar macrophages have a major role in the fibrotic reaction. Indeed, this cell type is able, when appropriately activated, to release a number of potent polypeptide growth factors for mesenchymal cells. In pneumoconiosis, these mediators mainly include fibrogenic cytokines (TNF-α, interleukin 1 [IL-1], and transforming growth factors α and β [TGFs-α and β]), growth factors such as platelet-derived growth factor (PDGF), the alveolar macrophage form of insulin-like growth factor (IGF) (Harvey and Crystal,1997), and fibronectin (Wagner et al., 1982; Begin et al., 1986; Rom et al., 1987; Rom, 1991).

MMPs and their tissue inhibitors (TIMPs) play an important role in the normal turnover of interstitial collagen. Patients with lung fibrosis have a decreased collagenolytic and an increased TIMPs activity in their lung parenchyma. It may be suggested that this phenomenon permits an accumulation of newly synthesized collagen at a faster rate than normal, thereby contributing to the fibrotic process (Pardo et al., 1992; Hayashi et al., 1996). Decreased expression of MMPs was also noticed

in the advanced phases of silicosis in the rat, contributing potentially to collagen accumulation and extension of progressive fibrosis (Perez-Ramos et al., 1999). Human and experimental observations suggest that the imbalance between plasminogen activators and their inhibitors (PAI 1 and 2) contributes as well to the exaggerated deposition of the extracellular matrix proteins (Li et al., 1991; Eitzman et al., 1996; Lardot et al., 1998a, 1998b).

14.2.3 Cancer

Chronic exposure to asbestos and silica (quartz) is associated with an increased elevated risk of lung cancer in humans and administration of these particles has been shown to result in the development of peripheral lung tumors in the rat (Johnson et al., 1987; International Agency for Research on Cancer [IARC], 1997; Calvert et al., 2003). Reactive oxygen species (ROS) and nitrogen species (RNS) are believed to play a major role in genotoxicity of particles, which may derive from their surface properties, the presence of transition metals, intracellular iron mobilization, and lipid preoccupation. These toxic molecules may cause DNA and cell damage, a prerequisite event for mutagenicity (Ding et al., 2002; Manning et al., 2002). In addition, *in vivo* investigations support

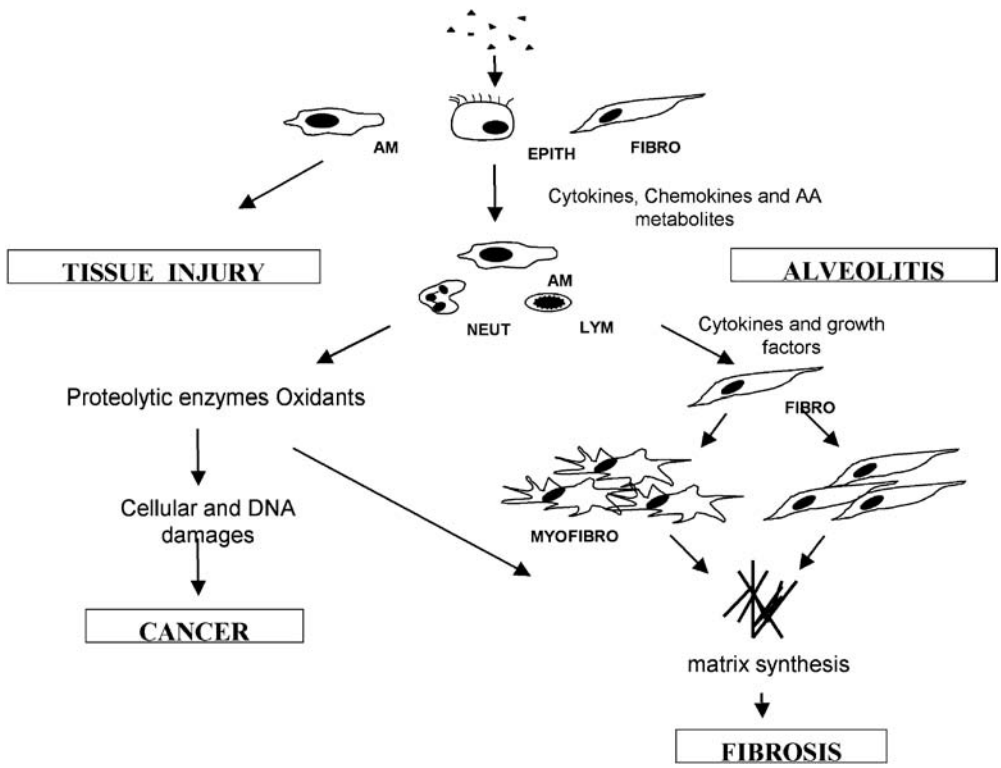


FIGURE 14.1 Major biological processes associated with injury and fibrosis of the lower respiratory tract in pneumoconiosis. Toxic dusts (5) stimulate alveolar macrophages (AM) but also epithelial cells (EPITH) and fibroblasts (FIBRO) to release several factors (chemokines, cytokines, and arachidonic acid [AA] metabolites), attracting leukocytes such as neutrophils (NEUT) and lymphocytes (LYM) into the lung. The activated alveolar macrophages and neutrophils produce a burden of oxidants and proteases that injure the alveolar wall. Oxidants can also induce cellular and DNA damage leading to cancer. Leukocytes also release growth factors and cytokines that stimulate lung fibroblasts to proliferate and differentiate into myofibroblasts (MYOFIBRO). The result is more mesenchymal cells and a larger mass of connective tissue matrix, characterizing lung fibrosis.

the existence of a relation between inflammation and mutagenicity. Using a hyposcenthine-guanine phosphoribosyl transferase (HPRT) mutagenicity assay, Driscoll and coworkers showed increased mutagenicity in alveolar epithelial cells of rats after exposure to silica (Driscoll et al., 1997). DNA damage in lung epithelial cells isolated from silica-exposed rats was also observed in another study (Knaapen et al., 2002a). These authors also emphasized the pivotal role of neutrophilic inflammation in particle-induced carcinogenesis because BAL cells from dust-exposed animals produced increased mutation rates in cocultures of rat lung epithelial cells, primarily as a result of oxidant production by neutrophils (Driscoll et al., 1997; Knaapen et al., 2002b). All these studies show a clear association between particle exposure and increased mutation. However, the precise mechanism leading to cancer upon particle exposure remains to be elucidated.

The hypothetical mechanisms by which inorganic particles induce interstitial lung disease and/or cancer are summarized in Figure 14.1.

14.3 PNEUMOCONIOSES AND CYTOKINES

14.3.1 Cytokine Networks

Cytokines constitute a group of low- molecular-weight proteins that are produced by many different cell types and, in general, act in a paracrine, autocrine, or juxtacrine fashion. The regulation of cytokine production is complex with transcriptional and translational controls, soluble receptor proteins that inhibit biological activity and naturally occurring receptor antagonists. An individual cytokine is able to stimulate the production of many cytokines, generating a network that interacts with other cell regulators such as hormones and neuropeptides. Conversely, the expression of a given cytokine is usually modulated by a variety of other cytokines. They have pleiotropic regulatory effects on hematopoietic and many other cell types and participate in host defense and repair processes (Oppenheim, 1994).

The most striking features of cytokines are the redundancy and the pleiotropism of their actions. In fact, structurally different cytokines show remarkable similarities in their actions and individual cytokines tend to exert a multitude of actions on different cells and tissues. For instance, cytokines such as TGF-β, IL-4, IL-10, IL-11, and IL-13 can all decrease the TNF-α production by lipopolysaccharide-stimulated monocytes/macrophages (Essner et al., 1989; de Waal Malefyt et al., 1991; Bogdan and Nathan, 1993; Minty et al., 1993; Trepicchio et al., 1996). TGF-β exerts a multiplicity of biological activities on most cells and regulates many cell physiological processes. TGF-β is a potent immune differentiation and immunosuppressive agent and plays a key role in the normal mammalian development and in malignant transformation and tumor development (Derynck, 1994).

In general, the effects of cytokines can be profoundly influenced by the environment in which they act and especially by the presence or absence of other biologically active agents such as other cytokines, hormones, growth factors, or prostaglandins. Target cells are likely to be exposed to a cocktail of several cytokines on top of other biologically active agents, with the resulting biological action reflecting various synergistic and antagonistic interactions of these agents. For example, IL-4 can down-regulate IL-1-β production by activated monocytes but the addition of interferon γ (IFN-γ) suppresses this down-modulation (Geiger et al., 1993).

Cytokines produce their actions by binding to specific high-affinity cell surface receptors. The modulation of the level of cytokine receptor expression may represent another important mechanism in the regulation of cytokine activity. For instance, IFN-γ stimulates the expression of TNF receptors and conversely TNF-α up-regulates IFN-γ binding (Aggarwal et al., 1985; Raitano and Korc, 1990). These diverse interactions and pleiotropic activities of cytokines are generally referred to as the "cytokine network" (Table 14.1).

In most cell types, constitutive production of cytokines is usually low or absent. Cytokine expression is often involved in maintaining tissue homeostasis after tissue injury or infection and an increased production of cytokines contributes to the initiation and resolution of local inflammation

TABLE 14.1 Molecular Philosophy of Cytokine Actions

Redundancy	Different cytokines may have similar actions
Pleiotropism	A cytokine tends to have multiple target cells and multiple actions
Synergism/Antagonism	Exposure of cells to two or more cytokines at a time may lead to qualitatively different responses
Cytokine cascade	A cytokine may increase (or decrease) the production of another cytokine
Receptor transmodulation	A cytokine may increase (or decrease) the expression of receptors for another cytokine

and tissue repair. However, in certain conditions, exaggerated expression of cytokines can be implicated in the pathogenesis of disease, especially in disorders associated with exuberant inflammation. For example, TNF-α has dichotomous effects, depending on its concentrations. At low concentrations, TNF-α serves to protect the host against pathogens and governs physiological events like circadian rhythm of body temperature, sleep, and appetite (Strieter et al., 1993). However, at high concentrations, TNF-α induces pathophysiological host alterations such as in septic shock (Tracey and Cerami, 1994).

14.3.2 Pro- and Anti-Inflammatory Cytokines

"Proinflammatory" cytokines are early mediators of response to injury. These molecules are induced and expressed rapidly after exposure to noxious agents or after the recognition by host defenses of infection or neoplasia. Prototype cytokines in this category include IL-1 and TNF-α. Production of TNF-α and/or IL-1 induces recruitment and activation of inflammatory cells (Dinarello and Wolff, 1993; Tracey and Cerami, 1994). Moreover, these cytokines are also active on the nonimmune cells such as fibroblasts, smooth muscles, epithelial, and endothelial cells (Standiford et al., 1990). One of the most profound effects of the early response to TNF-α and IL-1 is the induction of a secondary cascade of cytokines. Two of the most important cytokines in this cascade are IL-8/CXCL8 (one of the multiple chemokines) and IL-6. However, it is now clear that other proinflammatory peptides such as other chemokines, including monocyte chemotactic peptide (MCP) and macrophage inflammatory protein (MIP), which possess a high degree of cellular specificity for the recruitment of specific leukocytes, are essential in the development and the maintenance of the inflammatory reaction (Strieter and Kunkel, 1997). In the same way, different adhesion molecules on endothelial cells are tightly regulated by TNF-α and IL-1, including intercellular adhesion molecule (ICAM), endothelial leukocyte adhesion molecule (ELAM), and vascular cell adhesion molecule (VCAM), which are also important for leukocyte recruitment in the inflammatory response (Pilewski and Albelda, 1993).

The degree and persistence of inflammation in the lung and other tissues are influenced by the balance between these last proinflammatory factors and other processes that down-regulate inflammation. In this respect, several cytokines such as IL-10, TGF-β, IL-4, IL-11, and IL-13 exhibit anti-inflammatory activities in a number of *in vitro* and *in vivo* models. In particular, experiments using mice lacking the ability to synthesize TGF-β or IL-10 (Kuhn et al., 1993b; Shull et al., 1992) have well demonstrated the unique and essential function of these cytokines in regulating inflammatory responses. Indeed, TGF-β or IL-10 knockout mice develop abnormal phenotype characterized by dramatic inflammatory lesions in multiple organs, which results in lethal cardiopulmonary failure and inflammatory bowel disease, respectively. Additionally, the administration of both these cytokines attenuates the inflammatory response (Kuruvilla et al., 1991; Walley et al., 1996). This effect is at least in part mediated by attenuating proinflammatory mediators such as TNF-β, IL-1, chemokines, and prostaglandin E2 (PGE2). In the same way, IL-4, IL-11, and IL-13 are also effective in down-regulating expression of the prototype proinflammatory cytokines TNF-β and IL-1 by

monocytes and macrophages (Essner et al., 1989; Minty et al,. 1993; Trepicchio et al., 1996). All these cytokines are known to increase the production of soluble receptors for TNF-β or IL-1 in both humans and animals. Because these receptors are still able to fix the ligand, it has been postulated that they serve as molecules limiting the proactivity of IL-1 and TNF-α and thus limit the propagation of inflammation (Ulich et al., 1994). Finally, IL-1 receptor antagonist (IL-1Ra) is a naturally occurring cytokine and a member of the interleukin-1 family whose only function is to prevent a biological response to IL-1. Thus, IL-1Ra possesses strong anti-inflammatory properties (Dinarello, 2000).

14.3.3 Cell Subsets Defined by Cytokine Production

The existence of reciprocal relationships between T-cell-mediated and antibody-mediated immunity has been recognized for many years (Parish, 1972). In a seminal study, Mosmann et al. (1986) demonstrated that mouse T helper ($CD4^+$) cell clones can be classified into distinct populations on the basis of their patterns of cytokine production. On one hand, the $CD4^+$ T-cell clone that predominantly produce interleukin-2 (IL-2), IFN-γ, and TNF-β were called T-helper 1 (TH1 or type 1) clones. On the other hand the $CD4^+$ T-cell clones predominantly producing IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 were called T-helper 2 (TH2 or type 2) clones. Evidence rapidly accumulated showing that TH1 and TH2 clones differed in the type of immunological responses they stimulate. Whereas TH1 clones drive cell-mediated responses, TH2 clones are more potent helpers for antibody-mediated responses, providing an explanation for the observed dichotomy of immune responses (Mosmann and Coffman, 1989). The maturation of TH1 cells is mainly driven by IL-12 production by activated macrophages. The maturation of TH2-type cells requires stimulation by IL-4. Cytokines produced by TH1 and TH2 T cells can modulate type 1 and type 2 immune responses by mutually inhibiting the maturation of precursor T cells and suppressing the effector cells. For example, IL-12 and IFN-γ (TH1) have been shown to inhibit TH2 T-cell maturation. Conversely, the type 2 cytokines IL-4 and IL-10 have been shown to inhibit the maturation of TH1 T cells and the production of IFN-γ (Abbas et al., 1996; Liew, 2002).

The polarization of the immune system appears to be an essential event for efficient control of numerous diseases and has clearly been demonstrated to be of benefit to the elimination of infectious agents or tumoral cells (Liew, 2002). For example, human and animal studies have demonstrated the beneficial and protective role of TH1 cell population in infection with *Leishmania major*. By contrast, in parasite models such as helminth infection, studies showed that TH2 cells were frequently correlated with a protective immunity (Liew, 2002). Numerous studies have indicated the overt role of TH1 or TH2 cells in leading to numerous immune disorders and pathologies. For instance, allergic reactions involving IgE and mast cells could be due to the development of allergen-specific TH2 cells, whereas inflammatory autoimmune diseases were shown to be frequently associated with overexpansion of TH1 lymphocytes (Liew, 2002). Recent works indicate that the TH1/TH2 concept could be extended to other immune cell types, having distinct effector functions and cytokine phenotypes and thus playing a major role in the regulation of particular immune response. On the basis of cytokine production, it has been shown that macrophages, eosinophils, NK, $CD8^+$, and

TABLE 14.2 Cytokine Subsets

Cytokine class	Functions	Examples
PRO-INFLAMMATORY	increase inflammation	TNF-α, IL-1, IL-6, IFN-γ, IL-12p70
ANTI-INFLAMMATORY	dampen established inflammation	IL-10, IL-4, IL-13, TGF-β, sRIL-1, sRTNF-α, IL-1Ra
TYPE 1	modulate cellular immune response	IFN-γ, IL-2, IL-12p70
TYPE 2	modulate humoral immune response	IL-4, IL-5, IL-10, IL-13, TGF-β

B lymphocytes may also adopt type 1 or type 2 phenotypes, depending of immune circumstances (Mosmann, 2000).

The different subsets of cytokines are summarized in Table 14.2.

14.3.4 Roles of Cytokines in Pneumoconioses

14.3.4.1 Alveolitis and Cytokines

An extensive and growing number of data indicate that fibrogenic particles early activate the production of proinflammatory cytokines within the respiratory tract. For example, normal human and/or rat alveolar macrophages exposed *in vitro* to silica, asbestosis, or coal mine dust release TNF-α, IL-1, and IL-6 (Schmidt et al., 1984; Oghiso and Kubota, 1987; Dubois et al,. 1989; Driscoll et al., 1990a; Gosset et al., 1991; Perkins et al., 1993; Zhang et al., 1993; Huaux et al., 1995). Relatively innocuous dust, such as titanium dioxide, aluminum oxide, or tungsten carbide, however, is markedly less effective in stimulating this response (Driscoll et al., 1990a; Gosset et al., 1991; Savici et al., 1994; Huaux et al., 1995). The relevance of these *in vitro* observations is supported by *in vivo* studies showing that alveolar macrophages from animals exposed to silica or asbestos also overproduce TNF-α and IL-1 early after treatment (Oghiso and Kubota, 1986; Driscoll et al., 1990b, 1991; Li et al., 1993). "Inert" dusts such as titanium dioxide do not induce, in the rat, similarly increased release of TNF-α or IL-1 by alveolar macrophages. Other proinflammatory cytokines such as chemokines are also expressed after exposure to fibrogenic dusts. For example, expression of MIP-1 α/CCL3 and α/CCL4, MCP-1/CCL2, and MIP-2 mRNA and proteins is markedly increased in the lungs of rats or mice exposed to silica. The up-regulation of these chemokines precedes the influx of inflammatory cells into the respiratory airways (Driscoll et al., 1993; Driscoll, 1994; Driscoll et al., 1995, 1996; Yuen et al., 1996; Hubbard et al., 2002; Pryhuber et al., 2003). The proinflammatory cascade implies the activation of transcription factors such as NF-kB and AP-1, largely implicated in the regulation of genes encoding inflammatory cytokines. Indeed, *in vitro* and *in vivo* studies suggested that silica and asbestos, but not nonfibrogenic particles, interact with pulmonary epithelial cells and alveolar macrophages to cause directly or indirectly (via TNF or oxidant production) the activation of these transcription factors (Savici et al., 1994; Chen et al., 1995; Janssen et al., 1995; Hubbard et al., 2002; Ortiz et al., 2001).

Several lines of evidence support the view that TNF-α plays a key role in the recruitment of inflammatory cells induced by toxic dusts. (a) In silica-exposed rats, a significant positive correlation exists between *in vivo* activation of macrophages, TNF-α release, and the recruitment of neutrophils in the lung (Driscoll, 1995; Driscoll and Maurer, 1991). (b) A similar correlation has also been shown between TNF-α levels in bronchoalveolar lavage fluid and neutrophilic inflammation in the lungs of humans exposed to asbestos (Zhang et al., 1993). (c) Pretreatment of rats with a monoclonal antibody against TNF-α significantly attenuated the pulmonary recruitment of neutrophils and decreased lung MIP-2 expression in response to silica (Driscoll, 1994, 1995). Similarly, deficiency in TNF receptor I was associated with a defect of MIP-2 synthesis in silica-treated mice (Pryhuber et al., 2003). (d) Finally, TNF-α release facilitates the attachment of leukocytes to the endothelium of blood vessels by stimulating the expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) on vascular endothelial cells in humans or mice exposed to coal mine or silica dusts, respectively (Nario and Hubbard, 1996; Vanhee et al., 1996).

In addition to alveolar macrophages, it is now clear that respiratory epithelial and endothelial cells as well as mesenchymal cells also play a major role in the establishment of the alveolitis induced by inhaled particles. For example, *in vitro* exposure of human and rat epithelial cells to asbestos fibers or silica particles increases the production of inflammatory mediators like MIP-2, IL-8/CXCL8, and ICAM-1 (Driscoll et al. 1993, 1996; Rosenthal et al., 1994; Simeonova and Luster, 1996; Stringer et al,. 1996; Treadwell et al., 1996). Concurrently, neutrophils recruited in an area of inflammation can also contribute to amplifying alveolitis by secreting inflammatory factors such as TNF-α and IL-1 (Kusaka et al., 1990; Xing et al., 1994b).

Despite this wealth of evidence concerning the role of proinflammatory cytokines in the early response to mineral particles, the contribution of anti-inflammatory cytokines, such as IL-10, IL-4, IL-11, IL-13, and TGF-β, has been studied only recently. By blocking or increasing IL-10 in silica-induced lung inflammation, Driscoll and colleagues were able to increase or limit the acute pulmonary response to particles (acute toxicity, neutrophils recruitment, and MIP-2 expression) (Driscoll et al., 1998). These results were in accordance with the observations obtained in silica-treated IL-10-deficient mice where the amplitude of inflammation and its consequences were significantly increased (Huaux et al., 1998). Mice and hamsters have the ability to produce more IL-10 than the rat, suggesting that different species and probably strains have different predisposition to control inflammation and subsequent damages (Carter and Driscoll, 2001; Huaux et al., 1999a). Indeed silica-treated mice and hamsters developed limited inflammation in comparison with exposed rats. The exact role of IL-4, IL-11, IL-13, and TGF-β during particle-induced pulmonary inflammation has not yet been tested, but on the basis of their anti-inflammatory capacity they might have an important role in the control of inflammation.

14.3.4.2 Fibrosis and Cytokines

14.3.4.2.1 Growth Factors

TGF-β is associated with the development of fibrosis in humans and experimental animals. In humans, elevated TGF-β expression has been observed in asbestosis (Khalil et al., 1996), silicosis (Jagirdar et al., 1996), and CWP (Vanhee et al., 1994). In a variety of animal models of pulmonary fibrosis, induction of TGF-β has been demonstrated after exposure to silica and asbestos but also after treatment with other fibrogenic agents such as ionizing radiation or bleomycin (Phan and Kunkel, 1992; Williams et al., 1993; Finkelstein et al., 1994; Perdue and Brody, 1994; Mariani et al., 1996). Giri and colleagues have shown that pretreatment with anti-TGF-β antibodies significantly decreases pulmonary fibrosis in different animal models (Denis, 1994; Giri et al., 1993). In addition, adenovector-mediated gene transfer of active TGF-β1 induces the development of lung fibrosis (Sime et al., 1997). Moreover, extensive *in vitro* data have demonstrated the essential role of TGF-β in fibrosis. TGF-β directly stimulates fibroblast proliferation (Border and Noble, 1994), myofibroblast differentiation, and expression of extracellular matrix proteins such as collagen (Phan, 2002) and fibronectin (Roberts et al., 1988) and causes inactivation of proteases (Laiho et al., 1986). However, in some cell systems, TGF-β may act as an inhibitor of fibroblast proliferation (Battegay et al., 1990), which again illustrates the pleiotropy of cytokine action. Collectively, these studies conclusively demonstrate a key role of TGF-β in the pathogenesis of lung fibrosis.

TGF-α, which has a potent mitogenic activity for epithelial and mesenchymal cells, is also up-regulated in the fibrotic lungs of rats exposed to asbestos fibers or silica particles. TGF-α may thus be critical in directing the proliferation of type II pneumocytes that are associated with pneumoconiosis (Absher et al., 1993; Liu et al., 1996). Overexpression of TGF-α in transgenic mice disrupts alveolar morphogenesis and produces fibrotic lesions (Korfhagen et al., 1994).

Several human studies have shown that alveolar macrophages recovered from the epithelial surface of the lungs of individuals with asbestosis, silicosis, and CWP spontaneously release significant amounts of growth factors such as PDGF (Lesur et al,. 1992; Melloni et al., 1994; Vanhee et al., 1994, Harvey and Crystal, 1997), IGF (Melloni et al., 1994; Vanhee et al., 1994; Jagirdar et al., 1996), and fibroblast growth factor (FGF) (Lesur et al., 1992; Melloni et al., 1994; Hamada et al., 2000). Authors have incriminated these mediators in the exaggerated wound healing and the proliferative response of type II epithelial cells occurring in malignant pneumoconioses. PDGF-like, IGF-like, and FGF-like molecules are released by human or rat alveolar macrophages exposed *in vitro* to asbestos and silica (Bonner et al., 1991; Melloni et al., 1996). In the same *in vitro* conditions, inert particles such as titanium dioxide had no effect on growth factor production (Melloni et al., 1993). Alveolar macrophages from rats or sheep treated with silica or asbestos spontaneously release growth factors such as PDGF and FGF, early after exposure and several months thereafter (Adamson et al., 1991, 1997).

It is possible, however, that cells other than alveolar macrophages (mesenchymal cells themselves or epithelial cells) release such growth factors (Liu et al., 1997). Pulmonary inflammation and fibrosis were observed in transgenic mice overexpressing the PDGF gene under the control of the lung-specific surfactant protein C (SPC) promoter, appointing this growth factor as a pivotal mediator in fibrogenesis (Hoyle et al., 1999).

14.3.4.2.2 Pro- and Anti-inflammatory Cytokines

In addition to their proinflammatory activity, TNF-α and IL-1 α and β can induce the accumulation of extracellular matrix proteins such as collagen and fibronectin in lung fibroblast cultures (Goldring and Krane, 1987; Postlethwaite et al., 1988; Zhang et al., 1993). TNF-α and IL-1 can stimulate fibroblast proliferation by inducing them to secrete PDGF, which in turn triggers cells to enter the cell cycle (Raines et al., 1989; Battegay et al., 1990). Thus, it is not surprising that increased levels of TNF-α and IL-1 have been observed in both human and animal lungs under conditions of developing fibrosis such as silicosis (Hartmann et al., 1984; Struhar et al., 1989b; Driscoll et al., 1990b; Mohr et al., 1991), asbestosis (Perkins et al., 1993; Zhang et al,. 1993), and CWP (Borm et al., 1988; Lassalle et al., 1990; Vanhee et al., 1995b; Vallyathan et al., 2000). Collagen deposition in silica-induced lung fibrosis was markedly decreased by administration of an anti-TNF-α antibody in mice (Piguet et al., 1990). In addition, administration of soluble TNF receptor or IL-1 receptor antagonist (IL-1 Ra) significantly reduced the extent of fibrosis observed after administration of silica in mice (Piguet and Vesin, 1994; Piguet et al., 1995). Transgenic mice that overexpress TNF-α spontaneously develop lung fibrotic reactions and, at the opposite, double knockout mice for TNF-α/lymphotoxin-α are resistant to material-induced pulmonary fibrosis (Miyazaki et al., 1995; Piguet et al., 1997). Besides, limited fibrotic response induced by asbestos or silica was observed in TNF receptors I and/or II knockout mice when compared with corresponding wild-type mice (Liu et al., 1998; Ortiz et al., 1999, 2001; Pryhuber et al., 2003). Deficiency in IL-1 β protects mice from silica-induced fibrosis (Srivastava et al., 2002). Increasing lung content of TNF and IL-1 β by transferring adenoviral constructs in the lungs of rodents also demonstrated that both cytokines participate directly in the development of inflammation and of collagen deposition (Sime et al., 1998; Kolb et al., 2001). From these elegant studies, authors concluded that both these proinflammatory mediators may play a central role in the production of other profibrotic factors such as TGF-α and β and PDGF, but also in the balance between MMP and TIMP expression in favor of matrix deposition. Remarkably, several reports pointed out an association between polymorphism in TNF-α promoter and in IL-1RA gene and silicosis and CWP. The authors suggest that these polymorphisms may confer increased risk for the development of the disease (Yucesoy et al., 2001a, 2001b; Corbett et al., 2002). Taken together, these findings demonstrate a causal relationship between excessive or persistent expression of IL-1 and TNF-α and the establishment of lung fibrosis.

Chemokines are essential mediators not only in the initiation but also in the persistence of alveolitis. It has been suggested that chemokines are responsible for specific recruitment of macrophages, lymphocytes, and neutrophils observed in pneumoconiosis during chronic lesions. For instance, silica-induced fibrotic lesions in mice are associated with marked expression of MIP-1 α/CCL3 (macrophage/monocyte and neutrophil chemoattractant), MIP-2/CXCL2/3 (neutrophil chemoattractant), MCP-1/CCL2 (macrophage/monocyte chemoattractant), and IP-10/CXCL10 (T-cell chemoattractant) (Pryhuber et al., 2003). Alveolar macrophages cultured *in vitro* from asbestos-exposed individuals spontaneously released significant amounts of the neutrophil chemotaxin, IL-8/CXCL8 (Broser et al., 1996). In addition, production of IL-8/CXCL8 from blood monocytes was significantly increased in coal miners with pneumoconiosis compared with healthy controls (Kim et al., 1999). Patients with CWP have a marked pulmonary overproduction of MCP-1/CCL2, suggesting that this chemokine may be responsible, at least in part, for the recruitment of monocytes observed in pneumoconiosis (Boitelle et al., 1997). Osteopontin, another cytokine considered as a macrophage chemoattractant, was found to be associated with silicosis (Nau et al., 1997). Besides their strong chemotactic activity, however, recent literature has identified additional roles for chemokines

in the pathogenesis of pulmonary fibrosis. MCP-1/CCL2 can directly stimulate fibroblast collagen expression via specific receptors and endogenous up-regulation of TGF-β (Gharaee Kermani et al., 1996). Angiogenic activity during lung fibrotic process is also potentially an important event in the extension of lung fibrosis. IL-8/CXCL8 in contrast to IP-10/CXCL10 can promote this angiogenic activity (Strieter et al., 2002). Collectively, these data suggest that chemokines are important mediators in the pathogenesis of lung fibrosis by controlling pulmonary accumulation of immune cells but also certain functions of fibroblasts and other lung cells.

Because anti-inflammatory cytokines such as IL-10 are relatively effective in the control of inflammation, several authors have suggested that this type of cytokine could also be considered in regulating fibrosis. However, several lines of evidence have demonstrated that anti-inflammatory factors may possess profibrotic functions. First, in human lung fibrosis anti-inflammatory cytokines were associated with up-regulation of IL-10 and IL-13 expression by alveolar macrophages (Martinez et al., 1997; Hancock et al., 1998; Cohen et al., 1999). Animal models of pneumoconiosis revealed that IL-10 was specifically expressed by alveolar macrophages after silica and well correlated with the establishment of fibrosis (Huaux et al., 1999a). Targeted expression of IL-10, IL-11, or IL-13 in the murine airways via the CC10 promoter caused airway remodeling with fibrosis, collagen deposition, and accumulation of mesenchymal cells (Tang et al., 1996; Lee et al. 2001, 2002). These elegant models supported the view that these anti-inflammatory cytokines may play a crucial role in the fibrotic responses in airway disorders. In the same way, IL-10 knockout (KO) mice treated with silica were relatively protected from silica-induced fibrosis, denoting that in certain circumstances, IL-10 can be considered as a profibrotic mediator despite its anti-inflammatory functions (Huaux et al., 1998). Finally, TGF-β, a well-established key fibrogenic mediator, is also a powerful anti-inflammatory cytokine (Shull et al., 1992; Border and Noble,1994). These results challenge the dogma that persistent pro-inflammatory mediators and inflammation lead to fibrosis. These recent observations could be interpreted as follows. During the inflammatory response, expression of anti-inflammatory cytokines (TGF-β, IL-4, IL-10, IL-11, and IL-13) can limit both the recruitment of inflammatory cells and the activity of proinflammatory mediators such as TNF-α. With the time, when the fibrotic reaction is initiated and involves other cell components (mesenchymal cells, lymphocytes), the high amount of anti-inflammatory cytokines produced could, in addition to their anti-inflammatory action, also act as profibrotic mediators, conceivably by stimulating mesenchymal cells directly or indirectly. This hypothetical view again illustrates the ambiguity of the cytokine network and emphasizes the fact that cytokines may have different activities on different cell types, here on inflammatory and mesenchymal cells.

14.3.4.2.3 Type-1 and Type-2 Cytokines

The predominance of $CD4^+$ T cells (helper/inducer T cells) is increasing both in humans (Rom and Travis, 1992) and in experimental pneumoconiosis (Kumar, 1989; Struhar et al., 1989a; Kumar et al., 1990; Li et al., 1992; Ueki et al., 1994), and an essential participation of T lymphocytes in the pathogenesis of lung fibrosis induced by inorganic dusts has been indicated in several experimental studies (Hubbard, 1989; Corsini et al., 1994; Suzuki et al., 1996). However, the determination of the subtype of T-helper lymphocytes (TH1 or TH2) is still under investigation and has apparently led to conflicting data.

Interest in the balance between type 1 and type 2 immune response in lung fibrosis was initiated from *in vitro* studies showing an opposite effect between IFN-γ (TH1 cytokine) and IL-4 (TH2 cytokine) on certain fibroblast functions. Indeed, IFN-γ suppresses collagen production by fibroblasts in culture, acting at the transcriptional level to turn off collagen gene expression (Elias et al., 1987; Sempowski et al,. 1994a, 1996; Varga et al., 1990). Lung fibroblasts in culture, which normally respond vigorously to PDGF, were inhibited from dividing by IFN-γ treatment (Badgett et al., 1996). Gene transfer and expression of IFN-γ in fibroblasts induced a suppression of type I collagen expression (Jaffe et al., 1997). However, treatment with IFN-γ of alveolar macrophages, obtained from normal rats or from patients with interstitial lung disease, increased expression of

PDGF (Shaw et al., 1991; Badgett et al,. 1996). In addition, normal macrophages treated with IFN-γ showed an enhanced transcription, translation, and synthesis of fibronectin (Cofano et al., 1984). Profibrotic activity of IL-4 was also first described *in vitro* with fibroblast cell lines obtained from different organs. Indeed, IL-4 has been reported to stimulate fibroblast chemotaxis (Postlethwaite and Seyer, 1991), proliferation (Sempowski et al,. 1994a), and contraction (Liu et al., 2002). In addition, IL-4 can stimulate myofibroblast differentiation as well as fibroblast production of extracellular matrix components, including collagen, fibronectin, and tenascin (Makhluf et al., 1996; Mattey et al., 1997; Oriente et al., 2000; Postlethwaite et al., 1992; Sempowski et al., 1994a, 1994b). However, this up-regulation of fibroblast functions seemed indirect (via TGF-β production) and was not observed with all fibroblast types. For instance, IL-4 was relatively ineffective in promoting procollagen 1 gene expression and myofibroblast transformation in certain fibroblasts (Huaux et al., 2003; Richter et al., 2001).

In humans, interstitial lung diseases such as idiopathic fibrosis and TH2 cytokines such as IL-4, IL-5 (Wallace et al., 1995; Furuie et al., 1997), IL-10 (Moller et al., 1996; Martinez et al., 1997), and IL-13 (Hancock et al., 1998) were all increased in pathological lungs, suggesting a key role of the type 2 polarization in the pathogenesis of lung fibrosis. Unfortunately, only partial information concerning IFN-γ and IL-4 expression are available in human pneumoconiosis. Inflammatory cells, recovered from the lung of nonsmokers with an history of exposure to asbestos, spontaneously release IFN-γ especially in those without respiratory impairment, suggesting a potential antifibrogenic role for IFN-γ in the pneumoconiosis (Rom and Travis, 1992). In CWP, amounts of IFN-γ in BALF were decreased as compared with the IFN-γ in the control group (Lesur et al., 1994).

Although, lung fibrosis is thought to be a type 2-related disease, several experimental studies with rodents have demonstrated a concomitant presence of both immune responses during the establishment of pneumoconiosis. For instances, lung and lung-associated lymph nodes from silicotic mice and rats displayed an enhanced IFN-γ and IL-18 gene transcription (Davis et al., 1999, 2000; Garn et al., 2000). No similar increase was noted by analyzing IL-4 expression in lymphocytes (Davis et al., 1999). In contrast, another group showed that silicosis in mice is associated with an up-regulation of IL-4 mainly in alveolar macrophages (Arras et al., 2001). In addition, other type 2 cytokines such as IL-10 and IL-12p40 are synthesized by silica-activated macrophages present in silicotic lesions (Huaux et al., 1999a, 2002). The exact importance of both these existing responses in the pathogenesis of pneumoconiosis was recently investigated by analyzing particle-induced lung responses of IFN-γ or IL-4-deficient animals. Unfortunately, these studies showed conflicting conclusions (Davis et al., 2001; Desaki et al., 2002). Thus it is still difficult to discriminate between preferential type 1 or type 2 immune response in particle-induced lung fibrosis in animals. This clearly argues for a more complicated mechanism, wherein both types of responses could promote fibrosis. On the other hand, type 2 cytokines also participate in the extension of fibrosis because transgenic mice overexpressing pulmonary IL-10 and IL-13 present signs of fibrosis (Lee et al. 2001, 2002). Numerous type 2 cytokines such as IL-10, IL-4, and IL-13 possess anti-inflammatory activities, supporting the concept that type 2 and anti-inflammatory cytokines are profibrotic mediators.

Other cytokines may be involved in the pathogenesis of pneumoconioses but their role in the fibrotic disease is still unclear and debated.

- The potential role of IL-9 has been investigated by Arras and coauthors because this TH2 cytokine is involved in asthma reaction (Arras et al., 2001). Transgenic mice overexpressing IL-9 developed significantly less pulmonary fibrosis after administration of silica. The limitation of the fibrotic process was accompanied by numerous B/B1 lymphocytes through the pulmonary parenchyma. These observations confer to this cytokine antifibrotic functions. However, the exact mechanism by which IL-9 can down-regulate fibrosis still needs to be explored.
- IL-12p70 (p35/p40) and its related cytokines IL-12p40 (p40/p40) and IL-23 (p40/p19) are preferentially produced by macrophages and thus may play important roles in

pneumoconiosis. In a recent study, it was demonstrated that IL-12p40 but not IL-12p70 was clearly associated with the extension of experimental silicosis (Huaux et al., 1999b). IL-12p40-deficient mice developed significantly less silica-induced lung fibrosis than treated wild-type and IL-12p35 KO mice (Huaux et al., 2002). In addition, recombinant IL-12p40 administered to silicotic mice induced specific recruitment of macrophages into the lung, demonstrating that this cytokine is, at least in part, involved in the macrophage accumulation observed in pneumoconiosis (Huaux et al., 2002). In line with data observed in IL-12p40-deficient mice, administration of blocking IL-12p40 antibodies significantly diminished bleomycin-induced fibrosis in mice (Maeyama et al., 2001). In contrast, in the same model of lung fibrosis, treatment with recombinant IL-12p70 decreased the intensity of fibrosis, potentially via its capacity to stimulate IFN-γ (Keane et al., 2001). Further investigations are necessary to clarify the exact role of IL-12 and its different subunits in lung fibrosis, especially the potential role of IL-23.

- Using adenoviral-mediated gene transfer techniques for overexpressing granulocyte macrophage-colony stimulating factor (GM-CSF) in the lung of rats, it has been demonstrated that GM-CSF may play a direct role in pulmonary fibrogenesis at least through its capability to induce TGF-β expression in macrophages (Xing et al., 1996, 1997). However, these results were not confirmed by administration of GM-CSF or anti-GM-CSF in a pulmonary fibrosis model induced by bleomycin (Piguet et al., 1993). In contrast, authors have suggested that this cytokine may possess antifibrotic functions because deficient mice developed marked bleomycin-induced fibrosis (Moore et al., 2000). The beneficial effect of GM-CSF could be attributed to its capacity to stimulate the production of PGE2 (an antifibrotic molecule).
- In CWP, IL-6 was found overexpressed by BAL cells and by alveolar macrophages in culture and was enhanced in BALF (Lesur et al., 1994; Vanhee et al., 1995b), suggesting a

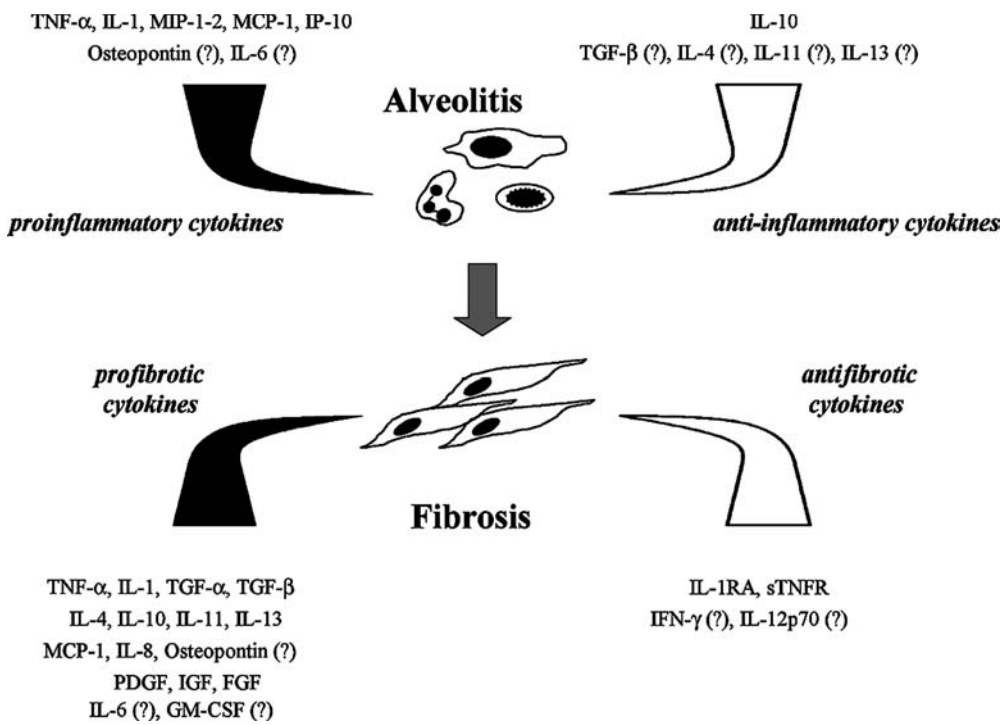


FIGURE 14.2 Activating and inhibiting cytokines in the alveolitis and fibrosis responses to inhaled particles.

profibrotic activity of IL-6. Overexpression of IL-6 by adenovector-mediated gene transfer results in a massive infiltration of the pulmonary parenchyma with lymphocytes (Xing et al., 1994a). IL-6 is considered as a growth factor for fibroblasts (Fries et al., 1994; Simeonova et al., 1997) and can inhibit collagen degradation (Bienkowski and Gotkin, 1995). An *in vivo* study, however, has suggested a protective effect of IL-6 in modulating the extent of fibrosis that follows acute lung injury (Denis, 1992).

- Osteopontin (OPN) expression was identified in the chronic stage of human silicosis and in the bleomycin-induced fibrosis model (Nau et al., 1997; Takahashi et al., 2001). Renal fibrosis after an acute ischemic insult is limited in OPN knockout mice (Persy et al., 2003). The authors of this study suggested that the reduced fibrotic response is potentially due to a lack in macrophage infiltration, essential in the propagation of fibrosis. Besides its activity on macrophage recruitment, a recent study has identified for this cytokine a new direct role in fibrosis. Indeed, recombinant OPN enhanced migration, adhesion, and PDGF-mediated proliferation of murine fibroblasts, demonstrating that OPN may also modulate fibroblast functions (Takahashi et al., 2001).

The known contributions of cytokines in both alveolitis and pulmonary fibrosis induced by mineral material are summarized in Figure 14.2.

14.4 CONCLUSION

Cytokines are of central importance in the regulation of immunity, inflammation, tissue remodeling, and embryonic development. They constitute a group of small proteins that regulate cell differentiation, proliferation, and cell secretory activity. Although cytokines play a key role in the immune system and normal physiology, uncontrolled or excessive cytokine production may contribute to the pathophysiology of acute and chronic disease. In particular, particle-induced pulmonary lesions are modulated and triggered by numerous cytokines produced in excess by pulmonary cells, especially alveolar macrophages but also epithelial cells and fibroblasts. They play critical roles in the chronic leukocyte accumulation and progressive fibrosis observed in pneumoconiosis. Proinflammatory cytokines such as TNF-α and IL-1 are key initiators of the lung response and play crucial roles in mediating inflammatory cell accumulation by inducing production of chemoattractants such as chemokines. The lung fibrotic response is also influenced by these proinflammatory cytokines and by growth factors including TGF-β, PDGF, epidermal growth factor, and FGF. Among these mediators, TGF-β appears to be an essential factor in the pathogenesis of fibrosis. There is a growing database suggesting that anti-inflammatory cytokines such as IL-10, IL-4, IL-11, and IL-13 have a detrimental activity during the establishment of lung fibrosis despite their capacity to control certain macrophage functions and the inflammatory process. The information is still insufficient to determine exactly if pneumoconiosis is preferentially associated with a specific type 1 or type 2 immune response. Future studies on the cytokine networks implicated in dust-induced alterations of lung structure and function are necessary to better understand basic mechanisms of pneumoconiosis.

REFERENCES

Abbas, A.K., Murphy, K.M., and Sher, A., Functional diversity of helper T lymphocytes, *Nature*, 383, 787–793, 1996.

Absher, M., Sjostrand, M., Baldor, L.C., Hemenway, D.R., and Kelley, J., Patterns of secretion of transforming growth factor-alpha (TGF- alpha) in experimental silicosis. Acute and subacute effects of cristobalite exposure in the rat, *Regul. Immunol.*, 5, 225–231, 1993.

Adamson, I.Y., Letourneau, H.L., and Bowden, D.H., Comparison of alveolar and interstitial macrophages in fibroblast stimulation after silica and long or short asbestos, *Lab. Investig.*, 64, 339–344, 1991.

Adamson, I.Y., Prieditis, H., and Young, L., Lung mesothelial cell and fibroblast responses to pleural and alveolar macrophage supernatants and to lavage fluids from crocidolite-exposed rats, *Am. J. Respir. Cell Mol. Biol.*, 16, 6, 650–656, 1997.

Aggarwal, B.B., Eessalu, T.E., and Hass, P.E., Characterization of receptors for human tumour necrosis factor and their regulation by gamma-interferon, *Nature*, 318, 665–667, 1985.

American Thoracic Society Committee, Adverse effects of crystalline silica exposure. American Thoracic Society Committee of the Scientific Assembly on Environmental and Occupational Health, *Am. J. Respir. Crit. Care Med.*, 155, 761–768, 1997.

Arras, M., Huaux, F., Vink, A., Delos, M., Coutelier, J.P., Many, M.C., Barbarin, V., Renauld, J.C., and Lison, D., Interleukin-9 reduces lung fibrosis and type 2 immune polarization induced by silica particles in a murine model, *Am. J. Respir. Cell Mol. Biol.*, 24, 368–375, 2001.

Badgett, A., Bonner, J.C., and Brody, A.R., Interferon-gamma modulates lung macrophage production of PDGF-BB and fibroblast growth, *J. Lipid Mediat. Cell Signal.*, 13, 1, 89–97, 1996.

Battegay, E.J., Raines, E.W., Seifert, R.A., Bowen Pope, D.F., and Ross, R., TGF-beta induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop, *Cell*, 63, 515–524, 1990.

Begin, R., Filion, R., and Ostiguy, G., Emphysema in silica- and asbestos-exposed workers seeking compensation. A CT scan study, *Chest*, 108, 3, 647–655, 1995.

Begin, R., Martel, M., Desmarais, Y., Drapeau, G., Boileau, R., Rola Pleszczynski, M., and Masse, S., Fibronectin and procollagen 3 levels in bronchoalveolar lavage of asbestos-exposed human subjects and sheep, *Chest*, 89, 237–243, 1986.

Begin, R., Ostiguy, G., Cantin, A., and Bergeron, D., Lung function in silica-exposed workers. A relationship to disease severity assessed by CT scan, *Chest*, 94, 539–545, 1988.

Begin, R., Ostiguy, G., Fillion, R., and Colman, N., Computed tomography scan in the early detection of silicosis, *Am. Rev. Respir. Dis.*, 144 (Part 1), 697–705, 1991.

Bienkowski, R.S. and Gotkin, M.G., Control of collagen deposition in mammalian lung, *Proc. Soc. Exp. Biol. Med.*, 209, 118–140, 1995.

Bitterman, P.B., Saltzman, L.E., Adelberg, S., Ferrans, V.J., and Crystal, R.G., Alveolar macrophage replication. One mechanism for the expansion of the mononuclear phagocyte population in the chronically inflamed lung, *J. Clin. Investig.*, 74, 460–469, 1984.

Blackford, J.A., Jr., Antonini, J.M., Castranova, V., and Dey, R.D., Intratracheal instillation of silica up-regulates inducible nitric oxide synthase gene expression and increases nitric oxide production in alveolar macrophages and neutrophils, *Am. J. Respir. Cell Mol. Biol.*, 11, 426–431, 1994.

Bogdan, C. and Nathan, C., Modulation of macrophage function by transforming growth factor beta, interleukin-4, and interleukin-10, *Ann. N. Y. Acad. Sci.*, 685, 713–739, 1993.

Boitelle, A., Gosset, P., Copin, M.C., Vanhee, D., Marquette, C.H., Wallaert, B., Gosselin, B., and Tonnel, A.B., MCP-1 secretion in lung from nonsmoking patients with coal worker's pneumoconiosis, *Eur. Respir. J.*, 10, 557–562, 1997.

Bonner, J.C., Osornio Vargas, A.R., Badgett, A., and Brody, A.R., Differential proliferation of rat lung fibroblasts induced by the platelet-derived growth factor-AA, -AB, and -BB isoforms secreted by rat alveolar macrophages, *Am. J. Respir. Cell Mol. Biol.*, 5, 539–547, 1991.

Border, W.A. and Noble, N.A., Transforming growth factor beta in tissue fibrosis, *N. Engl. J. Med.*, 331, 1286–1292, 1994.

Borges, V.M., Falcao, H., Leite-Junior, J.H., Alvim, L., Teixeira, G.P., Russo, M., Nobrega, A.F., Lopes, M.F., Rocco, P.M., Davidson, W.F., Linden, R., Yagita, H., Zin, W.A., and DosReis, G.A., Fas ligand triggers pulmonary silicosis, *J. Exp. Med.*, 194, 155–164, 2001.

Borm, P.J., Palmen, N., Engelen, J.J., and Buurman, W.A., Spontaneous and stimulated release of tumor necrosis factor- alpha (TNF) from blood monocytes of miners with coal workers' pneumoconiosis, *Am. Rev. Respir. Dis.*, 138, 1589–1594, 1988.

Borm, P.J., Schins, R., Janssen, Y.M., and Lenaerts, L., Molecular basis for differences in susceptibility to coal workers' pneumoconiosis, *Toxicol. Lett.*, 64–65, 767–772, 1992.

Broser, M., Zhang, Y., Aston, C., Harkin, T., and Rom, W.N., "Elevated interleukin-8 in the alveolitis of individuals with asbestos exposure, *Int. Arch. Occup. Environ. Health*, 68, 109–114, 1996.

Brown, D.M., Brown, G.M., Macnee, W., and Donaldson, K., Activated human peripheral blood neutrophils produce epithelial injury and fibronectin breakdown in vitro, *Inflammation*, 16, 21–30, 1992.

Burkhardt, A., Alveolitis and collapse in the pathogenesis of pulmonary fibrosis, *Am. Rev. Respir. Dis.*, 140, 513–524, 1989.

Calvert, G.M., Rice, F.L., Boiano, J.M., Sheehy, J.W., and Sanderson, W.T., Occupational silica exposure and risk of various diseases: an analysis using death certificates from 27 states of the United States, *Occup. Environ. Med.*, 60, 122–129, 2003.

Cantin, A., Allard, C., and Begin, R., Increased alveolar plasminogen activator in early asbestosis, *Am. Rev. Respir. Dis.*, 139, 604–609, 1989.

Carter, J.M. and Driscoll, K.E., The role of inflammation, oxidative stress, and proliferation in silica- induced lung disease: a species comparison, *J. Environ. Pathol. Toxicol. Oncol.*, 20 (Suppl. 1), 33–43, 2001.

Chen, F., Sun, S.C., Kuh, D.C., Gaydos, L.J., and Demers, L.M., Essential role of NF-kappa B activation in silica-induced inflammatory mediator production in macrophages, *Biochem. Biophys. Res. Commun.*, 214, 985–992, 1995.

Cofano, F., Comoglio, P. M., Landolfo, S., and Tarone, G., Mouse immune interferon enhances fibronectin production of elicited macrophages, *J. Immunol.*, 3102–3106, 1984.

Cohen, C., Fireman, E., Ganor, E., Man, A., Ribak, J., and Lerman, Y., Accelerated silicosis with mixed-dust pneumoconiosis in a hard-metal grinder, *J. Occup. Environ. Med.*, 41, 480–485, 1999.

Corbett, E.L., Mozzato-Chamay, N., Butterworth, A.E., De Cock, K.M., Williams, B.G., Churchyard, G.J., and Conway, D.J., Polymorphisms in the tumor necrosis factor-alpha gene promoter may predispose to severe silicosis in black South African miners, *Am. J. Respir. Crit. Care Med.*, 165, 690–693, 2002.

Corsini, E., Luster, M.I., Mahler, J., Craig, W.A., Blazka, M.E., and Rosenthal, G.J., A protective role for T lymphocytes in asbestos-induced pulmonary inflammation and collagen deposition, *Am. J. Respir. Cell Mol. Biol.*, 11, 531–539, 1994.

Crouch, E., Pathobiology of pulmonary fibrosis, *Am. J. Physiol.*, 259 (Part 1), L159–L184, 1990.

D'Armiento, J., Dalal, S.S., Okada, Y., Berg, R.A., and Chada, K., Collagenase expression in the lungs of transgenic mice causes pulmonary emphysema, *Cell*, 71, 955–961, 1992.

D'Ortho, M.P., Jarreau, P.H., Delacourt, C., Macquin Mavier, I., Levame, M., Pezet, S., Harf, A., and Lafuma, C., Matrix metalloproteinase and elastase activities in LPS-induced acute lung injury in guinea pigs, *Am. J. Physiol.*, 266 (Part 1), L209–L216, 1994.

Davis Smith, T., Silica, in *Occupational and Environmental Respiratory Disease*, 1st ed., Harber, P., Schenker, M.B., and Balmes, J.R., Eds., Mosby-Year Book, Inc, St. Louis, 1996, pp. 373–399.

Davis, G.S., Holmes, C.E., Pfeiffer, L.M., and Hemenway, D.R., Lymphocytes, lymphokines, and silicosis, *J. Environ. Pathol. Toxicol. Oncol.*, 20 (Suppl. 1), 53–65, 2001.

Davis, G.S., Pfeiffer, L.M., and Hemenway, D.R., Expansion of interferon-gamma-producing lung lymphocytes in mouse silicosis, *Am. J. Respir. Cell Mol. Biol.*, 20, 813–824, 1999.

Davis, G.S., Pfeiffer, L.M., and Hemenway, D.R., Interferon-gamma production by specific lung lymphocyte phenotypes in silicosis in mice, *Am. J. Respir. Cell Mol. Biol.*, 22, 491–501, 2000.

de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C.G., and de Vries, J.E., Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes, *J. Exp. Med.*, 174, 1209–1220, 1991.

Demers, L.M. and Kuhn, D.C., Influence of mineral dusts on metabolism of arachidonic acid by alveolar macrophage, *Environ. Health Perspect.*, 102 (Suppl. 10), 97–100, 1994.

Denis, M., Interleukin-6 in mouse hypersensitivity pneumonitis: changes in lung free cells following depletion of endogenous IL-6 or direct administration of IL-6, *J. Leukoc. Biol.*, 52, 197–201, 1992.

Denis, M., Neutralization of transforming growth factor-beta 1 in a mouse model of immune-induced lung fibrosis, *Immunology*, 82, 584–590, 1994.

Derynck, R., Transforming growth factor-beta, in *The Cytokine Handbook*, 2nd ed., Thomson, A. ed., Academic Press, London, 1994, pp. 319–342.

Desaki, M., Sugawara, I., Iwakura, Y., Yamamoto, K., and Takizawa, H., Role of interferon-gamma in the development of murine bronchus- associated lymphoid tissues induced by silica in vivo, *Toxicol. Appl. Pharmacol.*, 185, 1–7, 2002.

Dinarello, C.A., "The role of the interleukin-1-receptor antagonist in blocking inflammation mediated by interleukin-1", *N. Engl. J. Med.*, vol. 343, no. 10, pp. 732–734, 2000.

Dinarello, C.A. and Wolff, S.M., The role of interleukin-1 in disease, *N. Engl. J. Med.*, 328, 106–113, 1993. (Erratum 328, 744, 1993.)

Ding, M., Chen, F., Shi, X., Yucesoy, B., Mossman, B., and Vallyathan, V., Diseases caused by silica: mechanisms of injury and disease development, *Int. Immunopharmacol.*, 2, 173–182, 2002.

Driscoll, K.E., Macrophage inflammatory proteins: biology and role in pulmonary inflammation, *Exp. Lung Res.*, 20, 473–490, 1994.

Driscoll, K.E., Role of cytokines in pulmonary inflammation and fibrosis, in *Concept in Inhalation Toxicology*, 2nd ed., McClellan, R.O. and Henderson, R.F., Eds., Taylor & Francis, London, pp. 471–496, 1995.

Driscoll, K.E., Carter, J.M., Howard, B.W., Hassenbein, D., Burdick, M., Kunkel, S.L., and Strieter, R.M., Interleukin-10 regulates quartz-induced pulmonary inflammation in rats, *Am. J. Physiol*., 275 (Part 1), L887–L894, 1998.

Driscoll, K.E., Deyo, L.C., Carter, J.M., Howard, B.W., Hassenbein, D.G., and Bertram, T.A., Effects of particle exposure and particle-elicited inflammatory cells on mutation in rat alveolar epithelial cells, *Carcinogenesis*, 18, 423–430, 1997.

Driscoll, K.E., Hassenbein, D.G., Carter, J., Poynter, J., Asquith, T.N., Grant, R.A., Whitten, J., Purdon, M.P., and Takigiku, R., Macrophage inflammatory proteins 1 and 2: expression by rat alveolar macrophages, fibroblasts, and epithelial cells and in rat lung after mineral dust exposure, *Am. J. Respir. Cell Mol. Biol*., 8, 311–318, 1993.

Driscoll, K.E., Hassenbein, D.G., Howard, B.W., Isfort, R.J., Cody, D., Tindal, M.H., Suchanek, M., and Carter, J.M., Cloning, expression, and functional characterization of rat MIP- 2: a neutrophil chemoattractant and epithelial cell mitogen, *J. Leukoc. Biol*., 58, 359–364, 1995.

Driscoll, K.E., Higgins, J.M., Leytart, M.J., and Crosby, L., Differential effects of mineral dusts on the in vitro activation of alveolar macrophage eicosanoid and cytokine release, *Toxicol. In Vitro*, 4, 284–288, 1990a.

Driscoll, K.E., Howard, B.W., Carter, J.M., Asquith, T., Johnston, C., Detilleux, P., Kunkel, S.L., and Isfort, R.J., Alpha-quartz-induced chemokine expression by rat lung epithelial cells: effects of in vivo and in vitro particle exposure, *Am. J. Pathol*., 149, 1627–1637, 1996.

Driscoll, K.E., Lindenschmidt, R.C., Maurer, J.K., Higgins, J.M., and Ridder, G., Pulmonary response to silica or titanium dioxide: inflammatory cells, alveolar macrophage-derived cytokines, and histopathology, *Am. J. Respir. Cell Mol. Biol*., 2, 381–390, 1990b.

Driscoll, K.E., Lindenschmidt, R.C., Maurer, J.K., Perkins, L., Perkins, M., and Higgins, J., Pulmonary response to inhaled silica or titanium dioxide, *Toxicol. Appl. Pharmacol*., 111, 201–210, 1991.

Driscoll, K.E. and Maurer, J.K., Cytokine and growth factor release by alveolar macrophages: potential biomarkers of pulmonary toxicity, *Toxicol. Pathol*., 19 (Part 1), 398–405, 1991.

Dubois, C.M., Bissonnette, E., and Rola Pleszczynski, M., Asbestos fibers and silica particles stimulate rat alveolar macrophages to release tumor necrosis factor. Autoregulatory role of leukotriene B4, *Am. Rev. Respir. Dis*., 139, 1257–1264, 1989.

Eitzman, D.T., McCoy, R.D., Zheng, X., Fay, W.P., Shen, T., Ginsburg, D., and Simon, R.H., Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene, *J. Clin. Invest*., 97, 1, 232–237, 1996.

Elias, J.A., Jimenez, S.A., and Freundlich, B., Recombinant gamma, alpha, and beta interferon regulation of human lung fibroblast proliferation, *Am. Rev. Respir. Dis*., 135, 62–65, 1987.

Englen, M.D., Taylor, S.M., Laegreid, W.W., Silflow, R.M., and Leid, R.W., The effects of different silicas on arachidonic acid metabolism in alveolar macrophages, *Exp. Lung Res*., 16, 691–709, 1990.

Essner, R., Rhoades, K., McBride, W.H., Morton, D.L., and Economou, J.S., IL-4 down-regulates IL-1 and TNF gene expression in human monocytes, *J. Immunol*., 142, 3857–3861, 1989.

Ferry, G., Lonchampt, M., Pennel, L., de Nanteuil, G., Canet, E., and Tucker, G.C., Activation of MMP-9 by neutrophil elastase in an in vivo model of acute lung injury, *FEBS Lett*., 402, 111–115, 1997.

Finkelstein, J.N., Johnston, C.J., Baggs, R., and Rubin, P., Early alterations in extracellular matrix and transforming growth factor beta gene expression in mouse lung indicative of late radiation fibrosis, *Int. J. Radiat. Oncol. Biol. Phys*., 28, 621–631, 1994.

Flaherty, D.M., Monick, M.M., Carter, A.B., Peterson, M.W., and Hunninghake, G.W., Oxidant-mediated increases in redox factor-1 nuclear protein and activator protein-1 DNA binding in asbestos-treated macrophages, *J. Immunol*., 168, 5675–5681, 2002.

Franco, G. and Massola, A., Non-occupational pneumoconiosis at high altitude villages in central Ladakh, *Br. J. Ind. Med*., 49, 452–453, 1992.

Fries, K.M., Felch, M.E., and Phipps, R.P., Interleukin-6 is an autocrine growth factor for murine lung fibroblast subsets, *Am. J. Respir. Cell Mol. Biol*., 11, 552–560, 1994.

Furuie, H., Yamasaki, H., Suga, M., and Ando, M., Altered accessory cell function of alveolar macrophages: a possible mechanism for induction of Th2 secretory profile in idiopathic pulmonary fibrosis, *Eur. Respir. J*., 10, 787–794, 1997.

Garn, H., Friedetzky, A., Kirchner, A., Jager, R., and Gemsa, D., Experimental silicosis: a shift to a preferential IFN-gamma- based Th1 response in thoracic lymph nodes, *Am. J. Physiol. Lung Cell Mol. Physiol*., 278, L1221–L1230, 2000.

Geiger, T., Arnold, J., Rordorf, C., Henn, R., and Vosbeck, K., Interferon-gamma overcomes the glucocorticoid-mediated and the interleukin-4-mediated inhibition of interleukin-1 beta synthesis in human monocytes, *Lymphokine Cytokine. Res*., 12, 271–278, 1993.

Gharaee Kermani, M., Denholm, E. M., and Phan, S. H., Costimulation of fibroblast collagen and transforming growth factor beta1 gene expression by monocyte chemoattractant protein-1 via specific receptors, *J. Biol. Chem*., 271, 17779–17784, 1996.

Ghio, A. J., Kennedy, T. P., Whorton, A. R., Crumbliss, A. L., Hatch, G. E., and Hoidal, J. R., Role of surface complexed iron in oxidant generation and lung inflammation induced by silicates, *Am. J. Physiol*., 263(Part 1), L511–L518, 1992.

Giri, S. N., Hyde, D. M., and Hollinger, M. A., Effect of antibody to transforming growth factor beta on bleomycin induced accumulation of lung collagen in mice, *Thorax*, 48, 959–966, 1993.

Goldring, M. B. and Krane, S. M., Modulation by recombinant interleukin 1 of synthesis of types I and III collagens and associated procollagen mRNA levels in cultured human cells, *J. Biol. Chem*., 262, 16724–16729, 1987.

Gossart, S., Cambon, C., Orfila, C., Seguelas, M. H., Lepert, J. C., Rami, J., Carre, P., and Pipy, B., Reactive oxygen intermediates as regulators of TNF-alpha production in rat lung inflammation induced by silica, *J. Immunol*., 156, 1540–1548, 1996.

Gosset, P., Lassalle, P., Vanhee, D., Wallaert, B., Aerts, C., Voisin, C., and Tonnel, A. B., Production of tumor necrosis factor-alpha and interleukin-6 by human alveolar macrophages exposed in vitro to coal mine dust, *Am. J. Respir. Cell Mol. Biol*., 5, 431–436, 1991.

Gout, M., La silicose en France, *Travail Sante*, 550–551, 52–64, 1996.

Gozal, E., Ortiz, L. A., Zou, X., Burow, M. E., Lasky, J. A., and Friedman, M., Silica-induced apoptosis in murine macrophage: involvement of tumor necrosis factor-alpha and nuclear factor-kappaB activation, *Am. J. Respir. Cell Mol. Biol*., 27, 91–98, 2002.

Hamada, H., Vallyathan, V., Cool, C. D., Barker, E., Inoue, Y., and Newman, L. S., Mast cell basic fibroblast growth factor in silicosis, *Am. J. Respir. Crit. Care Med*., 161, 2026–2034, 2000.

Hancock, A., Armstrong, L., Gama, R., and Millar, A., Production of interleukin 13 by alveolar macrophages from normal and fibrotic lung, *Am. J. Respir. Cell Mol. Biol*., 18, 60–65, 1998.

Hartmann, D. P., Georgian, M. M., Oghiso, Y., and Kagan, E., Enhanced interleukin activity following asbestos inhalation, *Clin. Exp. Immunol*., 55, 643–650, 1984.

Harvey, B.-G. and Crystal, R. G., Pulmonary response to chronic inorganic dust exposure, in *The Lung*, 2nd ed., Vol. II, Crystal, R. G., West, J. B., Weibel, E. R., and Barnes, P. J., Eds., Lippincott-Raven, Philadelphia, PA, 1997, pp. 2339–2352.

Hayashi, T., Stetler Stevenson, W. G., Fleming, M. V., Fishback, N., Koss, M. N., Liotta, L. A., Ferrans, V. J., and Travis, W. D., Immunohistochemical study of metalloproteinases and their tissue inhibitors in the lungs of patients with diffuse alveolar damage and idiopathic pulmonary fibrosis, *Am. J. Pathol*., 149, 1241–1256, 1996.

Heppleston, A. G., Pathogenesis of mineral pneumoconioses, in *Occupational Lung Disorders*, 3rd ed., Parkes, R. W., Ed., Butterworth–Heinemann, Oxford, 1994, pp. 100–134.

Hoogsteden, H. C., van Dongen, J. J., van Hal, P. T., Delahaye, M., Hop, W., and Hilvering, C., Phenotype of blood monocytes and alveolar macrophages in interstitial lung disease, *Chest*, 95, 574–577, 1989.

Hoogsteden, H. C., van Hal, P. T., Wijkhuijs, J. M., Hop, W., and Hilvering, C., Differences in expression of monocyte/macrophage surface antigens in peripheral blood and bronchoalveolar lavage cells in interstitial lung diseases, *Lung*, 171, 149–160, 1993.

Hoyle, G. W., Li, J., Finkelstein, J. B., Eisenberg, T., Liu, J. Y., Lasky, J. A., Athas, G., Morris, G. F., and Brody, A. R., Emphysematous lesions, inflammation, and fibrosis in the lungs of transgenic mice overexpressing platelet-derived growth factor, *Am. J. Pathol*., 154, 1763–1775, 1999.

Huaux, F., Arras, M., Tomasi, D., Barbarin, V., Delos, M., Coutelier, J. P., Vink, A., Phan, S. H., Renauld, J. C., and Lison, D., A profibrotic function of IL-12p40 in experimental pulmonary fibrosis, *J. Immunol*., 169, 2653–2661, 2002.

Huaux, F., Arras, M., Vink, A., Renauld, J. C., and Lison, D., Soluble tumor necrosis factor (TNF) receptors p55 and p75 and interleukin-10 downregulate TNF-alpha activity during the lung response to silica particles in NMRI mice, *Am. J. Respir. Cell Mol. Biol*., 21, 137–145, 1999a.

Huaux, F., Lardot, C., Arras, M., Delos, M., Many, M.C., Coutelier, J.P., Buchet, J.P., Renauld, J.-C., and Lison, D., Lung fibrosis induced by silica particles in NMRI mice is associated with an upregulation of the p40 subunit of interleukin-12 and Th-2 manifestations, *Am. J. Respir. Cell Mol. Biol*., 20, 561–572, 1999b.

Huaux, F., Lasfargues, G., Lauwerys, R., and Lison, D., Lung toxicity of hard metal particles and production of interleukin-1, tumor necrosis factor-alpha, fibronectin, and cystatin-c by lung phagocytes, *Toxicol. Appl. Pharmacol*., 132, 53–62, 1995.

Huaux, F., Liu, T., McGarry, B., Ullenbruch, M., and Phan, S.H., Dual Roles of IL-4 in lung injury and fibrosis, *J. Immunol*., 170, 2083–2092, 2003.

Huaux, F., Louahed, J., Hudspith, B., Meredith, C., Delos, M., Renauld, J.-C., and Lison, D., Role of interleukin-10 (IL-10) in the lung response to silica in mice, *Am. J. Respir. Cell Mol. Biol*., 18, 51–59, 1998.

Hubbard, A.K., Role for T lymphocytes in silica-induced pulmonary inflammation, *Lab. Investig*., 61, 46–52, 1989.

Hubbard, A.K., Timblin, C.R., Shukla, A., Rincon, M., and Mossman, B.T., Activation of NF-kappaB-dependent gene expression by silica in lungs of luciferase reporter mice, *Am. J. Physiol Lung Cell Mol. Physiol.*, 282, L968–L975, 2002.

IARC, IARC Monograph on the evaluation of the carcinogenic risk of chemicals to humans, IARC Press, Vol. 68, 1997.

Jaffe, H.A., Gao, Z.C., Li, L.Y., and Varga, J., Suppression od type I collagen expression in fibroblasts by gene transfer and expression of interferon gamma, *Am. J. Respir. Crit. Care Med*., 155, A447, 1997.

Jagirdar, J., Begin, R., Dufresne, A., Goswami, S., Lee, T.C., and Rom, W.N., Transforming growth factor-beta (TGF-beta) in silicosis, *Am. J. Respir. Crit. Care Med*., 154 (Part 1), 1076–1081, 1996.

Janssen, Y.M., Barchowsky, A., Treadwell, M., Driscoll, K.E., and Mossman, B.T., Asbestos induces nuclear factor kappa B (NF-kappa B) DNA-binding activity and NF-kappa B-dependent gene expression in tracheal epithelial cells, *Proc. Natl. Acad. Sci. USA*, 92, 8458–8462, 1995.

Johnson, N.F., Smith, D.M., Sebring, R., and Holland, L.M., Silica-induced alveolar cell tumors in rats, *Am. J. Ind. Med*., 11, 93–107, 1987.

Kamp, D.W., Panduri, V., Weitzman, S.A., and Chandel, N., Asbestos-induced alveolar epithelial cell apoptosis: role of mitochondrial dysfunction caused by iron-derived free radicals, *Mol. Cell Biochem*., 234–235, 153–160, 2002.

Kawanami, O., Jiang, H.X., Mochimaru, H., Yoneyama, H., Kudoh, S., Ohkuni, H., Ooami, H., and Ferrans, V.J., Alveolar fibrosis and capillary alteration in experimental pulmonary silicosis in rats, *Am. J. Respir. Crit. Care Med*., 151, 1946–1955, 1995.

Keane, M.P., Belperio, J.A., Burdick, M.D., and Strieter, R.M., IL-12 attenuates bleomycin-induced pulmonary fibrosis, *Am. J. Physiol Lung Cell Mol. Physiol*, 281, L92–L97, 2001.

Khalil, N., O'Connor, R.N., Flanders, K.C., and Unruh, H., TGF-beta 1, but not TGF-beta 2 or TGF-beta 3, is differentially present in epithelial cells of advanced pulmonary fibrosis: an immunohistochemical study, *Am. J. Respir. Cell Mol. Biol*., 14, 131–138, 1996.

Kim, K.A., Lim, Y., Kim, J.H., Kim, E.K., Chang, H.S., Park, Y.M., and Ahn, B.Y., Potential biomarker of coal workers' pneumoconiosis, *Toxicol. Lett*., 108, 297–302, 1999.

Kipen, H.M., Lilis, R., Suzuki, Y., Valciukas, J.A., and Selikoff, I.J., Pulmonary fibrosis in asbestos insulation workers with lung cancer: a radiological and histopathological evaluation, *Br. J. Ind. Med*., 44, 96–100, 1987.

Knaapen, A.M., Albrecht, C., Becker, A., Hohr, D., Winzer, A., Haenen, G.R., Borm, P.J., and Schins, R.P., DNA damage in lung epithelial cells isolated from rats exposed to quartz: role of surface reactivity and neutrophilic inflammation, *Carcinogenesis*, 23, 1111–1120, 2002a.

Knaapen, A. M., Schins, R.P., Polat, D., Becker, A., and Borm, P.J., Mechanisms of neutrophil-induced DNA damage in respiratory tract epithelial cells, *Mol. Cell Biochem*., 234–235, 143–151, 2002b.

Kolb, M., Margetts, P.J., Anthony, D. C., Pitossi, F., and Gauldie, J., Transient expression of IL-1beta induces acute lung injury and chronic repair leading to pulmonary fibrosis, *J. Clin. Investig*., 107, 1529–1536, 2001.

Koren, H.S., Joyce, M., Devlin, R.B., Becker, S., Driscoll, K., and Madden, M.C., "Modulation of eicosanoid production by human alveolar macrophages exposed to silica in vitro, *Environ. Health Perspect*., 97, 77–83, 1992.

Korfhagen, T.R., Swantz, R.J., Wert, S.E., McCarty, J.M., Kerlakian, C.B., Glasser, S.W., and Whitsett, J.A., Respiratory epithelial cell expression of human transforming growth factor-alpha induces lung fibrosis in transgenic mice, *J. Clin. Investig*., 93, 1691–1699, 1994.

Kuhn, D. C., Griffith, J. W., Stauffer, J. L., Riling, S., and Demers, L. M., Characterization of alveolar macrophage eicosanoid production in a non-human primate model of mineral dust exposure, *Prostaglandins*, 46, 207–220, 1993a.

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K., and Muller, W., Interleukin-10-deficient mice develop chronic enterocolitis, *Cell*, 75, 263–274, 1993b.

Kumar, R. K., Quantitative immunohistologic assessment of lymphocyte populations in the pulmonary inflammatory response to intratracheal silica, *Am. J. Pathol*., 135, 605–614, 1989.

Kumar, R. K., Li, W., and O'Grady, R., Activation of lymphocytes in the pulmonary inflammatory response to silica, *Immunol. Investig*., 19, 363–372, 1990.

Kuruvilla, A. P., Shah, R., Hochwald, G. M., Liggitt, H. D., Palladino, M. A., and Thorbecke, G. J., Protective effect of transforming growth factor beta 1 on experimental autoimmune diseases in mice, *Proc. Natl. Acad. Sci. USA*,88, 2918–2921, 1991.

Kusaka, Y., Cullen, R. T., and Donaldson, K., Immunomodulation in mineral dust-exposed lungs: stimulatory effect and interleukin-1 release by neutrophils from quartz- elicited alveolitis, *Clin. Exp. Immunol*., 80, 293–298, 1990.

Laiho, M., Saksela, O., Andreasen, P. A., and Keski Oja, J., Enhanced production and extracellular deposition of the endothelial-type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factor-beta, *J. Cell Biol*., 103 (Part 1), 2403–2410, 1986.

Lardot, C., Heusterpreute, M., Mertens, P., Philippe, M., and Lison, D., Expression of plasminogen activator inhibitors type-1 and type-2 (PAI-1 and PAI-2) in the mouse lung after administration of crystalline silica, *Eur. Respir. J*., 11, 912–921, 1998a.

Lardot, C., Huaux, F., Broeckaert, F., Declerk, P. J., Delos, M., Fubini, B., and Lison, D., Role of urokinase in the fibrogenic response of the lung to mineral particles, *Am. J. Respir. Crit. Care Med*., 157, 617–628,1998b.

Lasfargues, G., Lison, D., Maldague, P., and Lauwerys, R., Comparative study of the acute lung toxicity of pure cobalt powder and cobalt-tungsten carbide mixture in rat, *Toxicol. Appl. Pharmacol*., 112, 41–50, 1992.

Lassalle, P., Gosset, P., Aerts, C., Fournier, E., Lafitte, J. J., Degreef, J. M., Wallaert, B., Tonnel, A. B., and Voisin, C., Abnormal secretion of interleukin-1 and tumor necrosis factor alpha by alveolar macrophages in coal worker's pneumoconiosis: comparison between simple pneumoconiosis and progressive massive fibrosis, *Exp. Lung Res*., 16, 73–80, 1990.

Lee, C. G., Homer, R. J., Cohn, L., Link, H., Jung, S., Craft, J. E., Graham, B. S., Johnson, T. R., and Elias, J. A., Transgenic overexpression of interleukin (IL)-10 in the lung causes mucus metaplasia, tissue inflammation, and airway remodeling via IL-13- dependent and -independent pathways, *J. Biol. Chem*., 277, 35466–35474, 2002.

Lee, C. G., Homer, R. J., Zhu, Z., Lanone, S., Wang, X., Koteliansky, V., Shipley, J. M., Gotwals, P., Noble, P., Chen, Q., Senior, R. M., and Elias, J. A., Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta(1), *J. Exp. Med*., 194, 809–821, 2001.

Lee, K. P. and Kelly, D. P., Translocation of particle-laden alveolar macrophages and intra- alveolar granuloma formation in rats exposed to Ludox colloidal amorphous silica by inhalation, *Toxicology*, 77, 205–222, 1993.

Lesur, O., Melloni, B., Cantin, A.M., and Begin, R., Silica-exposed lung fluids have a proliferative activity for type II epithelial cells: a study on human and sheep alveolar fluids, *Exp. Lung Res*., 18, 633–654, 1992.

Lesur, O. J., Mancini, N. M., Humbert, J. C., Chabot, F., and Polu, J. M., Interleukin-6, interferon-gamma, and phospholipid levels in the alveolar lining fluid of human lungs. Profiles in coal worker's pneumoconiosis and idiopathic pulmonary fibrosis, *Chest*, 106, 407–413, 1994.

Li, W., Kumar, R. K., O'Grady, R., and Velan, G. M., Role of lymphocytes in silicosis: regulation of secretion of macrophage-derived mitogenic activity for fibroblasts, *Int. J. Exp. Pathol*., 73, 793–800, 1992.

Li, X. Y., Brown, G. M., Lamb, D., and Donaldson, K., Increased production of plasminogen activator inhibitor in vitro by pleural leukocytes from rats intratracheally instilled with crocidolite asbestos, *Environ. Res*., 55, 135–144, 1991.

Li, X. Y., Lamb, D., and Donaldson, K., The production of TNF-alpha and IL-1-like activity by bronchoalveolar leucocytes after intratracheal instillation of crocidolite asbestos, *Int. J. Exp. Pathol*., 74, 403–410, 1993.

Liew, F. Y., T(H)1 and T(H)2 cells: a historical perspective, *Nat. Rev. Immunol*., 2, 55–60, 2002.

Limper, A. H. and Roman, J., Fibronectin. A versatile matrix protein with roles in thoracic development, repair and infection, *Chest*, 101, 1663–1673, 1992.

Liu, J. Y., Brass, D. M., Hoyle, G. W., and Brody, A. R., TNF-alpha receptor knockout mice are protected from the fibroproliferative effects of inhaled asbestos fibers, *Am. J. Pathol*., 153, 1839–1847, 1998.

Liu, J. Y., Morris, G. F., Lei, W. H., Corti, M., and Brody, A. R., Up-regulated expression of transforming growth factor-alpha in the bronchiolar-alveolar duct regions of asbestos-exposed rats, *Am. J. Pathol.,* 149, 205–217, 1996.

Liu, J. Y., Morris, G. F., Lei, W. H., Hart, C. E., Lasky, J. A., and Brody, A. R., Rapid activation of PDGF-A and -B expression at sites of lung injury in asbestos-exposed rats, *Am. J .Respir. Cell Mol. Biol*., 17, 129–140, 1997.

Liu, X., Kohyama, T., Wang, H., Zhu, Y. K., Wen, F. Q., Kim, H. J., Romberger, D. J., and Rennard, S. I., Th2 cytokine regulation of type I collagen gel contraction mediated by human lung mesenchymal cells, *Am. J. Physiol Lung Cell Mol. Physiol*, 282, L1049–L1056, 2002.

Maeyama, T., Kuwano, K., Kawasaki, M., Kunitake, R., Hagimoto, N., and Hara, N., Attenuation of bleomycin-induced pneumopathy in mice by monoclonal antibody to interleukin-12, *Am. J. Physiol Lung Cell Mol. Physiol*, 280, L1128–L1137, 2001.

Makhluf, H. A., Stepniakowska, J., Hoffman, S., Smith, E., LeRoy, E. C., and Trojanowska, M., IL-4 upregulates tenascin synthesis in scleroderma and healthy skin fibroblasts", *J. Investig. Dermatol*., 107, 856–859, 1996.

Manning, C. B., Vallyathan, V., and Mossman, B. T., Diseases caused by asbestos: mechanisms of injury and disease development, *Int. Immunopharmacol*., 2, 191–200, 2002.

Mariani, T. J., Roby, J. D., Mecham, R. P., Parks, W. C., Crouch, E., and Pierce, R. A., Localization of type I procollagen gene expression in silica- induced granulomatous lung disease and implication of transforming growth factor-beta as a mediator of fibrosis, *Am. J. Pathol*., 148, 151–164, 1996.

Martinez, J. A. B., King, T. E., Brown, K., Jennings, C. A., Borish, L., Mortenson, R. L., Khan, T. Z., Bost, T. W., and Riches, W. H., Increased expression of the interleukine-10 gene by alveolar macrophages in interstitial lung disease, *Am. J. Physiol*., 273 (Part 1), L676–L683, 1997.

Mattey, D. L., Dawes, P. T., Nixon, N. B., and Slater, H., Transforming growth factor beta 1 and interleukin 4 induced alpha smooth muscle actin expression and myofibroblast-like differentiation in human synovial fibroblasts in vitro: modulation by basic fibroblast growth factor, *Ann. Rheum. Dis*., 56, 426–431, 1997.

Melloni, B., Lesur, O., Bouhadiba, T., Cantin, A., and Begin, R., Partial characterization of the proliferative activity for fetal lung epithelial cells produced by silica-exposed alveolar macrophages, *J. Leukoc. Biol*., 55, 574–580, 1994.

Melloni, B., Lesur, O., Bouhadiba, T., Cantin, A., Martel, M., and Begin, R., Effect of exposure to silica on human alveolar macrophages in supporting growth activity in type II epithelial cells, *Thorax*, 51, 781–786, 1996.

Melloni, B., Lesur, O., Cantin, A., and Begin, R., Silica-exposed macrophages release a growth-promoting activity for type II pneumocytes, *J. Leukoc. Biol*., 53, 327–335, 1993.

Minty, A., Chalon, P., Derocq, J. M., Dumont, X., Guillemot, J. C., Kaghad, M., Labit, C., Leplatois, P., Liauzun, P., Miloux, B., Minty, C., Casellas, P., Loison, G., Lupker, J., Shine D., Ferrara, P., and Caput, D., Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses, *Nature*, 362, 248–250, 1993.

Miyazaki, Y., Araki, K., Vesin, C., Garcia, I., Kapanci, Y., Whitsett, J. A., Piguet, P. F., and Vassalli, P., Expression of a tumor necrosis factor-alpha transgene in murine lung causes lymphocytic and fibrosing alveolitis. A mouse model of progressive pulmonary fibrosis, *J. Clin. Invest*., 96, 250–259, 1995.

Mohr, C., Gemsa, D., Graebner, C., Hemenway, D. R., Leslie, K. O., Absher, P.M., and Davis, G. S., Systemic macrophage stimulation in rats with silicosis: enhanced release of tumor necrosis factor-alpha from alveolar and peritoneal macrophages, *Am. J. Respir. Cell Mol. Biol*., 5, 395–402, 1991.

Moller, D. R., Forman, J. D., Liu, M. C., Noble, P. W., Greenlee, B. M., Vyas, P., Holden, D. A., Forrester, J. M., Lazarus, A., Wysocka, M., Trinchieri, G., and Karp, C., Enhanced expression of IL-12 associated with Th1 cytokine profiles in active pulmonary sarcoidosis, *J. Immunol*., 156, 4952–4960, 1996.

Moore, B. B., Coffey, M. J., Christensen, P., Sitterding, S., Ngan, R., Wilke, C. A., McDonald, R., Phare, S. M., Peters-Golden, M., Paine, R., III, and Toews, G. B., GM-CSF regulates bleomycin- induced pulmonary fibrosis via a prostaglandin- dependent mechanism, *J. Immunol*., 165, 4032–4039, 2000.

Mosmann, T., Complexity or coherence? Cytokine secretion by B cells, *Nat. Immunol*., 1, 465–466, 2000.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., and Coffman, R. L., Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins, *J. Immunol*., 136, 2348–2357, 1986.

Mosmann, T.R. and Coffman, R.L., TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties, *Annu. Rev. Immunol.,* 7, 145–173, 1989.

Mossman, B.T., Bignon, J., Corn, M., Seaton, A., and Gee, J.B., Asbestos: scientific developments and implications for public policy, *Science,* 247, 294–301, 1990.

Nario, R.C. and Hubbard, A.K., Silica exposure increases expression of pulmonary intercellular adhesion molecule-1 (ICAM-1) in C57Bl/6 mice, *J. Toxicol. Environ. Health*, 49, 599–617, 1996.

Nau, G.J., Guilfoile, P., Chupp, G.L., Berman, J.S., Kim, S.J., Kornfeld, H., and Young, R.A., A chemoattractant cytokine associated with granulomas in tuberculosis and silicosis, *Proc. Natl. Acad. Sci. USA*, 94, 6414–6419, 1997.

Norboo, T., Angchuk, P.T., Yahya, M., Kamat, S.R., Pooley, F.D., Corrin, B., Kerr, I.H., Bruce, N., and Ball, K.P., Silicosis in a Himalayan village population: role of environmental dust, *Thorax*, 46, 341–343, 1991.

Oghiso, Y. and Kubota, Y., Enhanced interleukin 1 production by alveolar macrophages and increase in Ia-positive lung cells in silica-exposed rats, *Microbiol. Immunol*., 30, 1189–1198, 1986.

Oghiso, Y. and Kubota, Y., Interleukin 1 production and accessory cell function of rat alveolar macrophages exposed to mineral dust particles, *Microbiol. Immunol*., 31, 275–287, 1987.

Oppenheim, J.J., Foreword, in *The Cytokine Handbook*, 2nd ed., Thomson, A., Ed., Academic Press, London, 1994, p. XVII.

Oriente, A., Fedarko, N.S., Pacocha, S.E., Huang, S.K., Lichtenstein, L.M., and Essayan, D.M., Interleukin-13 modulates collagen homeostasis in human skin and keloid fibroblasts, *J. Pharmacol. Exp. Ther*., 292, 988–994, 2000.

Ortiz, L.A., Lasky, J., Gozal, E., Ruiz, V., Lungarella, G., Cavarra, E., Brody, A.R., Friedman, M., Pardo, A., and Selman, M., Tumor necrosis factor receptor deficiency alters matrix metalloproteinase 13/tissue inhibitor of metalloproteinase 1 expression in murine silicosis, *Am. J. Respir. Crit. Care Med*., 163, 244–252, 2001.

Ortiz, L.A., Lasky, J., Lungarella, G., Cavarra, E., Martorana, P., Banks, W.A., Peschon, J.J., Schmidts, H.L., Brody, A.R., and Friedman, M., Upregulation of the p75 but not the p55 TNF-alpha receptor mRNA after silica and bleomycin exposure and protection from lung injury in double receptor knockout mice, *Am. J. Respir. Cell Mol. Biol*., 20, 825–833, 1999.

Osornio-Vargas, A.R., Hernandez-Rodriguez, N.A., Yanez-Buruel, A.G., Ussler, W., Overby, L.H., and Brody, A.R., Lung cell toxicity experimentally induced by a mixed dust from Mexicali, Baja California, Mexico, *Environ. Res*., 56, 31–47, 1991.

Panduri, V., Weitzman, S.A., Chandel, N., and Kamp, D.W., The mitochondria-regulated death pathway mediates asbestos-induced alveolar epithelial cell apoptosis, *Am. J. Respir. Cell Mol. Biol*., 28, 241–248, 2003.

Pardo, A., Selman, M., Ramirez, R., Ramos, C., Montano, M., Stricklin, G., and Raghu, G., Production of collagenase and tissue inhibitor of metalloproteinases by fibroblasts derived from normal and fibrotic human lungs, *Chest,* 102, 1085–1089, 1992.

Parish, C.R., The relationship between humoral and cell-mediated immunity, *Transplant. Rev*., 13, 35–66, 1972.

Parkes, W.R., Non-fibrogenic ('inert') minerals and pneumoconiosis, in *Occupational Lung Disorders*, 3rd ed., Parkes, R.W., Ed., Butterworth-Heinemann, Oxford, 1998, pp. 253–284.

Perdue, T.D. and Brody, A.R., Distribution of transforming growth factor-beta 1, fibronectin, and smooth muscle actin in asbestos-induced pulmonary fibrosis in rats, *J. Histochem. Cytochem*., 42, 1061–1070, 1994.

Perez-Ramos, J., Lourdes Segura-Valdez, M., Vanda, B., Selman, M., and Pardo, A., "Matrix metalloproteinases 2, 9, and 13, and tissue inhibitors of metalloproteinases 1 and 2 in experimental lung silicosis, *Am. J. Respir. Crit Care Med*., 160, 1274–1282, 1999.

Perkins, R.C., Scheule, R.K., Hamilton, R., Gomes, G., Freidman, G., and Holian, A., Human alveolar macrophage cytokine release in response to in vitro and in vivo asbestos exposure, *Exp. Lung Res*., 19, 55–65, 1993.

Persy, V.P., Verhulst, A., Ysebaert, D.K., De Greef, K.E., and De Broe, M.E., "Reduced postischemic macrophage infiltration and interstitial fibrosis in osteopontin knockout mice, *Kidney Int*., 63, 543–553, 2003.

Phan, S.H., The myofibroblast in pulmonary fibrosis, *Chest*, 122 (Suppl), 286S–289S, 2002.

Phan, S.H. and Kunkel, S.L., Lung cytokine production in bleomycin-induced pulmonary fibrosis, *Exp. Lung Res*., 18, 29–43, 1992.

Piguet, P.F., Collart, M.A., Grau, G.E., Sappino, A.P., and Vassalli, P., Requirement of tumour necrosis factor for development of silica- induced pulmonary fibrosis, *Nature*, 344, 245–247, 1990.

Piguet, P.F., Grau, G.E., and de Kossodo, S., Role of granulocyte-macrophage colony-stimulating factor in pulmonary fibrosis induced in mice by bleomycin, *Exp. Lung Res*., 19, 579–587, 1993.

Piguet, P.F., Kaufman, S., Barazzone, C., Muller, M., Ryffel, B., and Eugster, H.P., Resistance of TNF/LT alpha double deficient mice to bleomycin-induced fibrosis, *Int. J. Exp. Pathol*., 78, 43–48, 1997.

Piguet, P.F., Tacchini Cottier, F., and Vesin, C., Administration of anti-TNF-alpha or anti-CD11a antibodies to normal adult mice decreases lung and bone collagen content: evidence for an effect on platelet consumption, *Am. J. Respir. Cell Mol. Biol*., 12, 227–231, 1995.

Piguet, P.F. and Vesin, C., Treatment by human recombinant soluble TNF receptor of pulmonary fibrosis induced by bleomycin or silica in mice, *Eur. Respir. J*., 7, 515–518, 1994.

Pilewski, J.M. and Albelda, S.M., Adhesion molecules in the lung. An overview, *Am. Rev. Respir. Dis*.,148 (Part 2), S31–S37, 1993.

Postlethwaite, A.E., Holness, M.A., Katai, H., and Raghow, R., Human fibroblasts synthesize elevated levels of extracellular matrix proteins in response to interleukin 4, *J. Clin. Investig*.,90, 1479–1485, 1992.

Postlethwaite, A.E., Raghow, R., Stricklin, G.P., Poppleton, H., Seyer, J.M., and Kang, A.H., Modulation of fibroblast functions by interleukin 1: increased steady-state accumulation of type I procollagen messenger RNAs and stimulation of other functions but not chemotaxis by human recombinant interleukin 1 alpha and beta, *J. Cell Biol*., 106, 311–318, 1988.

Postlethwaite, A.E. and Seyer, J.M., Fibroblast chemotaxis induction by human recombinant interleukin-4. Identification by synthetic peptide analysis of two chemotactic domains residing in amino acid sequences 70–88 and 89–122, *J. Clin. Investig*., 87, 2147–2152, 1991.

Pryhuber, G.S., Huyck, H.L., Baggs, R., Oberdorster, G., and Finkelstein, J.N., Induction of chemokines by low-dose intratracheal silica is reduced in TNFR I (p55) null mice", *Toxicol. Sci*., 72, 150–157, 2003.

Raines, E.W., Dower, S.K., and Ross, R., Interleukin-1 mitogenic activity for fibroblasts and smooth muscle cells is due to PDGF-AA, *Science*, 243, 393–396, 1989.

Raitano, A.B. and Korc, M., Tumor necrosis factor up-regulates gamma-interferon binding in a human carcinoma cell line, *J. Biol. Chem*., 265, 10466–10472, 1990.

Richter, A., Puddicombe, S.M., Lordan, J.L., Bucchieri, F., Wilson, S.J., Djukanovic, R., Dent, G., Holgate, S.T., and Davies, D.E., The contribution of interleukin (IL)-4 and IL-13 to the epithelial-mesenchymal trophic unit in asthma, *Am. J. Respir. Cell Mol. Biol*.,25, 3, 385–391. 2001,.

Roberts, C.J., Birkenmeier, T.M., McQuillan, J.J., Akiyama, S.K., Yamada, S.S., Chen, W.T., Yamada, K.M., and McDonald, J.A., Transforming growth factor beta stimulates the expression of fibronectin and of both subunits of the human fibronectin receptor by cultured human lung fibroblasts, *J. Biol. Chem*., 263, 4586–4592, 1988.

Robinson, B.W., Rose, A.H., James, A., Whitaker, D., and Musk, A.W., Alveolitis of pulmonary asbestosis. Bronchoalveolar lavage studies in crocidolite- and chrysotile-exposed individuals, *Chest*, 90, 396–402, 1986.

Rom, W.N., Relationship of inflammatory cell cytokines to disease severity in individuals with occupational inorganic dust exposure, *Am. J. Ind. Med*., 19, 15–27, 1991.

Rom, W.N., Bitterman, P.B., Rennard, S.I., Cantin, A., and Crystal, R.G., Characterization of the lower respiratory tract inflammation of nonsmoking individuals with interstitial lung disease associated with chronic inhalation of inorganic dusts, *Am. Rev. Respir. Dis*., 136, 1429–1434, 1987.

Rom, W.N. and Travis, W.D., Lymphocyte-macrophage alveolitis in nonsmoking individuals occupationally exposed to asbestos, *Chest*, 101, 779–786, 1992.

Rosenthal, G.J., Germolec, D.R., Blazka, M.E., Corsini, E., Simeonova, P., Pollock, P., Kong, L.Y., Kwon, J., and Luster, M.I., Asbestos stimulates IL-8 production from human lung epithelial cells, *J. Immunol*., 153, 3237–3244, 1994.

Savici, D., He, B., Geist, L.J., Monick, M.M., and Hunninghake, G.W., Silica increases tumor necrosis factor (TNF) production, in part, by upregulating the TNF promoter, *Exp. Lung Res*., 20, 613–625, 1994.

Schins, R.P., Duffin, R., Hohr, D., Knaapen, A.M., Shi, T., Weishaupt, C., Stone, V., Donaldson, K., and Borm, P.J., Surface modification of quartz inhibits toxicity, particle uptake, and oxidative DNA damage in human lung epithelial cells, *Chem. Res. Toxicol*., 15, 1166–1173, 2002a.

Schins, R.P., Knaapen, A.M., Cakmak, G.D., Shi, T., Weishaupt, C., and Borm, P.J., Oxidant-induced DNA damage by quartz in alveolar epithelial cells, *Mutat. Res*., 517, 77–86, 2002b.

Schmidt, J.A., Oliver, C.N., Lepe Zuniga, J.L., Green, I., and Gery, I., Silica-stimulated monocytes release fibroblast proliferation factors identical to interleukin 1. A potential role for interleukin 1 in the pathogenesis of silicosis, *J. Clin. Investig.,* 73, 1462–1472, 1984.

Sempowski, G.D., Beckmann, M.P., Derdak, S., and Phipps, R.P., Subsets of murine lung fibroblasts express membrane-bound and soluble IL-4 receptors. Role of IL-4 in enhancing fibroblast proliferation and collagen synthesis, *J. Immunol*., 152, 3606–3614, 1994b.

Sempowski, G.D., Beckmann, M.P., Derdak, S., and Phipps, R.P., Subsets of murine lung fibroblasts express membrane-bound and soluble IL-4 receptors. Role of IL-4 in enhancing fibroblast proliferation and collagen synthesis, *J. Immunol*., 152, 3606–3614, 1994a.

Sempowski, G.D., Derdak, S., and Phipps, R.P., Interleukin-4 and interferon-gamma discordantly regulate collagen biosynthesis by functionally distinct lung fibroblast subsets, *J. Cell Physiol*., 167, 290–296, 1996.

Shaw, R.J., Benedict, S.H., Clark, R.A., and King, T.E., Jr., Pathogenesis of pulmonary fibrosis in interstitial lung disease. Alveolar macrophage PDGF(B) gene activation and up-regulation by interferon gamma, *Am. Rev. Respir. Dis*., 143, 167–173, 1991.

Shih, J.F., Hunninghake, G.W., Goeken, N.E., Galvin, J.R., Merchant, J.A., and Schwartz, D.A., The relationship between HLA-A, B, DQ, and DR antigens and asbestos-induced lung disease, *Chest*, 104, 26–31, 1993.

Shull, M.M., Ormsby, I., Kier, A.B., Pawlowski, S., Diebold, R.J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., Annunziata, N., and Doetschnan, T., Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease, *Nature*, 359, 6397, 693–699, 1992.

Sime, P.J., Marr, R.A., Gauldie, D., Xing, Z., Hewlett, B.R., Graham, F.L., and Gauldie, J., Transfer of tumor necrosis factor-alpha to rat lung induces severe pulmonary inflammation and patchy interstitial fibrogenesis with induction of transforming growth factor-beta1 and myofibroblasts, *Am. J. Pathol*., 153, 825–832, 1998.

Sime, P.J., Xing, Z., Graham, F.L., Csaky, K.G., and Gauldie, J., Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung, *J. Clin. Investig*., 100, 768–776, 1997.

Simeonova, P.P. and Luster, M.I., Iron and reactive oxygen species in the asbestos-induced tumor necrosis factor-alpha response from alveolar macrophages, *Am. J. Respir. Cell Mol. Biol*., 12, 676–683, 1995.

Simeonova, P.P. and Luster, M.I. 1996, "Asbestos induction of nuclear transcription factors and interleukin 8 gene regulation", *Am. J. Respir. Cell Mol. Biol*., vol. 15, no. 6, pp. 787–795.

Simeonova, P.P., Toriumi, W., Kommineni, C., Erkan, M., Munson, A.E., Rom, W.N., and Luster, M.I., Molecular regulation of IL-6 activation by asbestos in lung epithelial cells: role of reactive oxygen species, *J. Immunol*., 159, 3921–3928, 1997.

Srivastava, K.D., Rom, W.N., Jagirdar, J., Yie, T.A., Gordon, T., and Tchou-Wong, K.M., Crucial role of interleukin-1beta and nitric oxide synthase in silica- induced inflammation and apoptosis in mice, *Am. J. Respir. Crit. Care Med*., 165, 527–533, 2002.

Standiford, T.J., Kunkel, S.L., Basha, M.A., Chensue, S.W., Lynch, J.P., III, Toews, G.B., Westwick, J., and Strieter, R.M., Interleukin-8 gene expression by a pulmonary epithelial cell line. A model for cytokine networks in the lung, *J. Clin. Investig*., 86, 1945–1953, 1990.

Steenland, K. and Brown, D., Silicosis among gold miners: exposure—response analyses and risk assessment, *Am. J. Public Health*, 85, 1372–1377, 1995.

Strieter, R.M., Belperio, J.A., and Keane, M.P., CXC chemokines in angiogenesis related to pulmonary fibrosis, *Chest*, 122 (Suppl), pp. 298S–301S, 2002.

Strieter, R.M. and Kunkel, S.L., Chemokines, in *The Lung*, 2nd ed., vol. I R.G. Crystal et al., eds., Lippincott-Raven, Philadelphia, PA, pp. 155–186. 1997,

Strieter, R.M., Kunkel, S.L., and Bone, R.C., Role of tumor necrosis factor-alpha in disease states and inflammation, *Crit. Care Med*., 21 (Suppl.), S447–S463, 1993.

Stringer, B., Imrich, A., and Kobzik, L., Lung epithelial cell (A549) interaction with unopsonized environmental particulates: quantitation of particle-specific binding and IL-8 production, *Exp. Lung Res*., 22, 495–508, 1996.

Struhar, D., Harbeck, R.J., and Mason, R.J., Lymphocyte populations in lung tissue, bronchoalveolar lavage fluid, and peripheral blood in rats at various times during the development of silicosis, *Am. Rev. Respir. Dis*., 139, 28–32, 1989a.

Struhar, D.J., Harbeck, R.J., Gegen, N., Kawada, H., and Mason, R.J., Increased expression of class II antigens of the major histocompatibility complex on alveolar macrophages and alveolar type II cells and interleukin-1 (IL-1) secretion from alveolar macrophages in an animal model of silicosis", *Clin. Exp. Immunol*., 77, 281–284, 1989b.

Suzuki, N., Ohta, K., Horiuchi, T., Takizawa, H., Ueda, T., Kuwabara, M., Shiga, J., and Ito, K., T lymphocytes and silica-induced pulmonary inflammation and fibrosis in mice, *Thorax*, 51, 1036–1042, 1996.

Takahashi, F., Takahashi, K., Okazaki, T., Maeda, K., Ienaga, H., Maeda, M., Kon, S., Uede, T., and Fukuchi, Y., Role of osteopontin in the pathogenesis of bleomycin-induced pulmonary fibrosis, *Am. J. Respir. Cell Mol. Biol*., 24, 264–271, 2001.

Takemura, T., Rom, W.N., Ferrans, V.J., and Crystal, R.G., Morphologic characterization of alveolar macrophages from subjects with occupational exposure to inorganic particles, *Am. Rev. Respir. Dis*., 140, 1674–1685, 1989.

Tang, W., Geba, G.P., Zheng, T., Ray, P., Homer, R.J., Kuhn, C., Flavell, R.A., and Elias, J.A., Targeted expression of IL-11 in the murine airway causes lymphocytic inflammation, bronchial remodeling, and airways obstruction, *J Clin. Investig*., 98, 2845–2853, 1996.

Thomas, G., Ando, T., Verma, K., and Kagan, E., Asbestos fibers and interferon-gamma up-regulate nitric oxide production in rat alveolar macrophages", *Am. J. Respir. Cell Mol. Biol*., 11, 707–715, 1994.

Torii, K., Iida, K., Miyazaki, Y., Saga, S., Kondoh, Y., Taniguchi, H., Taki, F., Takagi, K., Matsuyama, M., and Suzuki, R., Higher concentrations of matrix metalloproteinases in bronchoalveolar lavage fluid of patients with adult respiratory distress syndrome, *Am. J. Respir. Crit. Care Med*., 155, 43–46, 1997.

Tracey, K.J. and Cerami, A., Tumor necrosis factor: a pleiotropic cytokine and therapeutic target, *Annu. Rev. Med*., 45, 491–503, 1994.

Treadwell, M.D., Mossman, B.T., and Barchowsky, A., Increased neutrophil adherence to endothelial cells exposed to asbestos, *Toxicol. Appl. Pharmacol*., 139, 62–70, 1996.

Trepicchio, W.L., Bozza, M., Pedneault, G., and Dorner, A.J., Recombinant human IL-11 attenuates the inflammatory response through down-regulation of proinflammatory cytokine release and nitric oxide production, *J. Immunol*., 157, 3627–3634, 1996.

Ueki, A., Yamaguchi, M., Ueki, H., Watanabe, Y., Ohsawa, G., Kinugawa, K., Kawakami, Y., and Hyodoh, F., Polyclonal human T-cell activation by silicate in vitro, *Immunology*, 82, , 332–335, 1994.

Ulich, T.R., Yi, E.S., Yin, S., Smith, C., and Remick, D., Intratracheal administration of endotoxin and cytokines. VII. The soluble interleukin-1 receptor and the soluble tumor necrosis factor receptor II (p80) inhibit acute inflammation, *Clin. Immunol. Immunopathol*., 72, 137–140, 1994.

Vallyathan, V., Goins, M., Lapp, L.N., Pack, D., Leonard, S., Shi, X., and Castranova, V., Changes in bronchoalveolar lavage indices associated with radiographic classification in coal miners, *Am. J. Respir. Cell Mol. Biol*., 162, 958–965, 2000.

Vallyathan, V., Mega, J.F., Shi, X., and Dalal, N.S., Enhanced generation of free radicals from phagocytes induced by mineral dusts, *Am. J. Respir. Cell Mol. Biol*., 6, 404–413, 1992.

Vanhee, D., Gosset, P., Boitelle, A., Wallaert, B., and Tonnel, A.B., Cytokines and cytokine network in silicosis and coal workers' pneumoconiosis, *Eur. Respir. J*., 8, 834–842, 1995a.

Vanhee, D., Gosset, P., Marquette, C.H., Wallaert, B., Lafitte, J.J., Gosselin, B., Voisin, C., and Tonnel, A.B., Secretion and mRNA expression of TNF alpha and IL-6 in the lungs of pneumoconiosis patients," *Am. J. Respir. Crit. Care Med*., 152, 298–306, 1995b.

Vanhee, D., Gosset, P., Wallaert, B., Voisin, C., and Tonnel, A. B,. Mechanisms of fibrosis in coal workers' pneumoconiosis. Increased production of platelet-derived growth factor, insulin-like growth factor type I, and transforming growth factor beta and relationship to disease severity, *Am. J. Respir. Crit. Care Med*., 150, 1049–1055, 1994.

Vanhee, D., Molet, S., Gosset, P., Tillie Leblond, I., Boitelle, A., Wallaert, B., and Tonnel, A.B., Expression of leucocyte-endothelial adhesion molecules is limited to intercellular adhesion molecule-1 (ICAM-1) in the lung of pneumoconiotic patients: role of tumour necrosis factor-alpha (TNF-alpha), *Clin. Exp. Immunol*., 106, 541–548, 1996.

Varga, J., Olsen, A., Herhal, J., Constantine, G., Rosenbloom, J., and Jimenez, S.A., Interferon-gamma reverses the stimulation of collagen but not fibronectin gene expression by transforming growth factor-beta in normal human fibroblasts, *Eur. J. Clin. Investig*., 20, 487–493, 1990.

Wagner, G.R., Asbestosis and silicosis, *Lancet*, 349, 1311–1315, 1997.

Wagner, J.C., Burns, J., Munday, D.E., and McGee, J.O., Presence of fibronectin in pneumoconiotic lesions, *Thorax*, 37, 54–56, 1982.

Wallace, W. A., Ramage, E. A., Lamb, D., and Howie, S. E., A type 2 (Th2-like) pattern of immune response predominates in the pulmonary interstitium of patients with cryptogenic fibrosing alveolitis (CFA), *Clin. Exp. Immunol*., 101, 436–441, 1995.

Walley, K. R., Lukacs, N. W., Standiford, T. J., Strieter, R. M., and Kunkel, S. L., Balance of inflammatory cytokines related to severity and mortality of murine sepsis, *Infect. Immun*., 64, 4733–4738, 1996.

Weiss, S. J., Tissue destruction by neutrophils, *N. Engl. J. Med*., 320, 365–376, 1989.

WHO, Silicosis, World Health Organization Information, Fact Sheet 238, 2000.

Williams, A. O., Flanders, K. C., and Saffiotti, U., Immunohistochemical localization of transforming growth factor- beta 1 in rats with experimental silicosis, alveolar type II hyperplasia, and lung cancer, *Am. J. Pathol*., 142, 1831–1840, 1993.

Wolff, G. and Crystal, R. G., Biology of pulmonary fibrosis, in *The Lung*, 2nd ed., Vol. 2, Crystal, R. G., et al., Eds., Lippincott–Raven, Philadelphia, 1997, pp. 2509–2524.

Xing, Z., Braciak, T., Jordana, M., Croitoru, K., Graham, F. L., and Gauldie, J., Adenovirus-mediated cytokine gene transfer at tissue sites. Overexpression of IL-6 induces lymphocytic hyperplasia in the lung, *J. Immunol*., 153, 4059–4069, 1994a.

Xing, Z., Braciak, T., Ohkawara, Y., Sallenave, J. M., Foley, R., Sime, P. J., Jordana, M., Graham, F. L., and Gauldie, J., Gene transfer for cytokine functional studies in the lung: the multifunctional role of GM-CSF in pulmonary inflammation, *J. Leukoc. Biol*., 59, 481–488, 1996.

Xing, Z., Jordana, M., Kirpalani, H., Driscoll, K. E., Schall, T. J., and Gauldie, J., Cytokine expression by neutrophils and macrophages in vivo: endotoxin induces tumor necrosis factor-alpha, macrophage inflammatory protein-2, interleukin-1 beta, and interleukin-6 but not RANTES or transforming growth factor-beta 1 mRNA expression in acute lung inflammation, *Am. J. Respir. Cell Mol. Biol*., 10, 148–153, 1994b.

Xing, Z., Tremblay, G. M., Sime, P. J., and Gauldie, J., Overexpression of granulocyte-macrophage colony-stimulating factor induces pulmonary granulation tissue formation and fibrosis by induction of transforming growth factor-beta 1 and myofibroblast accumulation, *Am. J. Pathol*., 150, 59–66, 1997.

Yucesoy, B., Vallyathan, V., Landsittel, D. P., Sharp, D. S., Matheson, J., Burleson, F., and Luster, M. I., Polymorphisms of the IL-1 gene complex in coal miners with silicosis, *Am. J. Ind. Med*., 39, 286–291, 2001a.

Yucesoy, B., Vallyathan, V., Landsittel, D. P., Sharp, D. S., Weston, A., Burleson, G. R., Simeonova, P., McKinstry, M., and Luster, M. I., Association of tumor necrosis factor-alpha and interleukin-1 gene polymorphisms with silicosis, *Toxicol. Appl. Pharmacol*., 172, 75–82, 2001b.

Yuen, I. S., Hartsky, M. A., Snajdr, S. I., and Warheit, D. B., Time course of chemotactic factor generation and neutrophil recruitment in the lungs of dust-exposed rats, *Am. J. Respir. Cell Mol. Biol*., 15, 268–274, 1996.

Zhang, Y., Lee, T. C., Guillemin, B., Yu, M. C., and Rom, W. N., Enhanced IL-1 beta and tumor necrosis factor-alpha release and messenger RNA expression in macrophages from idiopathic pulmonary fibrosis or after asbestos exposure, *J. Immunol*., 150, 4188–4196, 1993.

15 Hormesis: Implications for Risk Assessment

Edward J. Calabrese

CONTENTS

15.1 INTRODUCTION

The field of toxicology has been strongly affected by the assumption that there are no treatment-related effects for noncarcinogens below the no observed adverse effect level (NOAEL). This assumption has had a profound effect on how studies are designed, including the number and spacing of doses, the types of hypotheses tested, and even the biological models selected. In the case of carcinogens it has not been feasible to conduct studies that attempt to estimate risks that approach and are less than about 1/100 because of the large number of animal resources needed. This condition also has had a significant impact on study design and model selection, as noted above. These two factors, the assumption of a threshold response for noncarcinogens and the lack of capacity to estimate and/or validate low-frequency cancer risks, tend to functionally converge, resulting in study designs for bioassays of noncarcinogens and carcinogens that are similar in terms of numbers and spacing of doses.

Despite the convergence of study designs in the assessment of noncarcinogens and carcinogens both utilize different dose–response assumptions. In noncarcinogens the threshold model is universally accepted whereas in carcinogens the assumption of linearity at low doses prevails. As for noncarcinogens a safe dosage has been assumed if it is below the NOAEL. In carcinogens no theoretical safe dose can exist because risk is proportionate to dose. Thus, in the assessment of carcinogens the concept of safe has given way to what is viewed as acceptable (e.g., one cancer to million per 70-year lifetime).

For the first three-fourths of the past century, in general, it was accepted that the threshold model strongly predominated in the assessment of dose–response relationships and could be applied to all end points measured including carcinogenesis. However, by the late 1950s the threshold assumption had given way to a linear no threshold (LNT) model for assessment of the risks of radiation-induced

cancer. This transition from a threshold evaluation for carcinogens to the LNT for radiation became inclusive of chemical carcinogens by the mid-1970s (National Academy of Sciences [NAS]/Safe Drinking Water Committee [SDWC], 1977).

The implementation of the LNT model into the risk assessment process proved to be significant in several ways. Most importantly, it dramatically increased the cost of both compliance and remediation for possible carcinogens as compared with what a threshold assumption would have yielded. The enormous costs imposed on industry and several governmental agencies has led to a long series of challenges to regulatory agencies such as the Environmental Protection Agency (EPA) to alter their positions on the LNT default assumption. In general, these challenges have asserted that linearity cannot be validated at low levels of risk and that adaptive responses are nearly universally present and active at ambient exposure levels. Thus, the weight of evidence would argue that linearity should not only not be assumed and used as the default but should be seen as a rare exception at most.

Although this line of argumentation has merit, it is often impossible to distinguish a linear from a threshold response when the study design only has two to four doses. In such cases it is common for the data to be compatible with both dose-response models. This lack of capacity to adequately distinguish the threshold model from the linear model for carcinogens brought new life to a long discredited dose–response model, the hormesis model, which is characterized by a J- or inverted U-shaped dose–response depending on the end point measured (Figure 15.1). In the case of disease incidence, such as cancer, the hormetic dose–response model, which would display a J-shaped curve, asserts that at low doses one would expect the risk to be lower than the controls while increasing to above control values as the dose increases. The inverted U-shaped dose–response would be seen when end points such as growth, longevity, or fecundity are reported.

The hormetic model offered several attractive features. First, it was compatible with the threshold model at the higher doses (i.e., above threshold). Secondly, the hormetic model can actually be tested in the experimental zone with an appropriate study design because one would expect the disease incidence to dip below the controls. Thus, in a fit of environmental exasperation with the extremely high costs of regulating carcinogens and given the attractive features of the hormetic model above, the hormesis hypothesis was essentially reborn in the mid-1980s.

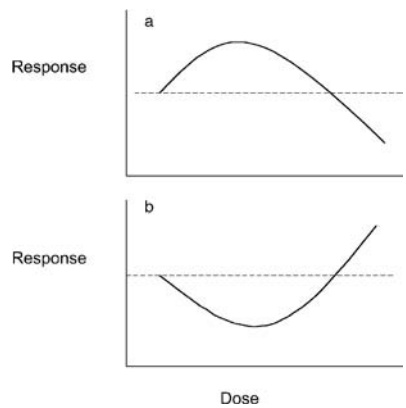


FIGURE 15.1 A generalized representation of the hormetic dose–response relationship. (a) The most common form of the hormetic dose–response curve depicting low-dose stimulatory and high-dose inhibitory response, the β- or inverted U-shaped curve. (b) The hormetic dose–response curve depicting low-dose reduction and high-dose enhancement of adverse effect, the J- or U-shaped curve.

Despite this recent rebirth the history of hormesis stretches back over a century to the 1880s when Hugo Schulz reported that low doses of various disinfectants enhanced the metabolism of yeasts but high doses were inhibitory. In the following quote, Schulz recounts the moment of discovery when what we call hormesis today was first observed.

> Since it could be foreseen that experiments on fermentation and putrescence in an institute of pathology would offer particularly good prospects for vigorous growth, I occupied myself as well as possible, in accordance with the state of our knowledge at the time, with this area. Sometimes, when working with substances that needed to be examined for their effectiveness in comparison to the inducers of yeast fermentation, initially working together with my assistant, Gottfried Hoffmann, I found in formic acid and also in other substances the marvelous occurrence that if I got below their indifference point, i.e., if, for example, I worked with less formic acid than was required in order to halt the appearance of its anti-fermentive property, that all at once the carbon dioxide production became distinctly higher than in the controls processed without the formic acid addition. I first thought, as is obvious, that there had been some kind of experimental or observation error. But the appearance of the overproduction continually repeated itself under the same conditions. First I did not know how to deal with it, and in any event at that time still did not realize that I had experimentally proved the first theorem of Arndt's fundamental law of biology. (From Schulz, 1923; 2003 translation.)

The discovery of this biphasic response by Schulz (1888) was significant not only for the reproducible nature of the dose–response but perhaps, more importantly, the interpretation that Schulz attached to the findings. That is, he considered these findings as providing the explanatory principle on which the medical practice of homeopathy was based. This interpretation proved to be important because Schulz embraced homeopathy throughout his long professional career and provided a focal point of opposition from the traditional medical establishment. This very close association of what would later be called hormesis with homeopathy proved to be a "scarlet letter" on the forehead of the new hormetic hypothesis that has undermined its acceptance even today, especially in the world of human-oriented toxicology, which originated in departments of pharmacology within traditional schools of medicine.

Despite its association with homeopathy and being the object of significant professional antipathy, the hypothesis of Schulz was confirmed and extended by many independent investigators who had no ostensible association with homeopathy or traditional medicine. In general, supportive findings were reported in research with plants and with microbes such as yeasts and fungi, and insects. These findings were substantial and published by scientists of extremely high regard. In fact, these findings were so common and expected that they became incorporated into leading plant (MacDougal, 1901; Peirce, 1909; Maximov, 1938) and microbiological textbooks (Salle, 1939; Clifton, 1957; Lamanna and Mallette, 1965) of the early and middle decades of the twentieth century (see Calabrese and Baldwin, 2000a through 2000e for a detailed review of the historical foundations of hormesis).

Even though the Schulz perspective, which became known as the Arndt–Schulz Law, had a relatively strong start in the final years of the nineteenth century and early decades of the twentieth century, it failed to prosper and become incorporated into modern toxicology. The reasons for this failure are many, including (1) the high-level opposition it received from major intellectual forces in the field of pharmacology, such as the profoundly influential A. J. Clark of the University of Edinburgh and his major text, the *Handbook of Pharmacology* (1937); (2) the principal interest in high-dose toxicology to determine safe concentrations of exposure to industrial workers and the need to protect people and food supplies from insects; (3) the ambiguous nature of what the significance of a modest low-dose stimulation was; (4) the failure of overzealous entrepreneurs to convert the low-dose stimulation into financial success; (5) the difficulty in replicating low-dose stimulatory findings especially with weak study designs; (6) the emerging concerns with agents such as radiation that appear to affect mutation rate in a linear fashion; (7) the failure to organize the supportive findings and to counter the arguments leveled against it.

The field of toxicology in the United States became notably better organized in the 1930s under the leadership of the National Research Council (NRC), which was providing guidance to the U.S. Government on radiation health effects. The concept of hormesis did not become incorporated into

the centrality of the field during this time, resulting in its absence from university teaching, research funding, and the agenda of the leaders in the field. As a result the concept of hormesis lost out in a classic musical chairs contest and took its place on the margins.

Despite marginalization of hormesis, hormetic dose–responses continued to be observed and reported in the literature. In 1943, researchers at the University of Idaho, who were studying the effects of extracts from the Red Cedar tree on fungal metabolism, reported a low-dose stimulation and a high-dose inhibition and called the phenomenon "hormesis," after the Greek word to excite (Southam and Ehrlich, 1943). These researchers appeared to be unaware of the earlier research on the topic and the term the Arndt–Schulz Law.

The choice of terms to label the low-dose stimulation, high-dose inhibition has been problematic over the decades. In fact, this phenomenon may be found with a wide range of "scientific" labels such as hormesis, Arndt–Schulz Law, Hueppe's Rule, biphasic, bimodal, bifunctional, U-shaped, J-shaped, stimulatory-inhibitory, ambivalent-dependent response, overcompensation, and others. The labels reflect a wide range of subbiological disciplines in which hormetic dose–responses have been reported and their lack of communication with each other. It also reflects the lack of a precise definition of the phenomenon.

Even though the hormetic concept has been controversial and marginalized, this phenomenon was recognized early on as being best described as a dose–time–response characterized by an initial disruption in homeostasis, followed by a modest overcompensation response. This characterization was first reported by Townsend in the late 1890s (Townsend, 1897) and repeatedly confirmed in multiple biological models throughout the twentieth century (Branham, 1929; Hektoen, 1920; Smith, 1935; Taliaferro and Taliaferro, 1951; Warren, 1945) (Figure 15.2). However, the lack of appreciation that the low-dose stimulation could result from an overcompensation response was an important blind spot. Compensatory responses are intended to reestablish homeostasis within a reasonable time after injury. The affected biological system allocates only a measured amount of resources to ensure the reestablishment of the homeostatic condition. This most likely accounts for the overwhelming observation that hormetic stimulatory responses (i.e., the overcompensation response) are modest and rarely exceed twice the control value and most often are less than 30 to 40% greater than control values. It was this lack of appreciation of the overcompensation nature of the stimulatory response that led some influential scientists of the early twentieth century to discount the Arndt–Schulz Law because they believed that it represents a direct stimulatory response rather than a compensatory process in response to injury (Warren, 1945; see Calabrese and Baldwin, 2000c).

The overcompensatory nature of the hormetic response was placed on more solid ground with research published in the late 1970s and 1980s by Stebbing (1982) and later by Calabrese (2001). Nonetheless, it is important to recognize that most studies do not have the capacity to assess the overcompensation mechanism hypothesis to explain hormetic responses due to either a lack of a temporal factor and/or an inadequate number and spacing of doses. Consequently, the majority of data to which "hormetic" effects are attributed lack temporal data, thereby making it impossible to ascribe a compensatory process/mechanism in these cases. Nevertheless, a large amount of data still exists to establish the reliability of the overcompensation interpretation.

The overcompensation hypothesis has provided a cogent explanation that can account for the modest magnitude of the stimulation and the relationship of the upper end of the stimulatory zone to the traditional NOAEL. That is, reparative compensatory responses would not be expected to grossly overshoot the homeostatic target and be extremely wasteful of resources. The modest stimulatory responses in the 20 to 50% above control zone, while challenging to establish in a statistical sense, are fully compatible with the underlying biological responsiveness of overcompensation responses in a broad range of biological systems (Calabrese, 2001). The continuity of the stimulatory response with the threshold for toxicity is consistent with the recognition that a dose too high to be compensated will manifest toxicity, as seen in the upper end (i.e., toxicity zone) of the hormetic dose–response relationship.

What makes the hormetic dose–response so biologically plausible and, in fact, compelling (e.g., compensatory response to low level damage, modest nature of the overcompensation response, continuity with the traditional threshold) makes it difficult to observe in traditional toxicological

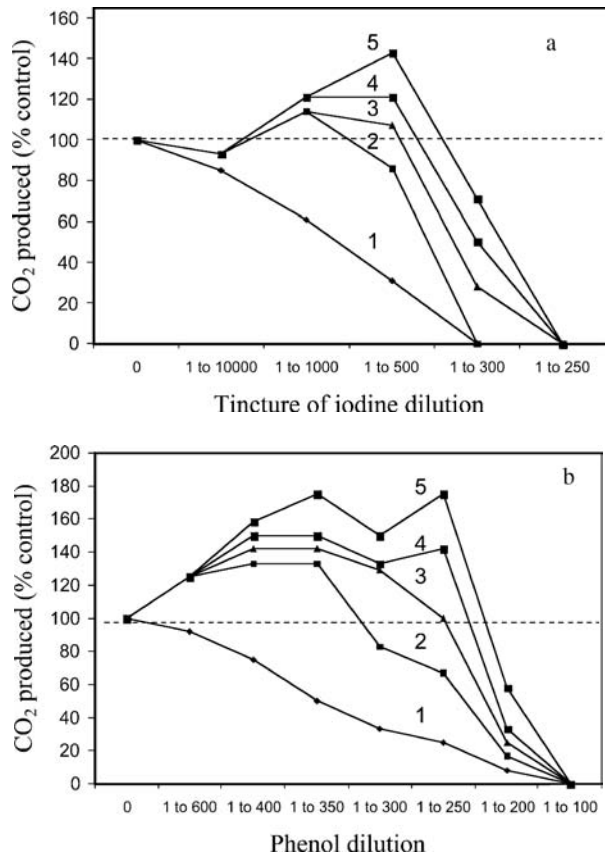


FIGURE 15.2 Representative dose–time–response relationships showing the effect of tincture of iodine dilutions (a) and the effect of phenol dilutions of CO_2 produced by baker's yeast (b). Numbers 1 through 5 indicate the time point in hours after the start of the experiment when the CO_2 measurement was made. (Data from Branham, 1929.)

testing and evaluation. That is, the response can be extremely difficult to distinguish from background variation without much greater numbers of subjects than normal, as well as without more doses that are properly spaced. These findings also have a greater need for replication than traditional high-dose toxicity studies. Consequently, to study possible hormetic effects will take more time and resources and still result in findings that have less certainty than high-dose studies. This type of situation has tended to discourage researchers from exploring the low-dose domain.

15.2 EVIDENCE FOR HORMESIS

Hormesis is a dose–response phenomenon and, as such, is not as easily proven as the case of a specific effect (e.g., mutation, tumor, serum enzyme change) would be. In hormesis what constitutes proof that it is real? In general, we do not speak of hormesis as having been unequivocally proven in any one specific study. In a specific experiment each end point can be evaluated for its consistency with the biphasic B curve based on the study design, magnitude of the stimulation, statistical significance, replication of findings, and mechanistic explanation. Collective findings addressing each of these demands would add to the weight of evidence to support a conclusion that a hormetic effect occurred. To make such judgments we have developed two independent databases to identify and assess possible hormetic effects (Calabrese and Baldwin, 1997, 2001, 2003). Each database had a different intent. One utilizes

only *a priori* evaluative criteria to capture a large number of potential examples of hormesis for evaluation (Calabrese and Baldwin, 1997). The second database has rigorous *a priori* entry and evaluative criteria to establish the frequency of hormetic responses in the toxicological literature (Calabrese and Baldwin, 2001, 2003). These respective databases include nearly 7,000 dose–responses with evidence indicating a hormetic response. The findings indicate that hormetic responses occur in essentially all types of biological models, including plants, microbes, a broad range of animal models, and humans including *in vitro* and *in vivo* systems. The range of end points (e.g., growth, disease incidence, cognitive function, immune response, etc.) displaying hormetic responses is extremely broad and appears to be without restriction. The agents capable of inducing hormetic responses are also extremely broad and occur within a vast range of chemical classes, again without known restriction. An assessment of the quantitative features of the hormetic dose–response indicates that it has a modest response with typical maximum stimulatory responses approximately 30 to 60% greater than the control, although infrequently in the literature this response exceeds twice the control values. This is also the case regardless of model, end point, or stressor agent. The width of the stimulatory response is usually within less than 10-fold of the dose designated as the NOAEL or zero equivalent point; however, 5 to 10% of the stimulatory width values have been as broad as 100-fold and about 2 to 4% have exceeded 1,000 while still maintaining the modest stimulatory amplitude.

As noted above, the width of the stimulatory response is typically less than a factor of 10 but can exceed well over 1,000-fold. The reason for such variability is uncertain. However, it is likely that the range of the stimulatory response is a function of the degree of heterogeneity within the population being studied based on experimental simulations that we have conducted. Because most biological models under investigation are highly homogeneous this may help to account for the narrow stimulatory range. The issue of the width of the stimulatory zone is of some practical importance because it might affect a concentration zone that physicians may seek to achieve or avoid. It might also affect the public health implications of the magnitude of an uncertainty factor (UF) in the risk assessment process.

The frequency of hormesis in the toxicological literature was estimated by using *a priori* entry and evaluation criteria. These criteria were applied to nearly 20,000 articles published over 30 years in three toxicological journals covering ecological- and pharmacological-oriented toxicology. Of importance is that only about 2% of the 21,000 articles had adequate study designs to assess whether hormesis was real. However, of those dose–responses that did satisfy the entry criteria about 40% also satisfied the evaluative criteria or functional definition of hormesis. Because the criteria for evaluation were considered highly rigorous, it is likely that the actual frequency is considerably higher than the 40%. Consistent with this perspective were observations that responses of doses below the NOAEL in the hormesis frequency data were nonrandomly distributed in a manner highly consistent with the hormesis hypothesis. Not only did these findings provide more support for the hormesis dose–response model but it also challenged the belief that the threshold and linear dose–response models were common in toxicology.

15.3 HORMESIS AND RISK ASSESSMENT

The implications of hormesis for the risk assessment process may be profound. Hormesis has the potential to affect the hazard assessment process, the derivation of the NOAEL, the size of the UF dealing with interindividual variation, assumptions and procedures for estimating cancer risk, and even the selection of the default risk assessment model in risk assessment.

15.4 HAZARD ASSESSMENT PROCESS

The hazard assessment process could be impacted by the acceptance of hormesis. Its acceptance could affect several features of the selection of animal models and end points to be measured. In the animal model and end point, a consideration of hormesis would require a careful assessment of the background incidence of end points of interest. With respect to diseases hormesis cannot be assessed

within an animal model with a negligible background disease incidence. The historical selection of animal models has typically considered susceptibility to infection, the capacity to develop the disease of interest such as cancer, a negligible to modest background tumor incidence to ensure adequate statistical power from a modest number of animals (e.g., 50 animals per concentration per sex), and a relatively short-lived species (to minimize costs and provide timely data).

To overcome the limitation of the use of too few animal models in hazard assessments that are responsive to an assessment of hormesis, it may be helpful to select animal models with differing and complementary patterns of disease incidence so that the hormetic model could be assessed across as broad a spectrum of end points as possible. This could mean that several mouse and rat models could be added to the range of animals currently being routinely used. It may also be necessary to incorporate a temporal component into the hazard assessment process because the hormetic response may follow an overcompensation mechanistic process that can only be studied properly by carefully timed interim samplings and sacrifices.

The extra resources, time, and additional data make the hormesis model a very unattractive option for it may adversely affect industry and government regulators who have pressure to move agents from the laboratory into the commercial sector. However, if these hazard assessment extra steps were taken the scientific database on which regulatory decisions could be made would be greatly strengthened, especially those dealing with a database consideration of which the toxicological model may be the most biologically credible. Nonetheless, the extra cost, time delay, and greater risk of finding new potential risks by adding new animal models and more dosages would likely make an improved scientific foundation take a backseat to a range of important practical considerations.

15.5 HORMESIS—NOAEL DERIVATIVE

The most significant goal in the hazard assessment process for noncarcinogens is the selection of the NOAEL, which has been defined by the EPA as the highest dose that is not statistically significantly different from the control group. Once the NOAEL is obtained then it is divided by appropriate UFs to derive the reference dose.

Estimation of the NOAEL generally employs a testing strategy that initially establishes highly toxic responses and then reduces the dose until a chronic NOAEL is obtained. That is, to derive a chronic NOAEL the testing process is designed to define a dose–response relationship that includes the NOAEL, lowest observable adverse effect level (LOAEL), and toxic responses exceeding the LOAEL such as a frank effects level (FEL). This process permits the direct estimation of the NOAEL as defined by the highest dosage not significantly different from the control group, its estimation using traditional UFs (i.e., LOAEL to NOAEL UF) if no NOAEL were observed experimentally, and/or its derivation via a benchmark dose (BMD) process. The EPA defines the BMD as a lower confidence limit on the effective dose associated with some defined level of effect (e.g., a 5% [BMD_{05}] or 10% [BMD_{10}] increase in response for a particular effect). Since the biostatistical model is only used to interpolate within the dose range of the experimental data, no assumptions about the presence (or absence) of a threshold are needed. This implies that any model fitting the data in an acceptable manner may be able to offer reasonable estimate of the BMD.

Although the NOAEL may be derived from any of the three procedures above (i.e., highest dose not differing from the control, LOAEL/UF, and BMD), the estimation of the NOAEL may be affected by the presence of U-shaped (i.e., hormetic) dose–responses in two ways: (1) doses within the hormetic range may affect the BMD derivation process or (2) a dose within the hormetic range may be selected as the NOAEL. In the BMD derivation, the presence of hormetic responses would tend to flatten the model-based dose–response relationship, resulting in a higher exposure to achieve the $BMD_{05/10}$ response. As for the traditional NOAEL methodology (i.e., where the highest dose is not statistically significantly different from the control and is designated the NOAEL), such a dose with a value either modestly greater or less than the zero equivalent point (ZEP) (i.e., value equal to the control value) would be designated the NOAEL. For example, Figure 15.3 depicts a stylized dose–response relationship in which a

U-shaped dose–response is illustrated. In this case, the highest dose represents a statistically significant toxic response and is designated the LOAEL. The second highest dose shows a response lower than the control response. By definition (i.e., the highest dose not statistically significantly different from the control with respect to an increased adverse response incidence),[1] this dosage would serve as the highest NOAEL for reference dose (RfD) derivation purposes and yet would be in the hormetic zone. In these two cases at least, the concept of NOAEL as determined by the BMD is therefore influenced by the presence of hormetic-like responses (U-shaped data), whereas being part of the hormetic response curve (i.e., traditional NOAEL derivation process), respectively. However, in many and probably the majority of cases where the number of doses is limited and emphasizes the high-dose response, the dose–response will not have U-shaped data and therefore will not be influenced by an hormetic response during BMD derivation. In addition, a dose exceeding the ZEP value is often selected as the NOAEL and therefore would not be in the hormetic zone. Nonetheless, it is necessary to recognize the relationship of hormesis to current NOAEL derivation processes because it may affect methods for RfD derivation that attempt to optimize population-based hormetic responses.

The strategy of starting at the upper-dose range in short-term toxicological studies and gradually defining a chronic NOAEL is intended to assess hazard potential across a broad spectrum of dosages and exposure durations. This traditional methodology used to derive the NOAEL has been incorporated into the vast majority of hazardous assessment schemes. The high- to low-dose testing process is not the only way that the NOAEL may be derived. In fact, one could start at the opposite end of the dose spectrum and work up to higher doses until the NOAEL and LOAEL are estimated. Therefore, it would be toxicologically possible to define the NOAEL from either side of the dose-testing spectrum (i.e., from very low or very high dosages). However, starting at the very low dosage end to proceed to the NOAEL is not attractive because society needs to define the major potential threats an agent may pose rather than the more modest and theoretical benefits (or harms) of a rather limited nature (i.e., hormetic response) as well as the NOAEL. These responses are determined in the domain of the traditional hazard assessment testing protocol. Although hormesis-oriented testing methods that ensure the incorporation of a wide dose range and large number of doses could be employed to derive a more reliable NOAEL than with current high-to-low dose testing, the hazard assessment process requires

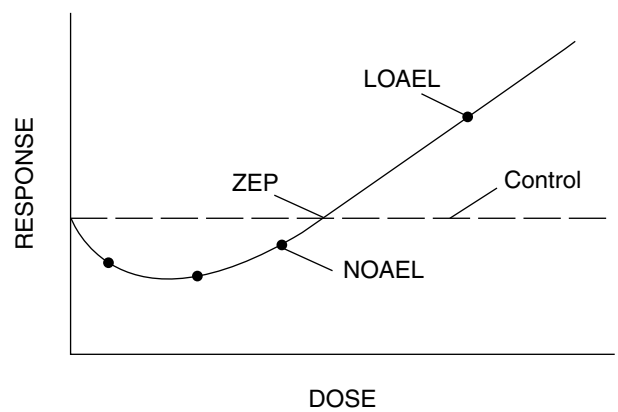


FIGURE 15.3 Stylized dose–response relationship depicting a U-shaped dose–response where the LOAEL is represented by the highest dose and the NOAEL is represented by a dose displaying a response lower than the control (i.e., in the hormetic zone). (Source: Calabrese and Baldwin, 1998.)

[1] However, this response could be statistically significantly different from the control in the hormetic zone. As long as the response was believed to be of a potentially beneficial nature, the issue of its being statistically significantly different from the control would not affect NOAEL designation for the dose–response.

that the full range of toxic end points be reasonably defined. This clearly favors the traditional testing protocol in which dose number has been limited and high doses emphasized. Thus, hormesis would not be changing the principal goal of defining toxic potential.

As reasonable as this argument for high-to-low end hazard testing might appear, it is not without its biases or limitations. By deriving a NOAEL from the traditional high-to-low dose-testing strategy possible hormetic responses may be frequently missed. This would have implications for the derivation of a BMD because some precision would be lost in defining the low-dose zone. Nonetheless, regardless of how the NOAEL is derived, recognition of the hormesis dose–response in toxicological testing can have a significant impact on RfD derivation.

15.6 THE DOSE–RESPONSE CONTINUUM AND THE RFD PROCESS

An analysis of published data from numerous experiments displaying hormetic dose–response relationships involving a wide range of biological models, end points, and chemical agents offers critical features of the quantitative nature of the dose–response relationship (Calabrese et al., 1999). Knowledge derived from this assessment of the hormetic dose–response relationship can be used in the derivation of the RfD. Based on several thousand dose–responses the stimulatory dose range of the dose–response curve varies from severalfold to several orders of magnitude with the majority approaching 10-fold. These observations provide a quantitative point of reference for application to the assessment of the RfD derivation process.

In deriving RfDs for noncancer effects UFs are typically employed, depending on the nature of the available data. The following example will illustrate how an RfD may be derived based on a hypothetical study (Calabrese, 1996). Assume that a lifetime mouse study was conducted on agent X using an unexposed control and six dosages (0, 2, 4, 8, 16, 32, and 64 mg/kg/day). The end point represented in the hypothetical study is median life span (Figures 15.4 and 15.5). The data indicate a dose–response relationship closely approximating the hormetic curve with low doses enhancing survival and high doses being progressively detrimental (i.e., decreased longevity). Using traditional definitions 16 mg/kg/day treatment represents the LOAEL, whereas the 8 mg/kg/day treatment is the NOAEL. Based on such a lifetime study, the RfD derivation process would generally employ at least two UFs, the inter- and intraspecies UFs. The interspecies UF assumes for public health protection purposes that the average human may be 10-fold more sensitive than the average mouse. Because the mouse NOAEL was 8 mg/kg/day as in the present hypothetical study, the NOAEL for the average human would be 0.8 mg/kg/day based on an interspecies UF of 10. Moreover, the expected shape of the entire dose–response curve (for the toxic response and the low-dose enhancement of longevity) for humans is similar to the mouse curve but simply displaced to the left by the interspecies UF. The RfD procedure would typically apply a 10-fold UF to address normal human variation (i.e., an intraspecies UF). Despite the assumption that there is considerable variation in human response, the basic hormetic dose–response is still assumed to be appropriate if shifted to the left for the so-called high-risk subsegment of the population.

Figure 15.5, which represents the range of adverse and beneficial effects in the traditional UF-RfD approach, reveals that the average human will display beneficial effects (i.e., enhanced longevity) when exposed in the range of ~0.08 to ~0.8 mg/kg/day, but adverse effects would be expected above the NOAEL of 0.8 mg/kg/day. However, the RfD of 0.08 mg/kg/day will provide assurances that the high-risk subgroup typically assumed by EPA and all less susceptible individuals will not develop adverse health effects. Beneficial effects in the high-risk subsegment from agent X would occur with an exposure of ~0.008 to ~0.08 mg/kg/day, assuming an identical distribution of responses as non-high-risk (i.e., average) humans.

The present assessment indicates that if the exposure is limited to a maximum of 0.08 mg/kg/day (i.e., the standard derived RfD) both normal and high-risk segments of the population will not experience adverse effects. However, a strong majority of the population including both average and high-risk groups will not experience the beneficial effect (i.e., increased longevity).

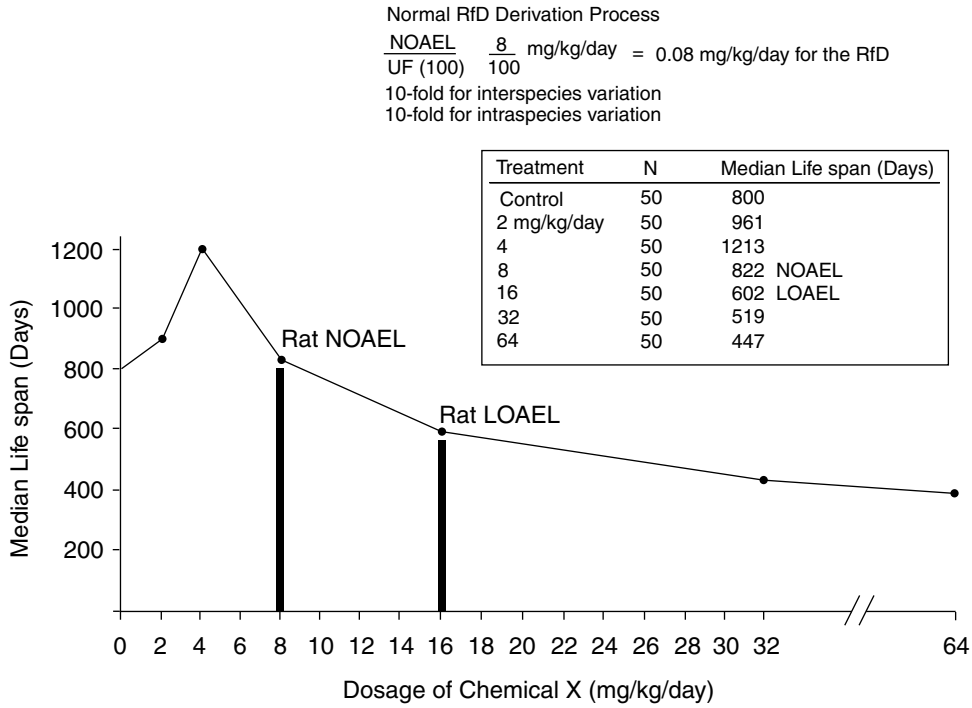


FIGURE 15.4 The effect of chemical X on the median life span of a rodent strain. (Source: Calabrese, 1996.)

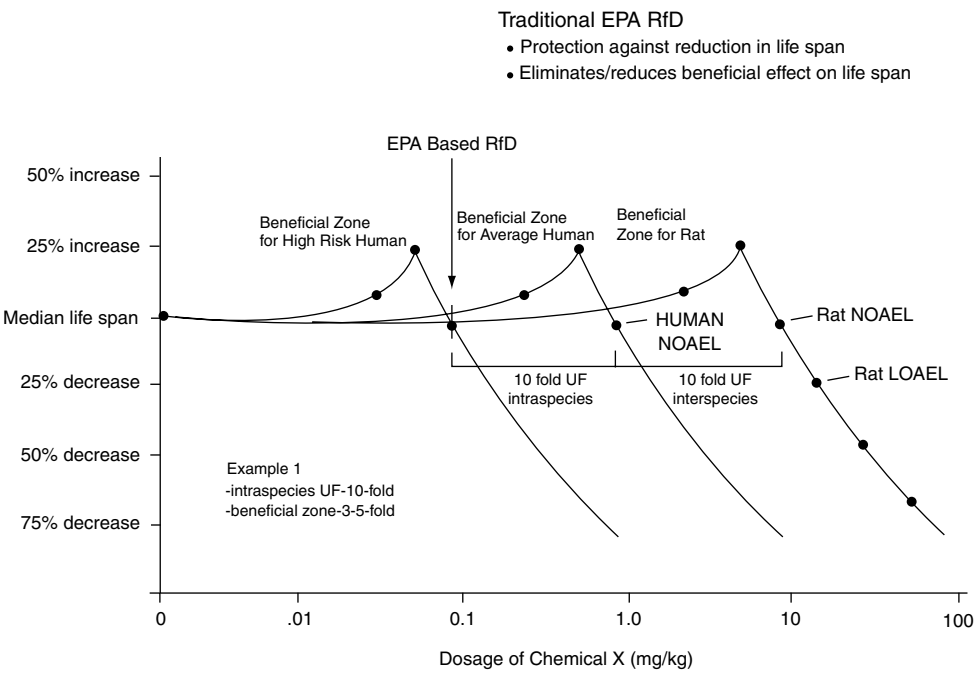


FIGURE 15.5 EPA RfD derivation process affects beneficial effect depending on the size of the beneficial zone and the size of the intraspecies UF. (Source: Calabrese, 1996.)

How would this concept of risk/benefit be employed by regulatory agencies to establish optimal risk management actions? In the case of this hypothetical example, a range of goals may be identified:

- Prevent compound-induced decreased longevity in most individuals, including sensitive individuals (i.e., the traditional RfD).
- Select the RfD that maximizes the number of days lived by the entire population.
- Select dosage that optimizes risks and benefits for the high-risk groups.

The EPA risk assessment process uses multiple UFs for noncancer toxicity depending on available data, applies these factors to the critical toxic effect (often the most sensitive end point in the most sensitive study) as well as using studies that define the full spectrum of the toxicity dose–response continuum, while often ignoring possible beneficial effects at lower doses. These collective and mutually reinforcing decisions have created a type of RfD that theoretically prevents the harmful effects of chemical exposure to all members of the population, while excluding dosages beneficial to the general population. However, regulatory agencies, such as EPA, have had to alter standard RfD derivation procedures to account for essential and/or beneficial effects of toxic agents by taking into account the entire dose–response continuum. For example, the regulation of fluoride in drinking water recognizes beneficial dental effects at low doses and a variety of adverse effects at higher doses (Calabrese, 1978). Likewise, the beneficial and harmful effects seen for essential but toxic elements such as copper, selenium, and sodium have been addressed by the EPA in an RfD derivation process designed to ensure benefits while preventing adverse effects.

Regulatory agencies have adopted procedures (e.g., RfD derivation process) that avoid making health trade-offs among different segments of the population. For example, the EPA could regulate under the assumption that most people, even those at recognized increased risk, may be protected from adverse effects. Although this situation would still be the case, this paper challenges how estimated beneficial effects could be incorporated into a population-based risk assessment. For the methodology presented here, however, it is not possible in a regulatory sense to optimize benefits for both the average- and high-risk segments of the population at the same time.

15.7 HIGH-RISK GROUPS

The risk assessment process attempts to address concerns for subsegments of the population considered at increased risk for experiencing adverse effects from toxic substances. Factors that can affect risk may be age, diet, gender, and the presence of any preexisting diseases (Calabrese, 1978, 1980, 1981, 1983, 1985). Assessment of the hormesis database has revealed that hormetic effects commonly occur in those individuals considered at high risk. This is also the case for species displaying enhanced susceptibility to toxic substances. In such cases, therefore, the causes of the observed enhanced susceptibility cannot be attributed to the lack of an hormetic response. However, instances exist where a hormetic response was lacking in a high-risk group that was associated with the enhanced susceptibility (Calabrese and Baldwin, 2002). Nonetheless, the assessment of hormetic responses as a function of developmental status, age, gender, diet, and preexisting disease status represents an area of considerable biological and public health significance.

15.8 MIXTURES

The issue of mixture toxicology and the risk assessment process is complex (Calabrese, 1991). The EPA has developed a methodology to address issues of dose and response additivity and toxic equivalent factors for estimating effects of complex mixtures and has incorporated these concepts into traditional dose–response models such as threshold and linearity.

In the case of hormetic responses several authors have raised the question of mixture toxicology and hormesis. Hormetic dose–responses have been reported for complex mixtures such as

tobacco smoke (Bonassi et al., 2003), petroleum hydrocarbons (Laughlin et al., 1981), and waste plant effluence (Walsh and Alexander, 1980; Walsh and Bahner, 1980). In fact, so common was the occurrence of a low-dose stimulation, high-dose inhibition that the EPA scientists studying waste plant effluent created the term SC20 to stand for the concentration that causes at 20% stimulation (Walsh and Alexander, 1980; Walsh and Bahner, 1980). Thus, the hormetic dose–response may be seen in studies with extremely complex mixtures. In addition, research dealing with several agents simultaneously has also revealed hormetic dose–responses (Pagano, 1982, 1986; Bae et al., 2001). Although no obvious reason exists to believe that hormetic dose–responses will not occur in mixture toxicology studies, this remains an underresearched area.

15.9 PRACTICAL IMPLICATIONS OF HORMESIS FOR QUANTITATIVE RISK ASSESSMENT

15.9.1 Hormesis and Carcinogens

Sielken and Stevenson (1998) identified seven ways in which the concept of hormesis could affect quantitative risk assessment (Table 15.1). These seven factors provide an important and specific framework for how the current approaches for both chemical and radiation cancer risk assessment modeling could both include and take advantage of the concept of hormesis. Many of these recommended changes also reflect a less biased approach. For example, the parameters in the multistage model for regulatory purposes are typically required to be nonnegative values. This restriction determines that the probabilities in the multistage model be increasing as the dose increases. These nonnegativity restrictions for the parameters in the multistage model could be removed so that the fitted model reflects the shape of the reported dose–response data. In addition, such a change would allow the multistage model enhanced flexibility to address hormetic effects seen in published data. When a hormetic effect is present the risk at low dose would be less than for the control group. The lower bound on the "added" risk in the case of hormesis is no longer the zero risk as currently assumed but would be negative. Both upper and lower bounds of uncertainty should be incorporated.

15.9.2 Hormesis as the Default Model in Risk Assessment

The EPA uses the threshold and linearity at low dose concepts as risk assessment default models for noncarcinogens and carcinogens, respectively. However, a case can be made that the hormetic model

TABLE 15.1 Implications of Hormesis for Quantitative Risk Assessment

Dose-response models need greater flexibility to fit the observed shape of the dose-response data; such models should not be constructed to be forced to always be linearly decreasing at low doses.
Hazard assessment evaluations need to incorporate greater opportunity to identify the hormetic portion of the dose-response relationship.
New dose metrics should be used that incorporate age or time dependence on the dose level rather than a lifetime average daily dose or its analog for a shorter time period.
Low-dose risk characterization should include the likelihood of beneficial effects and the likelihood that a dose level has reasonable certainty of no appreciable adverse health effects.
Exposure assessments should fully characterize the distribution of actual doses from exposure rather than the just upper bounds.
Uncertainty characterizations should include both upper and lower bounds.
Risk should be characterized in terms of the net effect of a dose on health instead of a single dose's effect on a single disease endpoint (i.e., total mortality rather than a specific type of fatal disease).

Note: Adapted from Sielken and Stevenson (1998).

should replace these models based on its generalizability, its high frequency in the toxicological literature, and its enhanced capacity to account for toxicological dose–response data as compared with its rival models (see Calabrese, 2004 for a detailed assessment of this issue).

If the hormetic dose–response model were to become the default dose–response model it would have highly significant implications for the risk assessment process, especially the process dealing with carcinogens. At present it appears that hormetic dose–responses are dealt with on a case-by-case basis. However, given the extra time and expense needed to study and prove hormesis, consideration on a case-by-case basis would result in very few cases of hormesis being brought forward. Therefore, to take advantage of the hormetic phenomenon it would have to be considered as the default.

ACKNOWLEDGMENTS

This work was sponsored by the Air Force Office of Scientific Research, Air Force Material Command, USAF, under Grant F49620–01–1-0164. The U.S. Government is authorized to reproduce and distribute for governmental purposes notwithstanding any copyright notation thereon. The views and conclusions contained herein are those of the authors and should not be interpreted as necessarily representing the official policies or endorsement, either expressed or implied, of the Air Force Office of Scientific Research or the U.S. Government.

REFERENCES

Bae, D-S., Gennings, C., Carter, W. H., Yang, R. S. H., and Campain, J. A., Toxicological interactions among arsenic, cadmium, chromium, and lead in human keratinocytes, *Toxicol. Sci.,* 63, 132–142, 2001.

Bonassi, S., Neri, M., Lando, C., Ceppi, M., Lin, Y.-P., Chang, W. P., Holland, N., Kirsch-Volders, M., Zeiger, E., Fenech, M., and The HUMN collaborative group, Effect of smoking habit on the frequency of micronuclei in human lymphocytes: results from the Human MicroNucleus project, *Mutat. Res.,* 543, 155–166, 2003.

Branham, S. E., The effects of certain chemical compounds upon the course of gas production by baker's yeast, *J. Bacterial.,* 18, 247–264, 1929.

Calabrese, E. J., *Methodological Approaches for Deriving Environmental and Occupational Health Standards,* John Wiley and Sons, New York, 1978, pp. 162–165.

Calabrese, E.J., *Nutrition and Environmental Health: The Influence of Nutritional Status on Pollutant Toxicity, Vol. 1, the Vitamins*, John Wiley and Sons, Inc., New York, 1980, pp. 600.

Calabrese, E.J., *Nutrition and Environmental Health: The Influence of Nutritional Status on Pollutant Toxicity, Vol. II., The Mineral and Macronutrients*, John Wiley and Sons, Inc., New York, 1981, pp. 500.

Calabrese, E.J., *Principles of Animal Extrapolation*, John Wiley and Sons, Inc., New York, 1983, pp. 603.

Calabrese, E.J., *Toxic Susceptibilities: Male and Female Differences*, John Wiley and Sons, Inc., New York, 1985, pp. 350.

Calabrese, E. J., Lack of total independence of UFs. Implications for the size of the total UF for selected malathion toxic endpoints, in *Proceedings of APHIS Labat-Anderson Malathion Workshop,* 1991, pp. 8.

Calabrese, E. J., Expanding the reference dose concept to incorporate and optimize beneficial effects while preventing toxic responses from nonessential toxicants, *Regul. Toxicol. Pharmacol.,* 24, S68–S75, 1996.

Calabrese, E. J., Overcompensation stimulation: a mechanism for hormetic effects, *Crit. Rev. Toxicol.,* 31, 425–470, 2001.

Calabrese, E. J., Hormesis: from marginalization to mainstream. A case for hormesis as the default dose response model in risk assessment, *Toxicol. Appl. Pharmacol.,* in Press.

Calabrese, E. J. and Baldwin, L. A., The dose determines the stimulation (and poison): development of a chemical hormesis database, *Int. J. Toxicol.,* 15, 545–559, 1997.

Calabrese, E. J. and Baldwin, L. A., Hormesis as a default parameter in RfD derivation, *Hum. Exp. Toxicol.,* 17, 444–447, 1998.

Calabrese, E. J. and Baldwin, L. A., Chemical hormesis: its historical foundations as a biological hypothesis, *Hum. Exp. Toxicol.,* 19, 2–31, 2000a.

Calabrese, E. J. and Baldwin, L. A., The marginalization of hormesis, *Hum. Exp. Toxicol.,* 19, 32–40, 2000b.

Calabrese, E. J. and Baldwin, L. A., Radiation hormesis: its historical foundations as a biological hypothesis, *Hum. Exp. Toxicol.,* 19, 41–75, 2000c.

Calabrese, E. J. and Baldwin, L. A., Radiation hormesis: the demise of a legitimate hypothesis, *Hum. Exp. Toxicol.,* 19, 76–84, 2000d.

Calabrese, E. J. and Baldwin, L. A., Tales of two similar hypotheses: the rise and fall of chemical and radiation hormesis, *Hum. Exp. Toxicol.,* 19, 85–97, 2000e.

Calabrese, E. J. and Baldwin, L. A., The frequency of U-shaped dose-responses in the toxicological literature, *Toxicol. Sci.,* 62, 330–338, 2001.

Calabrese, E. J. and Baldwin, L. A., Hormesis and high-risk groups, *Regul. Toxicol. Pharmacol.,* 35, 414–428, 2002.

Calabrese, E. J. and Baldwin, L. A., The hormetic dose response model is more common than the threshold model in toxicology, *Toxicol. Sci.,* 71, 246–250, 2003.

Calabrese, E. J., Baldwin, L. A., and Holland, C. D., Hormesis: a highly generalizable and reproducible phenomenon with important implications for risk assessment, *Risk Anal.,* 19, 261–281, 1999.

Clark, A. J., *Handbook of Experimental Pharmacology*, Verlig Von Julius Springer, Berlin, 1937.

Clifton, C. E., Death of bacteria, in *Introduction to Bacterial Physiology,* McGraw-Hill Book Company, Inc., New York, 1957.

Hektoen, L., Further observations on the effects of roentgenization and splenectomy on antibody production, *J. Infect. Dis.,* 27, 23–30, 1920.

Lamanna, C. and Mallette, M. F., *Basic Bacteriology: Its Biological and Chemical Background,* 3rd ed., The Williams & Wilkins Co., Baltimore, 1965.

Laughlin, R. B., Ng, J., and Guard, H. E., Hormesis: a response to low environmental concentrations of petroleum hydrocarbons, *Science,* 211, 705–707, 1981.

MacDougal, D. T., *Practical Text-Book of Plant Physiology,* Longmans, Green, and Co., New York, 1901.

Maximov, N. A., *Plant Physiology,* 2nd English ed., Harvey, R. B. and Murneek, A. E., Eds., McGraw-Hill Book Co., New York, 1938.

National Academy of Sciences (NAS), Safe Drinking Water Committee, *Drinking Water and Health,* NAS, Washington, DC, 1977, pp. 38–39.

Pagano, G., Cipollaro, M., Corsale, G., Esposito, A., Ragucci, E., Giordano, G. G., and Trieff, N. M., The sea urchin: bioassay for the assessment of damage from environmental contaminants, in *Community Toxicity Testing,* ASTM STP 920, Cairns, J., Jr., Ed., American Society for Testing and materials, Philadelphia, 1986. pp. 66–92.

Pagano, G., Esposito, A., and Giordano, G. G., Fertilization and larval development in sea urchins following exposure of gametes and embryos to cadmium, *Arch. Environ. Contam. Toxicol.,* 11, 47–55, 1982.

Peirce, G. J., *A Text-Book of Plant Physiology,* 2nd ed., Henry Holt and Company, New York, 1909.

Salle, A. J., Influence of environment upon bacteria. In: *Fundamental Principles of Bacteriology with Laboratory Exercises,* McGraw-Hill Book Company, Inc., New York, 1939.

Schulz, H., Uber Hefegifte, *Pflueg. Arch. Gesamte Physiol. Menschen Tiere,* 42, 517–541, 1888.

Schulz, H., NIH Library Translation: NIH-98–134: Contemporary medicine as presented by its practitioners themselves, Leipzig, 1923: 217–250 (T. Crump, trans.), *Nonlinearity Biol. Toxicol. Med.,* 1, 295–318, 2003.

Sielken, R. L., Jr. and Stevenson, D. E., Some implications for quantitative risk assessment if hormesis exists, *Hum. Exp. Toxicol.,* 17, 259–262, 1998. Smith, E. C., Effects of ultra-violet radiation and temperature on Fusarium. II. Stimulation, *Bull. Torrey Bot. Club,* 62, 151–164, 1935.

Southam, C. M. and Ehrlich, J., Effect of extract of western red cedar heartwood on certain wood decaying fungi in culture, *Phytopathology,* 33, 517–524, 1943.

Stebbing, A. R. D., Hormesis: the stimulation of growth by low levels of inhibitors, *Sci. Total Environ.,* 22, 213–234, 1982.

Taliaferro, W. H. and Taliaferro, L. G., Effect of X-rays on immunity: a review, *J. Immunol.,* 66, 181–212, 1951.

Townsend, C. O., The correlation of growth under the influence of injuries, *Ann. Bot.,* 11, 509–532, 1897.

Walsh, G. E. and Alexander, S. V., A marine algal bioassay method: Results with pesticides and industrial wastes, *Water Air Soil Pollut.,* 13, 45–55, 1980.

Walsh, G. E. and Bahner, L. H., Toxicity of textile mill effluents to freshwater and estuarine algae, crustaceans and fishes, *Environ. Pollut. Ser. A Ecol. Biol.,* 21, 169–179, 1980.

Warren, S., The histopathy of radiation lestions, *Physiol. Rev.,* 25, 225–238, 1945.

16 Nasal Toxicology

John B. Morris

CONTENTS

16.1 INTRODUCTION

Interest in nasal toxicity blossomed after the discovery that formaldehyde was a nasal carcinogen in the rodent. It has now become apparent that the nasal tissues are a common target site for airborne toxicant-induced damage. Indeed, induction of nasal lesions in rodent inhalation toxicity studies forms the basis for many current U.S. Environmental Protection Agency (EPA) inhalation exposure guidelines. Typical lesions in rodent inhalation studies include epithelial necrosis, degeneration, hyperplasia, metaplasia, and for some compounds, neoplasia. Several excellent reviews of nasal histopathology and pathogenesis of toxicant-induced nasal lesions have been published (Harkema, 1992, 1999; Morgan, 1995; Hardisty et al., 1999).

The nasal cavity is also an important site for initiation of respiratory reflex responses to inspired irritants. Stimulation of nasal trigeminal nerves in rodents results in a characteristic change in breathing pattern (slowed respiration due to a pause at the onset of expiration) termed sensory irritation (Alarie, 1973). The sensory irritation response is often quantified in the mouse. Such murine data form the basis for approximately one-half of the current occupational exposure guidelines in the United States (Schaper, 1993).

Nasal irritation to indoor air pollutants represents a very common complaint of humans. In addition to causing a sensation of itching or pain, nasal irritants stimulate a wide range of reflex responses (Baraniuk, 1994; Shusterman, 2003). The common nasal diseases in humans are rhinitis (allergic and nonallergic) and sinusitis. The role of pollutants in the etiology of these diseases is unknown (and is not considered herein). Note, however, that individuals with allergic rhinitis may be more responsive to irritants than healthy individuals (Shusterman et al., 2003; Bascom et al., 1991; Shusterma et al., 2005). Because of the inaccessibility of nasal tissues human conditions are usually evaluated functionally rather than histopathologically. This is particularly true for olfactory tissue. In contrast, rodent nasal toxicity data are defined in large part by histopathological rather than functional criteria. This has made direct cross-species comparisons and extrapolations difficult.

This chapter will highlight basic biochemical and inhalation toxicological aspects of nasal toxicity in the rodent with an emphasis on the responses to inspired vapors and on the relationships between dosimetry and response. Because nasal irritation represents a common response of humans, some detail on nasal irritant reflex responses in rodents and humans is also provided. Examples are provided to illustrate basic principles, not with the intent of providing an exhaustive survey of the nasal toxicity literature. When appropriate review articles are cited to guide the reader to more comprehensive information.

Knowledge of the relationship between delivered dose and response is absolutely essential for a comprehensive understanding of the toxicity and risk assessment of inspired materials that produce nasal injury. Indeed, the U.S. EPA inhalation reference concentration (RfC) (U.S. EPA, 1994) explicitly incorporates inhalation dosimetric adjustments for interspecies extrapolations as part of the quantitative risk assessment process. Because of its widespread use, this chapter describes the RfC methodology relative to risk assessment of nasal toxicants.

The RfC is an estimate (with uncertainty) of a continuous inhalation exposure to human populations (including sensitive subgroups) that is likely to be without appreciable risk of deleterious noncancer health effects during a lifetime (U.S. EPA, 1994). Derivation of the RfC is a several-step process. The database is evaluated to determine the critical (e.g., most sensitive) effect, concentration–response relationships are assessed, and the principal study on which to base the RfC is selected. Either a no observable adverse effect level (NOAEL), a lowest observed adverse effect level (LOAEL), or BMD (benchmark dose) may be used (U.S. EPA, 1994; Jarabek, 1995; Andersen and Jarabek, 2001). Duration adjustment factors, based on a linear concentration–time assumption are used to adjust from the typical inhalation toxicity study exposure regimen (6 h/d, 5 d/week) to continuous 24 h/d, 7 d/week exposure. A dosimetric adjustment factor is then used (see below) to allow for species extrapolation from the laboratory animal used in the principal study to the human. Using this dosimetric adjustment factor the exposure concentration in rodent studies (exposure duration adjusted) is converted to a human equivalent concentration. Finally, as is often done, uncertainty factors are used with 3- or 10-fold factors for extrapolations from animals to average humans, average to sensitive humans, subchronic to chronic exposures, etc. The animal-to-average-human factor implicitly includes both pharmacokinetic and pharmacodynamic uncertainties. In the RfC process an uncertainty factor of 3, rather than 10, is often used for the uncertainty associated with this extrapolation because the RfC derivation already incorporates dosimetric adjustment factors to account for pharmacokinetic (e.g., delivered dose) differences across species lines. Use of more rigorous pharmacokinetic modeling rather than the default RfC approach may support the use of a lower uncertainty factor.

16.2 NASAL STRUCTURE

16.2.1 Gross Anatomy

Similarities and differences occur in the structure of the nasal cavity among mammalian species. A schematic diagram is provided in Figure 16.1. (This figure also includes airflow patterns and nomenclature that is used for the pharmacokinetic modeling that is described below.) Air enters the nasal cavity from the external nares into the nasal vestibule and exits the nasal cavity via the nasopharyngeal duct. Although humans and some other mammals are capable of bypassing the nose during mouth breathing, the rodent is an obligatory nose breather (Proctor and Chang, 1983).

In mammals the nasal cavity is separated into right and left halves by the cartilaginous septum. The hard and soft palate form the base of the nasal cavity. The shape of the nasal cavity is determined by the shape of the head and, in particular, in rodents, by the placement of the teeth. Bony structures, termed turbinates, protrude into the airspace from the lateral walls of the nasal cavity, which serve to increase the nasal cavity surface area. Although not a perfect analogy, the nasal cavity might best be considered to have a parallel plate structure rather than the cylindrical structure of the lower airways. The shapes of the turbinates differ in complexity among species. The anterior turbinates of most

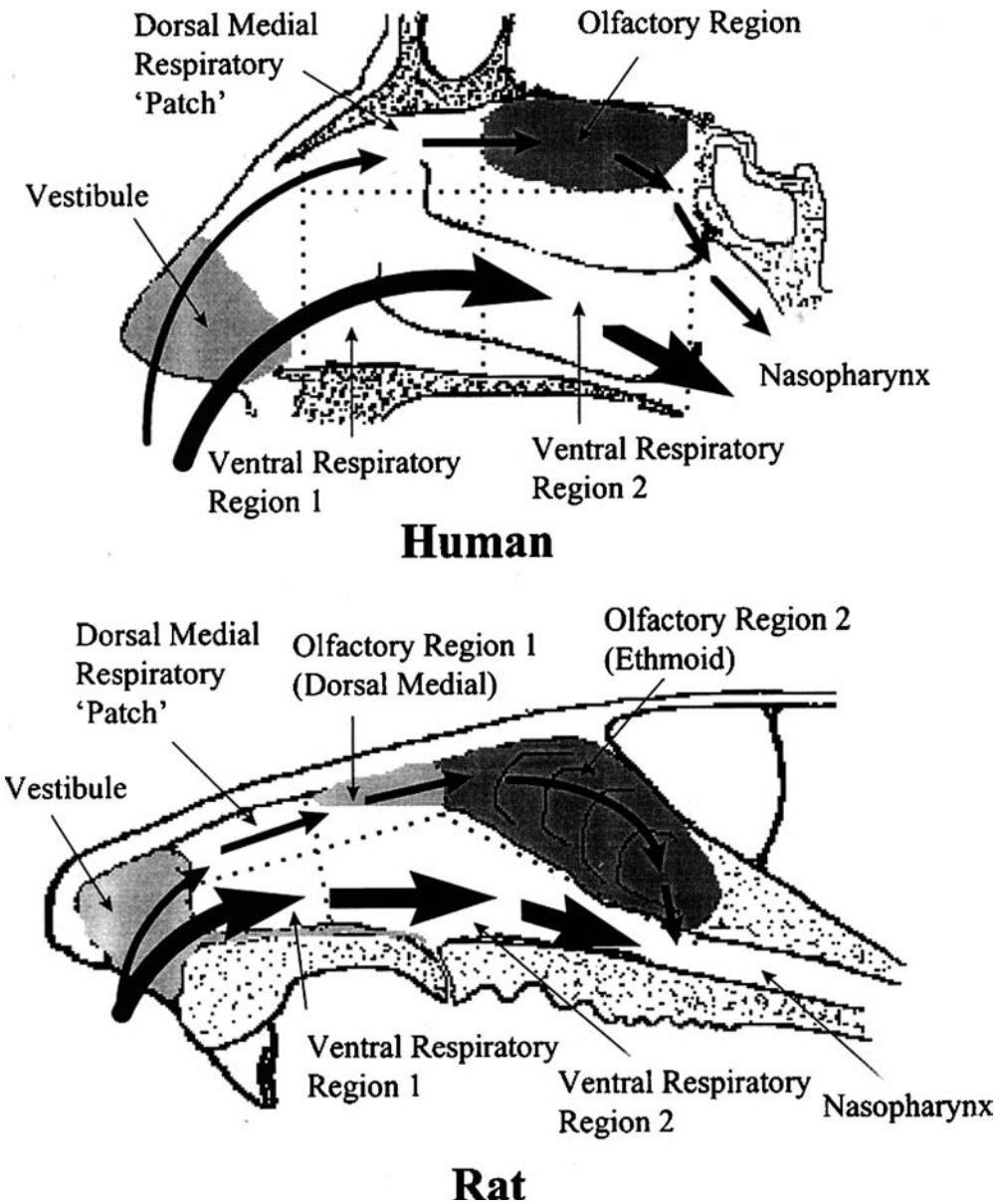


FIGURE 16.1 Schematic diagram of the nasal passages (from Frederick et al., 1998) depicting gross structure, epithelial types, and principal airflow patterns. Squamous epithelium (light grey), respiratory/transitional epithelium (white), and olfactory epithelium (dark grey) are depicted (see text for details). In both species air enters the nose through the anterior nasal vestibule, passes over the turbinates, and exits via the nasopharynx. The airstream patterns, as defined by computational fluid dynamic models, are depicted by the dark arrows. For dosimetry-modeling purposes the nose is often separated into various compartments. The scheme used by Frederick et al. (1998) is shown and consists of the vestibule, two ventral respiratory regions, a dorsal medial "patch" of respiratory epithelium, and two olfactory regions.

species tend to be fairly simple, whereas the posterior turbinates of nonprimates are quite complex (Proctor and Chang, 1983; Schreider, 1983; Harkema, 1992; Gross and Morgan, 1992).

In humans and other primates the nasal cavity has a simple structure. Three flat turbinates are present on each side, termed the superior turbinate, the middle turbinate, and the inferior turbinate. The total surface area of the human nasal cavity is estimated to be approximately 160–180 cm^2 (Proctor and Chang, 1983; Schreider, 1983; Gross and Morgan, 1992). At a minute ventilation rate of 8 l/min this corresponds to a surface area to ventilation rate ratio of ~2 cm^2/l/min. In the rodent the anterior portion of the nasal cavity contains two turbinates, the dorsal nasal turbinate and the ventral maxilloturbinate. These turbinates have a simple scroll structure. Posterior to these turbinates are the complex multiscrolled ethmoturbinates. Approximately one-half the surface area of the nasal cavity of the mouse and rat is contributed by ethmoturbinates. The total nasal cavity surface area is approximately 0.3 and 1.3 cm^2 in the mouse and rat, respectively (Schreider, 1983; Gross and Morgan, 1992). At minute ventilation rates of 0.05 and 0.2 l/min in the rat and mouse, surface area to ventilation ratios of approximately 6 are calculated for these species. These ratios are considerably higher in the rodent than the human, but this comparison is misleading because the nasal cavity is not uniformly ventilated (see below).

The narrowest portion of the nasal passages is located at the anterior aspect of the anterior turbinates. This is termed the ostium internum or nasal valve in humans (Proctor, 1977; Proctor and Chang, 1983; Harkema, 1999). Because of the low cross-sectional area, the highest airflow velocities are in this region. Tissues in the nasal cavity are highly erectile. The degree of tissue swelling can dramatically influence the airflow patterns through the nose. Indeed, in humans and other mammals the two sides of the nasal cavity may not be equally ventilated. Cyclical swelling of tissues on the right or left side of the nose obstructs airflow through one versus the other side of the nose. Over the course of several minutes to hours the pattern is reversed, with the alternate side of the nose being obstructed (Bojsen-Moller and Fahrenkrug, 1971; Fisher et al., 1993; Shusterman, 2003; Huang et al., 2003). Although the nasal cycle would have considerable impact on dosimetry of inhaled materials, especially during short-term exposures, it is rarely considered in evaluation of lesions.

It has long been known that the airflow patterns through the nose are quite complex (Dawes, 1952). This represents an important phenomenon relative to nasal toxicology because it influences the delivery of inspired materials within the nose. Two lines of investigation suggest that the bulk of the flow in the rodent nose follows ventral pathways over the simple structured naso- and maxillary turbinates and exits via the nasopharyngeal duct without ever passing over the complex ethomoturbinates (see Figure 16.1). Based on modeling of nasal uptake efficiencies of metabolized vapors in comparison with the regional distribution of nasal biotransformation enzymes it was estimated that ~10% of the airflow followed the dorsal pathway in the rat nose (Morris et al., 1993). Computational fluid dynamic modeling, based on conservation of momentum within the air phase, predict that 10–15% of the flow follows this pathway in this species (Kimbell et al., 1993, 1997). Thus, two fundamentally different approaches predict the same flow patterns. These modeling efforts suggest that only 10–15% of the inspired airstream follows a dorsal pathway and flows over the complex ethmoturbinates. Thus, whereas the structure of the ethmoturbinates in the rat is quite different than in the human, little of the inspired air passes through this region. A similar flow pattern is thought to exist in the human nose, with only a small fraction of the inspired air passing over the superior olfactory mucosa-lined turbinate (Hahn et al., 1993; Keyhani et al., 1995; Subramaniam et al., 1998). Thus, the complex structure of the ethmoturbinates in rodents may be of minimal relevance toward assessment of human risk from animal studies for materials that do not produce their effects in that site because the vast majority of the inspired air bypasses this region of the nose.

16.2.2 Cellular Composition

The cellular structure of nasal tissues has been described in detail (Morgan, 1995; Harkema, 1992, 1999). Four distinct principal types of epithelium line the nasal cavity. From anterior to posterior

these are: squamous epithelium, noncilicated cuboidal/columnar epithelium (also termed transitional epithelium), respiratory epithelium, and olfactory epithelium (see Figure 16.1). The vestibule and anterior portions of the nasal cavity are lined with squamous epithelium. The primary function of this epithelium is likely to offer protection of underlying tissues from deleterious materials. Posterior to the squamous epithelium is the nonciliated cuboidal/columnar transitional epithelium. This epithelium contains nonciliated cuboidal or columnar cells and has a scarcity of mucus-producing cells. The precise function of these epithelia is unknown. The respiratory epithelium is a pseudostratified columnar mucociliated epithelium whose structure is similar to that of the large pulmonary airways. Both ciliated cells and mucosubstance-producing goblet cells are present. Submucosal mucous secretory glands are also present. As in the lower airways, bacteria and other insoluble particulate matter may become entrapped in the mucous lining layer and can be quickly cleared from the nose. The olfactory mucosa contains the olfactory sensory and supporting cells. A few immotile cilia are present in olfactory sensory cells. Typical ciliated cells are not present. Bowman's glands are present in the lamina propria, which secrete mucosubstances into ducts that lead to the tissue surface. Lympoepithelium and nasal-associated lymphoid tissues are also present at focal sites within the nasal cavity (Harkema, 1999).

The basic structures of the epithelia are fairly similar in rodents and in the human; however, the proportions of the types differ (see Figure 16.1). This is particularly true for the olfactory epithelium. In the human, olfactory epithelium is present only on the superior turbinate and covers about 5–10% of the total nasal cavity surface area. In the rodent, olfactory mucosa covers approximately 50% of the surface area (Harkema, 1992, 1999; Morgan, 1995). It is present in the posterior portions of the ethmoturbinates. (The "leading edge" anterior portions of the ethmoturbinates is lined with respiratory epithelium.) A "tongue" of olfactory mucosa also lines the dorsal medial aspect of each side of the nasal cavity of the rodent extending toward the vestibule from the ethmoturbinates (see Figure 16.1). This region is sensitive to injury (Hardisty et al., 1999). The more anterior portions of the dorsal medial aspects of the nasal cavity are lined by respiratory epithelium; this is sometimes termed the dorsal medial respiratory patch (Frederick et al., 1998, 2002). Thus, in both the human and the rodent, air must first pass over squamous, transitional, and/or respiratory epithelium before reaching olfactory epithelium.

The anterior portions of the nasal cavity are richly perfused and contain a dense network of blood vessels. The posterior olfactory-lined portions of the nasal cavity contain much less vasculature (Morgan, 1995; Frederick et al., 1998). In the anterior portions, the vasculature is stratified with a superficial capillary layer that penetrates to, but not through, the basement membrane (Dawes and Prichard, 1953). Deeper layers of blood vessels contain venous sinuses and provide the erectile function of the nasal mucosa (see below). The nose is also richly innervated. The predominant innervation is from the trigeminal nerve (cranial nerve V) and the olfactory nerves, which enter through the cribiform plate (Shusterman, 2003). The olfactory nerves are specialized for the detection of odors. Trigeminal nerves provide the "common chemical sense," e.g., the detection of noxious materials. The trigeminal nerves contain partially myelinated Aδ fibers, which are thought to be involved primarily in mechanosensation, and also C fibers, which are thought to be involved primarily in chemosensation (Carr and Undem, 2001; Tai and Baraniuk, 2002; Shusterman, 2003).

16.3 NASAL FUNCTION

In addition to the detection of odiferous and noxious chemicals the nasal cavity is specialized for the conditioning of the inspired airstream. This represents an important respiratory defense mechanism. Inspired air is heated and humidified during transit through the nose and is cooled and dehumidified during exhalation. This latter process may be quite important in desert mammals. Nasal heating/cooling may also be important in temperature regulation of warm-blooded animals. Indeed, the lack of nasal turbinates in dinosaurs has been cited to support the conclusion they were cold blooded (Fischman, 1995).

The parallel platelike structure of the nose with a large tissue–air interfacial surface area and narrow air passages (see above) is well suited for the efficient transfer of heat and water vapor to and from the inspired airstream. In the human, air is extensively conditioned (toward 37ºC, 100% relative humidity) during passage through the nose under quiet respiration. At higher flow rates conditioning may not be complete; final conditioning occurs in the lower airways under these conditions (Proctor, 1977; Proctor and Chang, 1983). During exercise the human resorts to mouth breathing and the humidification capacity of the nose is bypassed. Some individuals breath through their mouths even when not exercising. As obligate nose breathers rodents are incapable of breathing through their mouths. Complete obstruction of the nasal passages leads to asphyxiation in such species. There is considerable resistance to airflow through the nose, approximately 50% or more of the total airways flow resistance is from the nasal passages. The fact that obligate nose breathers expend this amount of energy to breath through the nose emphasizes the biological importance of nasal air conditioning.

The ultimate source of heat and water for conditioning of the airstream is via the nasal circulation. It is thought that the rate-limiting step in the conditioning process is the heating of the air. Thus, air is maintained at 100% relative humidity as it is heated during transit through the nasal passages (Hanna and Scherer, 1986). The nose is richly perfused, receiving approximately 1% of the cardiac output. As highlighted above, superficial capillary loops penetrate to the basement membrane. These vessels are likely the most important with respect to the heating and humidifying capacity of the nose. The nose quickly adjusts to dramatic changes in environmental conditions. For example, humans may quickly move from subfreezing dry outdoor conditions into heated and humidified homes with little overt nasal symptomatology, except perhaps for a transient rhinorhea. This highlights the fact that the nasal vasculature is highly dynamic. It not appropriate to think of the nasal cavity as merely a static channel through which air must pass; the nasal cavity is a highly dynamic site that rapidly responds to changing environmental conditions.

In addition to heating and humidifying the airstream, the nasal passages also serve to scrub particulate and gas-phase pollutants from the air. By decreasing the airborne concentration of potential deleterious substances, this serves to protect the lower airways; however, this process serves to place the nasal tissues at risk for chemical-induced injury. This process is discussed in detail below under nasal dosimetry.

The nose is also a sensory organ. Olfactory structures detect the presence of chemicals with odors; sensory trigeminal nerves detect the presence of noxious chemicals. These are two distinct senses. Noxious chemicals can be detected as being present in either the right or left nose (or both), whereas there is not a lateral discrimination of odors. Moreover, anosmic individuals are capable of detecting irritants (Cometto-Muniz and Cain, 1998). From a toxicological perspective the primary interest in olfactory function may lie in the fact that various toxicants can impair this process. Whether or not an unpleasant odor represents a "toxic" effect is beyond the scope of the current text. Trigeminal senses are toxicologically important. By acting as a warning system these senses can serve to limit exposure to potentially noxious materials. In addition to initiating avoidance responses, stimulation of nasal trigeminal nerves initiates a variety of reflex responses that are likely defensive in nature in healthy individuals, but may be deleterious in certain disease states such as rhinitis or asthma (see below).

16.4 NASAL DOSIMETRY

Both particulate and gaseous pollutants can be efficiently scrubbed from the airstream in the nasal passages. This process has been termed "deposition," "extraction," and/or "uptake." Given the small mass of nasal tissues, efficient uptake of airborne toxicants in the nasal cavity can lead to high local delivered dosage rates. The most appropriate dosimeter for comparisons of delivered dose to nasal tissues is subject to debate. "Dose" might be expressed in a variety of manners, including deposited mass per unit surface area (or per unit tissue weight), flux (mass/min) normalized to tissue surface

area or weight, or steady-state tissue concentration. The most preferable metric may depend on the chemical/toxicological properties of the toxicant. Because the nasal cavity is not uniformly ventilated (see above) it should be recognized that normalizations based on the entire nasal cavity surface area are simplistic.

Several reviews have discussed particulate deposition in animals and humans (Schreider, 1983; Schlesinger, 1985; Miller, 1999). Mathematical models have been developed to predict and contrast nasal particulate dosimetry in rodents compared with humans (U.S. EPA, 1994). Particulate deposition is irreversible; once deposited, particles are not reentrained into the airstream. Particle size is the primary physical chemical characteristic influencing deposition efficiency in the nose. For particles greater than 0.5 μm (MMAD), inertial impaction represents the primary deposition mechanism. Thus, the greater the particle size, the greater the deposition in the nose. In the human nasal deposition is essentially 100% for particles 10 μm (MMAD) or greater with less efficient deposition for smaller particles. In general, inertial deposition of particles is of greater efficiency in the rodent than the human nose. For example, particles of 3 μm (MMAD) or greater deposit with very high efficiency in the nose of the rodent (Raabe et al., 1988), whereas particles of this size generally deposit within the lower airways of the humans. Because rodents are obligate nose breathers and nasal deposition cannot be circumvented, it is very difficult to approximate human particulate regional deposition patterns in the rodent. The narrowest portion of the nasal passages is at the anterior aspect of the turbinates, thus this is the region with the highest flow velocities (Proctor, 1977; Proctor and Chang, 1983) and the region in which inertial impaction is the greatest. Although not as well defined as for large particles, extremely small particles may deposit with great efficiency in the nose as well, because of diffusion. Thus, the nasal deposition efficiency for ultrafine particles may be quite large (Gerde et al., 1991).

To account for species differences in deposited doses a regional deposited dose ratio (RDDR) adjustment factor is used in the RfC derivation for particulate toxicants that cause nasal toxicity (U.S. EPA, 1994). Through this factor the critical exposure concentration is converted to the human equivalent concentration (HEC). The default value for this ratio incorporates prediction of the deposition efficiencies (based on particle size) and surface area to ventilation rate ratios for the laboratory animal and humans. Because the entire nasal cavity surface area is used, calculation of this ratio is based on the implicit assumption that aerosol deposits uniformly throughout the nose. This is a conservative assumption, which is appropriate for default parameters. Based on the differences in ventilation rates and surface areas of the human and rodent nose (see above) the RDDR is typically 0.3 or less. Thus, the human equivalent concentration is often 3-fold less than the animal exposure concentration from which it was derived. Particle deposition is not uniform throughout the nose; therefore, a careful consideration of effective surface areas across species lines for species extrapolations might lead to an improved risk estimation.

Particles can be cleared from the nose by a variety of routes. Soluble particles likely dissolve quickly in nasal mucus and diffuse quickly across the thin air–blood barrier and are removed by the nasal bloodstream. Certainly particulate cocaine is rapidly absorbed across the nasal mucosa. Insoluble particles become entrapped in the mucous lining layer and are quickly cleared to the pharynx where they are swallowed. Particles that deposit in the very anterior portions of the nose may be cleared to the nares where they can be removed by preening in animals and by mechanical means in the human (Proctor, 1977; Proctor and Chang, 1983; Morgan et al., 1984; Harkema, 1999). Mucociliary clearance of the respiratory epithelial-lined portions of the nasal cavity may be complete in a matter of minutes. The clearance mechanisms of the olfactory portions of the nasal cavity are not well understood. This region is coated with a mucous lining layer, but functional cilia are absent. Some particulate matter (and dissolved materials) is taken up by the olfactory neurons and into the olfactory bulb of the brain via retrograde transport mechanisms (Brenneman et al., 2000). This pathway may be biologically relevant in that the blood–brain barrier is bypassed.

Uptake of gases or vapors in the nasal tissues is complex. Unlike particulate deposition, vapor uptake can be a reversible process with vapor molecules entering and exiting nasal tissues. The

theoretical understanding of vapor uptake has been reviewed (Dahl, 1990; Medinsky et al., 1999; Medinsky and Bond, 2001; Bogdanffy and Sarangapani, 2003). The reader is referred to these sources for a thorough discussion of vapor uptake processes. Because of the cyclic nature of breathing an absorption/desorption process can occur in which vapor molecules enter the nasal mucus during inspiration but are desorbed back into the airstream during exhalation, when vapor-depleted air that is returning from the deep lungs passes back through the nose. This may be particularly important for nonreactive, soluble vapors (Dahl, 1990; Medinsky and Bond, 2001).

Important physical-chemical properties determining uptake efficiency are vapor solubility (expressed as an air–tissue partition coefficient) and reactivity. Reactivity includes enzymatically mediated pathways (CYP450, etc.) and direct chemical reaction (e.g., reaction of ozone with tissue/mucus substrates). In general, the higher the solubility the greater the potential for efficient nasal uptake. Similarly the greater the reactivity, the greater the potential for efficient nasal uptake. Gases and vapors that are both soluble and reactive can be scrubbed from the air with near total efficiency within the nose. This is true for both laboratory animals and humans. For example, greater than 99% scrubbing of sulfur dioxide or hydrogen fluoride has been observed in laboratory animals (Frank et al., 1969; Morris and Smith, 1982). Greater than 95% uptake of sulfur dioxide (Speizer and Frank, 1966) or chlorine (Nodelman and Ultman, 1999) occurs in the human nose. Vapors with limited solubility and low reaction rates (either direct or metabolic) are scrubbed with low efficiency. For example nasal uptake efficiencies of 10% or less have been observed experimentally for xylene and bromobenzene (Morris, 1993).

After uptake in the nasal cavity, gas/vapor molecules can be cleared by a variety of pathways (Morris, 1995, 2001; Medinsky et al., 1999; Bogdanffy and Sarangapani, 2003). Mucociliary clearance is one potential route of removal. Mucus flow rates are not large (10 μl/min in the rat) (Morgan et al., 1984); therefore, although dissolution in mucus and transport to the pharynx may occur, it is unlikely to represent a significant clearance pathway for soluble nonreactive materials. Reactive gases and vapors may bind to proteins or other components in mucus, as has been shown for formaldehyde (Bogdanffy et al., 1987). In this case mucociliary clearance may represent an important route of removal. Gas/vapor molecules can quickly diffuse from the mucous lining layer into the underlying tissues where they can be cleared by the bloodstream or by local metabolism or reaction. The nasal cavity is richly perfused (see above); circulatory clearance can be efficient. For example, blood fluoride levels are increased after exposure of the isolated upper respiratory tract to hydrogen fluoride (Morris and Smith, 1982). Once in the circulation toxicant is distributed to other organs including the lung. Frank et al. (1969) suggest that $^{35}SO_2$ could be absorbed into the bloodstream in the nose, transported to the lungs via the bloodstream, and exhaled. Therefore, the presence of pulmonary effects after inhalation exposure does not provide absolute proof that inhaled toxicant entered the lungs via the airstream.

The nose has a large xenobiotic metabolizing potential. On a per cell basis, the specific activity of many pathways may be equivalent in the nose and the liver. The reader is referred to several comprehensive reviews of nasal metabolic potential (Dahl and Hadley, 1991; Reed, 1993; Thornton-Manning and Dahl, 1997; Bogdanffy and Keller, 1999). Expression of biotransformation enzymes is not uniform throughout the nose; each enzyme shows a distinct cellular distribution. As for the liver, the potential exists for significant first-pass metabolism of toxicant and removal of metabolite(s) via the vasculature. This may be particularly true for vapors that are substrates for high-capacity metabolic systems. Two toxicologically important, high-capacity pathways are carboxylesterase and aldehyde dehydrogenase (see below). Cytochrome P450 (CYP450) is also expressed in nasal tissues. In general, the activity of CYP450 is higher in the olfactory mucosa than in other sites of the nose (Dahl and Hadley, 1991; Reed, 1993; Bogdanffy and Keller, 1999). Although a low-capacity pathway, CYP450 represents a toxicologically important metabolic route in the nose. These enzymes may result in the local production of reactive metabolites of inspired vapors. Because the isoenzyme patterns (and phase II conjugation patterns) of the nose differ from those of other tissues, it is possible that some blood-borne toxicants are selectively activated in nasal tissues leading to nasal

toxicity (Lofberg et al., 1982; Belinsky et al., 1986; Genter et al., 1994). Consequently, it is possible to envision a scenario in which an inspired low-solubility vapor is absorbed in the lungs (rather than the nose), is transported to the olfactory mucosa via the circulation, and produces injury at that site because of local activation.

Gas uptake in the rodent nose is often assessed in anesthetized animals via use of the surgically isolated upper respiratory tract. This methodology has been recently reviewed (Morris, 1999). Uptake is measured under nonphysiological airflow conditions (constant velocity unidirectional flow). Mathematic modeling approaches can be used to estimate uptake in normal breathing conditions. Due to the paucity of information on gas/vapor scrubbing by the human nose it is difficult to make precise estimates of species differences in nasal dosimetry of these materials. A methodology for measuring organic vapor uptake in the human nose has recently been reported (Thrall et al., 2003), suggesting that human vapor uptake data may become more available. The inorganic gas, sulfur dioxide, is scrubbed with 95% or greater efficiency by the human nose and with 99% or greater efficiency by the nose of the dog (Speizer and Frank 1966; Frank et al., 1969). Ozone deposits are scrubbed with approximately 50% efficiency in the nose of the guinea pig and rabbit (Miller et al., 1979) and with similar efficiency in the human nose (Hu et al., 1994).

Nasal dosimetric considerations form an important component of risk assessment for nasal toxicants. A regional deposited gas ratio (RDGR) is used for species extrapolations for gases and vapors as part of the RfC process (U.S. EPA, 1994). Consistent with our understanding of gas/vapor uptake processes, gases and vapors are separated into three categories based on solubility and reactivity. Category 1 gases and vapors are highly soluble and reactive for which little penetration of parent compound into tissues and the bloodstream is anticipated. Many, if not most, of the gases and vapors with upper respiratory tract toxicity that have been evaluated by the RfC process are classified as category 1 (Andersen and Jarabek, 2001). Category 2 gases and vapors are less soluble and/or reactive and some penetration to the bloodstream is expected. Category 3 gases and vapors are poorly soluble and slowly reactive. Significant accumulation of these materials is expected in the bloodstream. These latter gases are those for which physiologically based pharmacokinetic modeling approaches are often used, and species extrapolations should be based on the partition coefficients in each species. Conservative default assumptions are used for the category 1 and 2 gases, based on normalization of dose per time per unit surface area. As highlighted for particles, this adjustment factor is often 0.3 or less, often resulting in a human equivalent concentration 3-fold less than the exposure concentration in animal studies. As highlighted below nasal toxicants exhibit regionally specific toxicity; thus, the implicit assumption of uniform deposition across the nose is likely simplistic. The collection of mechanistic data and use of a more sophisticated dosimetric modeling approach would support the use of an alternate, more biologically based risk assessment approach (Andersen and Jarabek, 2001).

Mathematic models for nasal vapor uptake in humans and rodents have recently been developed (Morris et al., 1993; Bogdanffy et al., 1999; Andersen et al., 1999; Kimbell et al., 2001; Frederick et al., 2002). The current state-of-the-art approach is to use a hybrid model relying on computational fluid dynamic approaches to model vapor transport in the air phase and include, if appropriate, physiologically based pharmacokinetic approaches to model vapor disposition and metabolism within the tissue phases. This modeling approach may have great value for quantitative risk assessment of nasal toxicants.

Some of the potential complexities of vapor dosimetry relative to high- to low-dose and species extrapolations are exemplified by the studies of the author on nasal dosimetry of acetaldehyde. The uptake efficiency of this vapor is highly concentration dependent, with moderate to high (~70%) uptake efficiencies being observed at an inspired concentration of 1 ppm compared with low (~25%) efficiency at 1,000 ppm (Morris and Blanchard, 1992). The inspired concentration dependence on uptake efficiency was abolished in animals pretreated with an acetaldehyde dehydrogenase inhibitor (Stanek and Morris, 1999) providing strong evidence that reduced uptake at high concentrations was due to the capacity limitation of this enzyme. Because the expression levels of

aldehyde dehydrogenase may differ across species, it is anticipated that complicated relationships would be observed for uptake of acetaldehyde at various inspired concentrations across species. Greatly differing uptake efficiencies and relative dosimetric relationships were observed depending on the inspired concentration (1, 10, 100, or 1,000 ppm) and species (mouse, rat, hamster, and guinea pig). In fact, even the rank order of most efficient to least efficient species with respect to acetaldehyde scrubbing capacity differed depending on which exposure concentration was being examined (Morris, 1997). These studies highlight the potential complexities of nasal deposition and the potential weaknesses in extrapolation of toxic effects across species lines based simply on exposure concentration as the dosimeter.

16.5 RESPONSES OF THE NOSE

16.5.1 Reflex Responses

The respiratory tract is richly innervated with sensory nerves that arise from the trigeminal ganglia. Two classes of sensory nerves are thought to be important in respiratory tract reflex mechanisms: C fibers and Aδ fibers. There is no unambiguous functional criteria with which to separate these classes of sensory nerves. C fibers are thin unmyelinated nerves that are thought to be sensitive to chemical stimulation (Alarie, 1973; Nielsen, 1991; Carr and Undem, 2001). Aδ fibers are thicker and partially myelinated. The expression of neuropeptide mediators (see below) differs between nerve types, with neuropeptides being expressed primarily in C not Aδ fibers; however, this pattern may be altered by allergic airway disease (Undem et al., 2000; Carr and Undem, 2001) or by exposure to toxic agents (Hunter et al., 2000).

"Sensory irritants" are agents that stimulate upper airway trigeminal sensory nerves and produce a characteristic burning or painful sensation (Alarie, 1973). At high concentrations, an apneic response is produced. In rodents, the sensory irritation response is quantitated by the specific changes in breathing pattern that occur. Specifically, stimulation of trigeminal nerves by sensory irritants results in a decreased breathing frequency due to the induction of a pause (e.g., a brief apneic period) during expiration. The RD_{50} is the concentration of irritant that reduces breathing frequency to 50% of preexposure levels (Alarie, 1973). This has been a useful empirical approach for establishing occupation exposure guidelines in the absence of human data (Alarie, 1981; Bos et al., 1991; Schaper, 1993; Dalton, 2001). It has been suggested that occupational exposure guidelines be set at 3% of the RD_{50} in mice if human data are unavailable and if sensory irritation is the most sensitive end point (Schaper, 1993). Chemicals with widely differing structures can act as sensory irritants (Alarie, 1973; Nielsen, 1991; Bos et al., 1991). Efforts have been made to develop structure–activity modeling for sensory irritants (Abraham et al., 1998; Alarie et al., 1998). This is a challenging goal because sensory irritants may act on different sites on one receptor, may act through stimulation of several receptors, and may act directly (as the parent compound) or indirectly after metabolic activation.

Stimulation of sensory nerves may result in generation of an action potential and transmission of impulses to the respiratory centers of the brain stem with initiation of central responses (Figure 16.2). As discussed above, central responses include a sensation of itching or pain, as well as apnea, cough, or sneezing in humans, and "sensory irritation" in rodents (Alarie, 1973; Baraniuk, 1994; Shusterman, 2003). Stimulation of sensory nerves may activate parasympathetic efferent neurons resulting in release of acetylcholine and vasoactive intestinal protein (VIP) within the nasal mucosa. These mediators induce vasodilation, nasal congestion, and mucous secretion (Baraniuk 1994; Shusterman, 2003). The nasal mucosa is an erectile tissue. Nasal congestion is a condition in which the nasal tissues swell and occlude the nasal airway leading to airflow obstruction. Dilation of vessels deep in nasal tissue results in expansion of the nasal venous sinusoids and causes this swelling. Mucous secretion is caused by initiation of glandular exocytosis from the submucosal glands. The exocytosed material drains through the ducts into the nasal lumen.

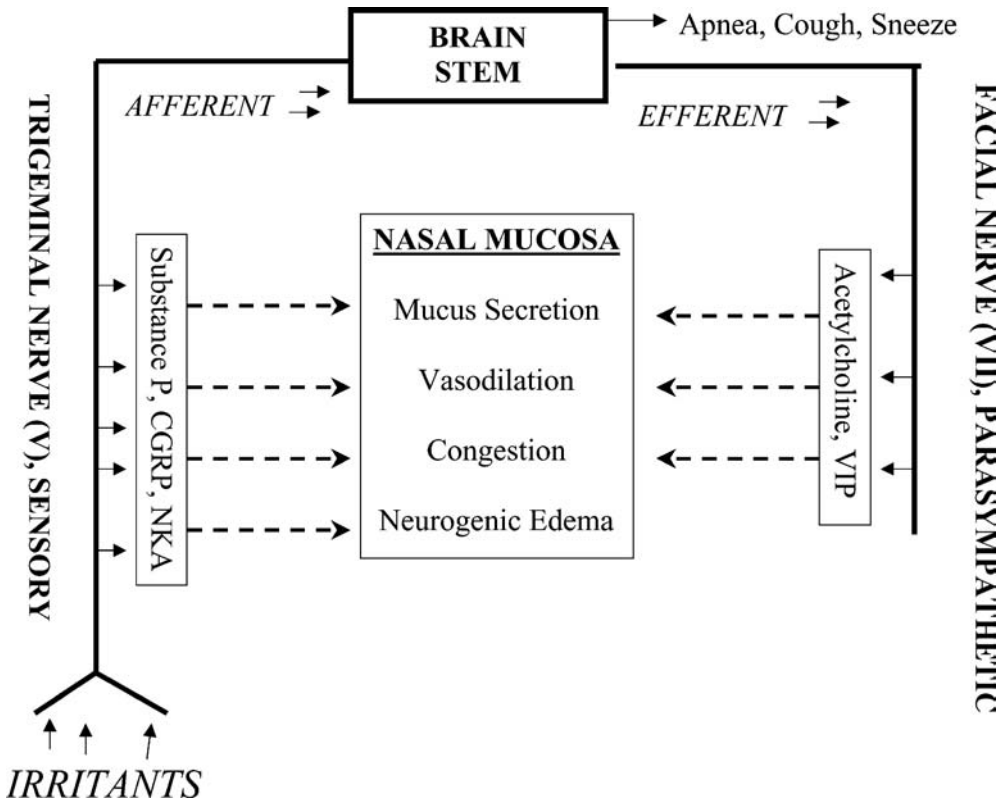


FIGURE 16.2 Schematic diagram of potential mechanisms of sensory nerve mediated nasal reflex responses to irritants. Activation of afferent sensory nerves can stimulate respiratory centers in the brain stem and cause the sensation of tickling and pain, can cause cough and changes in breathing patterns, and can initiate efferent parasympathetic nervous system–mediated responses. Sensory nerves activation can also result in the release of potent neuropeptide mediators in nasal tissues. The local nasal responses (mucus secretion, vasodilation, congestion, neurogenic edema) to irritants reflect the balance of parasympathetic and sensory nerve mediators (see text for details).

Extravasation of plasma in superficial capillaries may also contribute fluid and proteinaceous material to mucus (Baraniuk, 1994). All three of these processes, congestion, mucus release, and fluid secretion, can serve to diminish the dimensions of the nasal airspace and therefore cause nasal obstruction, in particular, in rodents.

Stimulation of sensory nerve terminals also results in a wave of depolarization that extends throughout the branched rami of the nerve, a process known as antidromal stimulation (Baraniuk, 1994; Barnes, 1996). This results in the release of neuropeptides in nasal tissues. These potent mediators include substance P and calcitonin gene-related peptide (CGRP) among others. Responses induced by these mediators include vasodilation, nasal congestion, mucus secretion, and neurogenic edema (Barnes et al., 1991a, 1991b). Vasodilation of the superficial capillaries results in increased blood flow, which may aid in removal of irritants from nasal tissues but does not cause nasal congestion and airflow obstruction (Widdicombe, 1990). This process may aid in rhinorhea (Baraniuk, 1994). In contrast, dilation of the vessels in the deep tissue results in pooling of blood in the venous sinusoids and nasal congestion as described above. Neurogenic edema is a process in which the endothelial cells of the postcapillary venules contract, resulting in increased capillary permeability. The basement membrane may become exposed as well, allowing for migration of leukocytes into

the nasal mucosa. Thus, the development of a neurogenic edema is the first step in the initiation of an inflammatory response (Baraniuk, 1994). Neuropeptides are rapidly degraded by tissue peptidases, in particular, neutral endopeptidase. These enzymes may be inhibited in inflammatory conditions or by tobacco smoke (Piedimonte, 1995). This may provide a potential mechanism for the enhanced sensitivity to irritants that is sometimes reported by individuals.

The response pattern to a specific irritant will depend on the interplay between parasympathetic activation and cholinergic mediator release with sensory nerve activation and antidromally mediated neuropeptide release. The overall balance may depend on the chemical properties of the irritant, the degree of stimulation of C versus Aδ fibers, the species (rodent versus human), and disease status. Unfortunately, few of these parameters are well defined.

Shusterman et al. (2003) has examined the responses of healthy and rhinitic humans to chlorine gas. A nasal congestive response was observed at exposure concentrations that produced minimal, if any, sensation of irritation, suggesting that local responses can occur in the absence of marked perception of the presence of an irritant. The physiological mechanisms responsible for the nasal congestive response to chlorine are not known. Interestingly, an enhanced congestive response to chlorine was observed in subjects with rhinitis. Sanico et al. (1999) have reported an enhanced responsiveness to capsaicin in rhinitic subjects. These results suggest that irritant sensitivity can by modulated by allergic airway disease.

Work in the author's laboratory has focused on reflex responses of the rodent to inspired irritants. In the rat the electrophilic irritant vapor acrolein rapidly induces multiple nasal responses including sensory irritation ($RD_{50} \sim 6$ ppm), vasodilation, plasma protein extravasation (neurogenic edema), and increased nasal airflow resistance (Morris et al., 1999). All these responses were diminished by chemical-induced degeneration of sensory nerves. Of these, the most sensitive response was vasodilation, which at low-exposure concentrations (<2 ppm) was observed in the absence of a change in nasal airflow resistance, suggesting it was due to dilation of the superficial capillaries. Subsequent studies revealed that vasodilation was the most sensitive response of the rat to inspired ethyl acrylate, acetaldehyde, and acetic acid vapors (Morris, 2002; Stanek et al., 2001). The RD50 for acetaldehyde is 3700 ppm yet acetaldehyde-induced nasal vasodilation was observed at exposure concentrations as low as 50 ppm, suggesting local effects may occur in the absence of centrally mediated responses (Stanek et al., 2001). This is perhaps analogous to the response pattern observed for chlorine in the human (Shusterman et al., 2003). The mediators responsible for the vasodilatory response in the rat are not known, but likely candidates include CGRP and nitric oxide (unpublished observations).

In the mouse, sensory irritation and increased nasal airflow resistance are the most sensitive responses to inspired acrolein and acetic acid (Morris et al., 2003). These responses are diminished by chemical-induced degeneration of sensory fibers. The absence of an effect of atropine on the response to acetic acid suggests that parasympathetic activation may not play an essential role in this response in this species. Heightened responses to these irritants were observed in mice with ovalbumin-induced allergic airway disease. Thus, enhanced nasal sensitivity to irritants in allergic airway disease has been documented in both humans (Shusterman et al., 2005) and animals (Morris et al., 2003). The sensory irritation response to both acrolein and acetic acid was enhanced in diseased-animals; however, the flow-resistance response was increased only by acetic acid not by acrolein. The mechanisms responsible for the heightened responsiveness are not known, but the comparison of these two irritants suggests that differing chemical-specific pathways may be modulated differentially in allergic airway disease.

As highlighted by the brief summary above, the overall response of the nose to an inspired irritant may be complex. Measurement of sensory irritation via quantitation of respiratory depression has been a useful empirical tool for assessing irritant potency (Schaper, 1993) but serves to obscure the complexity of the nasal response patterns. Because nasal symptoms are a common response of the human to pollutants, these represent toxicologically important response pathways. Nasal reflex responses appear to be protective in nature. For example, nasal discomfort may result in avoidance

behavior. Increased mucous secretion may serve to enhance clearance of irritants. Increased blood flow may serve to remove irritant from nasal tissues (but will enhance systemic delivery of these materials). A pause during expiration may enhance desorption of vapor and subsequent exhalation. It is likely that nasal reflex responses will alter nasal dosimetric patterns. Increased blood flow will serve to enhance nasal uptake of inspired water-soluble vapors (Morris, 2001; Morris et al., 2003). Nasal congestion will no doubt alter airflow patterns in the nose and alter regional delivery of airborne materials. Therefore, it is important to recognize that the microdosimetric patterns that occur during exposure to toxic levels of an irritant may differ from those that occur during exposure to no-effect levels of that irritant. These responses may appear relatively benign in healthy individuals, but the responses are augmented in allergic airway disease and can result in the exacerbation of symptoms. Moreover, although beyond the scope of this text, the pathogenic similarities between rhinitis and asthma may suggest that enhanced nasal responsiveness to irritants in rhinitis may be the harbinger of enhanced responsiveness of the lower airways in individuals with asthma.

16.5.2 Cytotoxic Injury

Cytotoxic agents can produce acute nasal injury. Often such agents produce characteristic patterns of lesions within the nose (Morgan, 1995). Lesion distribution in the nasal mucosa depends on toxicokinetic (delivered dose) and toxicodynamic (tissue sensitivity) factors. The focus of this text is on the toxicokinetic and toxicodynamic factors and their interplay relative to nasal lesion distribution. The histopathological characterization of common nasal lesions in rodent studies is well described (Harkema, 1992, 1999; Morgan, 1995; Hardisty et al., 1999) and will not be described herein. In assessing the potential importance of toxicokinetic versus toxicodynamic factors, it is essential to realize that scrubbing of inspired toxicant anteriorly in the nose results in diminished delivery to posterior sites (e.g., ethmoturbinates). Therefore, delivered dosage rates must be lower in posterior then anterior sites, and the demonstration of selective injury in the olfactory epithelium provides strong *a priori* evidence that toxicodynamic factors are critical. Toxicants can produce injury in squamous epithelial-lined anterior nose, in transitional and/or respiratory mucosa epithelium of the nasomaxilary turbinate region, or the olfactory mucosa of the ethmoturbinates. Examples of toxicants that injure these regions are provided below.

16.5.2.1 Squamous Epithelial Injury

The most anterior portions of the nasal cavity are lined with stratified squamous epithelium. Because of the difficulty in obtaining appropriate histological sections the nasal vestibule is rarely studied in detail (Gross et al., 1994). The squamous epithelium of the nasal vestibule is thought to be relatively resistant to toxic insult. Therefore, it would be predicted that only those agents that deposit with very high efficiency in that region would produce injury in this site. The reasons for this prediction are 2-fold. First, high delivered dosage rates would be needed to result in injury to resistant stratified squamous epithelium. Second, efficient uptake in the vestibule reduces the amount of toxicant that penetrates to the transitional, respiratory, or olfactory epithelium, thus limiting the potential for injury in these typically more sensitive tissues.

Efficient nasal uptake would only be anticipated for compounds that are highly water soluble and highly reactive (see above). The inorganic acid, hydrogen fluoride, possesses these properties. Inhalation exposure to this acid results in injury of the most anterior portions of the nose with a strong anterior-to-posterior gradient. Specifically, short-term ($<$1 h) exposure of the rat to near-lethal levels of hydrogen fluoride results in mucosal and submucosal necrosis and acute inflammation in the nasal vestibule, with no apparent injury in the other areas of the nose (Rosenholtz et al., 1963). Hydrogen fluoride is scrubbed from the airstream with extremely high efficiency ($>$99%) in the rat nose (Morris and Smith, 1982). The focal nature of these lesions would suggest that most of the material deposits in the nasal vestibule.

Glutaraldehyde, a highly water-soluble, highly reactive aldehyde can also produce squamous epithelial injury (Gross et al., 1994; van Birgelen et al., 2000). In addition this aldehyde also produces injury in the most anterior portions of the transitional and respiratory epithelium. Epithelial erosion and ulceration were observed in mice and rats at early time points (<1 week) during a 13-week inhalation study. As the exposure progresses, a delayed neutrophilic infiltration and squamous metaplasia occur. Cell replication rates are elevated, suggesting that a regenerative cell-proliferative response occurred (Gross et al., 1994). In these obligate nose-breathing rodents, these responses lead to nasal obstruction and asphyxia. Inhalation exposure to formaldehyde (see below) produces a similar type of response except that it is localized more posteriorly in the nose (Monticello et al., 1991). Both formaldehyde and glutaraldehyde cause a similar distribution of nasal lesions when instilled as aqueous solutions (St. Clair et al., 1990). This comparison suggests the anterior distribution of glutaraldehyde-induced lesions is attributable to high delivered dosage rates to this region during inhalation exposure.

Reactive and soluble gases often cause squamous epithelial injury. These are categorized in the RfC process as category 1 gases and vapors and the default species extrapolation is based on a ventilation-to-surface-area ratio for the laboratory animal compared with the human (see above). The default surface areas include all areas of the nose, including the extensive ethmoturbinate regions of the rodent. This provides a conservative estimate; however, because the injury is confined to the squamous epithelial lined anterior areas of the nose, an improvement in the extrapolation might be achieved by use of squamous epithelium-lined surface areas in both species as a surrogate for the effective surface areas.

16.5.2.2 Transitional Epithelial Injury

Transitional epithelium is present immediately posterior to the squamous epithelium and anterior to the mucus-lined respiratory epithelium. Ozone targets this epithelium (Harkema et al., 1997; Cho et al., 1999). The precise mechanisms for this pattern are not known. It has been postulated that nasal mucus acts as an antioxidant and serves to protect against the toxic effects of ozone (Harkema et al., 1997). Because the mucous lining layer covering the respiratory epithelium may be better developed than that covering the transitional epithelium, the underlying transitional epithelium may be at greater risk. Although speculative, as exemplified by the regional injury patterns for ozone, the characteristics of the mucous lining layer over transitional versus respiratory epithelia may differ, a factor that could be important for certain toxicants.

Ozone-induced nasal injury has been observed in the monkey and rat (Harkema et al. 1987a, 1987b; Cho et al., 1999). Similar lesions have been observed in people living in Mexico City, which has high-ozone pollution (Calderon Gardicuenas et al., 1992, 1995), but direct attribution of the injury to ozone per se is not possible. Acute exposure to high-ozone concentrations to human volunteers results in nasal inflammation (McBride et al., 1994). The progression of ozone-induced transitional epithelial lesions in the rat has been described (Cho et al., 1994). After acute cell injury, a regenerative hyperplasia occurs with an increase in the numbers of mucus-producing cells, a process termed mucous cell metaplasia. Pretreatment with an anti-inflammatory steroid can diminish the metaplastic response, suggesting that the initiation of an inflammatory response may be involved in the pathogenesis of this lesion (Hotchkiss et al., 1998). Mucous cell metaplasia has been observed in animal models and in humans (Harkema et al., 1987a, 1997; Calderon-Garciduenas et al., 1992, 1995). This response may be analogous to the goblet cell hyperplasia that is induced in lower airways by sulfur dioxide (Janey et al., 1991; Reed, 1978). An increased mucous barrier might serve to protect the underlying epithelium from inspired toxicants; however, excessive mucus production may be pathogenic.

16.5.2.3 Respiratory Epithelial Injury

Perhaps the best studied nasal toxicant is formaldehyde. Significant work on formaldehyde dosimetry and toxicity has ensued since the discovery that it is a rodent nasal carcinogen. Morgan noted that this

aldehyde produces a very site-specific pattern of injury and was also perhaps the first to appreciate that this might be reflective of inhalation dosimetric factors (Morgan and Monticello, 1989, 1990). A detailed review of this literature is beyond the scope of this chapter. The reader is referred to several excellent reviews (Morgan, 1977; Heck et al., 1990; Feron et al., 2001). In the rodent, formaldehyde forms DNA-protein crosslinks, which forms a useful tissue biomarker for delivered dose and may be mechanistically involved in formaldehyde carcinogenesis (Heck et al., 1990). DNA-protein crosslink levels are not uniformly distributed in the nose but are highest in specific regions of the nasomaxilary turbinates (Casanova et al., 1991, 1994). Acute and subacute exposure of the rat to formaldehyde can result in nasal respiratory epithelial necrosis and inflammation. The early cytotoxic response is followed by regenerative cell proliferation. With longer exposure times (up to 2 years) respiratory epithelial metaplasia, dysplasia, and squamaous cell carcinoma are observed. The concentration–response curve for formaldehyde-induced tumor formation in the rat nose is extremely nonlinear, and increased tumor incidence rates are only observed at exposure concentrations that are cytotoxic and cause regenerative cell proliferation (Morgan, 1977; Heck et al., 1990; Feron et al., 2001). It is thought that the regenerative cell proliferation is a key step in the carcinogenic process because the enhanced cell division rates result in less time for DNA repair, possible fixation of mutations, and/or clonal expansion of mutated cells (Morgan, 1977; Feron et al., 2001; Conolly et al., 2003). Whereas cytotoxicity and regenerative proliferation may be necessary steps in formaldehyde carcinogenesis, they are not sufficient to cause nasal tumor formation. Many cytotoxic compounds produce chronic nasal injury without a concomitant carcinogenic response (Feron et al., 2001).

Our understanding of the nasal dosimetry of formaldehyde is quite detailed and has lead to fundamental advances in our understanding of nasal functioning. Using a computations fluid dynamic approach, Kimbell et al. (1993) modeled formaldehyde uptake in the rodent nose. Predicted areas with high-deposition rates corresponded well with areas with the greatest delivered dosage, as assessed by DNA-protein crosslinks (Hubal et al., 1997), and greatest injury, as assessed by cytotoxicity and regenerative cell proliferation (Kimbell et al., 1997). Recently these efforts have been extended to the monkey nose, and again, good correlation was observed between the local nasal cell proliferation rates in formaldehyde-exposed monkeys and the predicted formaldehyde deposition flux in those regions (Kimbell et al., 2001). Most recently these modeling efforts have been extended to the human nose (Conolly et al., 2003).

As a category 1 gas, default species extrapolations for the RfC process for formaldehyde would be based on the ventilation-to-surface-area ratios for the rat and human. The extensive research on formaldehyde provides an elegant example of how dosimetry modeling can be used to gain insights into regional respiratory tract injury and to provide a strong biological basis for interspecies extrapolation and risk assessment of nasal toxicants. Basing species extrapolations on the use of the modeling approaches to predict regional depositional fluxes among species leads to a greatly improved risk assessment. Recently this modeling approach has been extended to predict local deposition fluxes within the ethmoturbinates of the olfactory toxicant hydrogen sulfide (Moulin et al., 2002). Again a reasonable correlation was observed between areas of high-deposition flux and regions of greatest injury in the ethmoturbinates. This suggests that this modeling approach may be useful for olfactory-selective toxicants in addition to the respiratory mucosal toxicants such as formaldehyde.

16.5.2.4 Olfactory Mucosal Injury

Many agents produce injury to the nasal olfactory epithelium. Because the delivered dosage rates of inspired agents to the posterior olfactory epithelium-lined turbinates must be less than to the anterior respiratory epithelium-lined turbinates, tissue sensitivity factors must be important for olfactory selective toxicants. Thus, review of olfactory toxicity encompasses many of the classical toxicological concepts such as metabolic activation, detoxication, etc.

Weak organic acids such as acrylic or acetic acid appear to target the olfactory mucosa of the rodent. For example, olfactory sustentacular cell necrosis, olfactory neuronal degeneration, and

desquamation occur after acute exposure of the rat to acrylic acid (Miller et al., 1981; Frederick et al., 1998). No injury is observed in the adjacent respiratory mucosal-lined regions. These lesions show an anterior-to-posterior gradient within the olfactory mucosa of the nose with the greatest injury being observed in the dorsal medial meatus (Miller et al., 1981; Hardisty et al., 1999). The modeling of rodent nasal airflow patterns predicts that only the air in the dorsal medial airstream flows over olfactory mucosa (Kimbell et al., 1993, 1997). Therefore, the highest delivered dosage rates would be anticipated to be over those regions where injury is the most severe. The olfactory mucosal injury can be reproduced *in vitro* by short-term (2-h) incubation of nasal explants with acrylic acid at concentrations of 0.5 m*M* or greater (Frederick et al., 1998). At these concentrations acrylic acid does not produce injury in nasal respiratory epithelial explants. Cells of the olfactory mucosa seem to be particularly sensitive to injury, thus explaining why olfactory injury is observed in the absence of respiratory epithelial injury in rodents exposed to weak organic acids.

As a category 1 vapor, the ventilation-to-surface-area ratios for the rat and human would be used for the default derivation of the RfC for acrylic acid. A computational fluid dynamics and physiologically based inhalation model has been established for interspecies extrapolation of acrylic acid toxicity in the rodent and human (Frederick et al., 1998). This model incorporates air-phase diffusion of the acid- and tissue-phase disposition of the acid in a compartmentalized nose and makes specific predictions relative to olfactory tissue acid concentrations over a range of airborne exposure concentrations. This model predicts that, at equal airborne concentrations, the tissue levels of acrylic acid in the rat exceed those in the human nose, the opposite of what is predicted by the default RfC methodology.

The high sensitivity to acids would suggest that olfactory epithelium would be sensitive to injury from materials that are metabolized to acids within that site, which appears to be the case. Carboxylesterases act to hydrolyze esters to their respective acids and alcohols. These are high-capacity enzymes that are highly expressed in nasal tissues (Bogdanffy and Keller, 1999) with the activity of carboxyleterases being higher in olfactory than in respiratory mucosa. Pretreatment of rodents with carboxylesterase inhibitors dramatically reduces nasal uptake rates of inspired ester vapors (Morris, 1990; Morris and Frederick, 1995; Bogdanffy et al., 1999), providing strong evidence that these vapors are indeed extensively metabolized by carboxylesterases in nasal tissues *in vivo.* Further evidence is provided by the fact that alcohol metabolite, ethanol, is present in air exiting the isolated nasal cavity of rats exposed to either ethyl acetate (Morris, 1990) or ethyl acrylate (Morris and Frederick, 1995). It has been shown that olfactory selective injury results from acute, subchronic, or chronic injury to a variety of ester vapors (Hardisty et al., 1999) characterized by sustentacular cell necrosis and olfactory neuronal degeneration with regeneration and/or atrophy depending on the length of exposure and exposure concentration. A strong anterior-to-posterior olfactory lesion gradient is observed with the most severe injury being present in the dorsal medial meatus (Hardistry et al., 1999). Again, the similarity of the lesions between acids and esters is striking. Moreover, the lesions observed in rat nasal olfactory explants treated with ethyl acrylate are virtually identical with those caused by acrylic acid (Frederick et al., 2002). The high levels of carboxylesterase and the sensitivity of olfactory mucosa to acid-induced injury likely account for the selective injury of this mucosa by inspired esters.

Given the weight of the evidence supporting the carboxylesterase-dependent mechanism of action of esters vapors, quantitative risk assessment of ester-induced olfactory injury would best be based on parameters related to acid production rates. This requires formulation of models that incorporate tissue disposition factors such as diffusion to the bloodstream and vascular clearance, and nonlinear (e.g., Michealis–Menten) metabolism kinetics. A physiologically based pharmacokinetic model represents a convenient, widely used approach to approximate these processes. Models have been developed for vinyl acetate (Bogdanffy et al., 1999), ethyl acrylate (Frederick et al., 2002), and methyl methacrylate (Andersen et al., 1999). All models incorporated metabolic data for the rat and the human to facilitate quantitative species extrapolations. The vinyl acetate model incorporates terms to predict changes in tissue pH induced by vinyl acetate. As a proof of concept it has been

shown that vinyl acetate reduces hepatocellular pH *in vitro* through a carboxylesterase-dependent mechanism (Bogdanffy, 2002). The ethyl acrylate model is a hybrid model that incorporates a computational fluid dynamic approach to model the air-phase behavior and a physiologically based pharmacokinetic approach for tissue disposition. This model is of the same form as used for acrylic acid (Frederick et al., 1998) and provides a template that could be easily extended to cover other vapors. The methyl methacrylate model utilizes a clearance/extraction approach more analogous to those used for hepatic models. These modeling approaches exemplify state-of-the-art techniques on which to base species extrapolations and quantitative risk assessment.

In addition to carboxylesterase, aldehyde dehydrogenase is another enzyme that is highly expressed in nasal tissues that results in the formation of acidic metabolites. This author has suggested that aldehyde dehydrogenase-dependent formation of acetic acid from acetaldehyde may be important in the olfactory toxicity and carcinogenecity of this aldehyde (Morris, 1997). In the rodent, acetaldehyde causes olfactory necrosis, degeneration, and tumorigenesis (Wouterson et al., 1986), depending on the exposure concentration and duration. In contrast to formaldehyde, only limited respiratory epithelial damage is observed. Because of its lower water solubility and reactivity, a lower deposition flux over respiratory epithelium is anticipated for acetaldehyde versus formaldehyde. This, coupled with a role for aldehyde dehydrogenase-dependent acid production, may provide potential explanations for the olfactory selectivity of acetaldehyde.

Cytochromes P450 (CYP450s) are widely expressed in nasal tissues (Dahl and Hadley, 1991; Bogdanffy and Keller, 1999; Reed, 1993; Thornton-Manning and Dahl, 1997; Ding and Kaminsky, 2003). CYP450s known to be present in human nasal tissues include CYP2A6, 2A13, 2C, and 3A. Because of the difficulty in obtaining nasal tissues, thorough investigations of CYP540 expression have not been performed (Ding and Kaminsky, 2003). In general, these enzymes are expressed in higher levels in olfactory than respiratory mucosa. CYP450 activation of compounds to metabolically active forms by these enzymes is important in the olfactory toxicity of a variety of compounds, including methyl bromide (Hurtt et al., 1988), styrene (Cruzan et al., 2002), chloroform (Mery et al., 1994), and bromobenzene (Brittebo et al., 1991). Several agents produce nasal olfactory injury when administered parenterally, including dichlobenil (Brittebo et al., 1991), acetaminophen (Jeffrey and Haschek, 1988), and β,β′-iminodiproprionitrile (Genter et al., 1992). Local activation by olfactory CYP450 is thought to be responsible for the injury induced by these compounds. Injury can be produced by these compounds in a variety of cells and structures, including epithelium, Bowman's glands in the deep olfactory tissue, and the underlying bone.

The fact that olfactory injury can be produced after parenteral administration highlights the possibility that nasal olfactory injury in inhalation toxicity tests may not be a result of direct delivery of toxicant from the airstream. As exemplified by chloroform, suggestive information on the route of delivery of toxicant can be inferred from the distribution of injury within the ethmoturbinates. For many inhaled compounds, a strong anterior-to-posterior pattern of injury is observed in the olfactory mucosa (Hardisty et al., 1999). In contrast, inhaled chloroform-induced injury is most severe in the ventral lateral portions of the ethmoturbinates and in cells deep within the lamina propria. Because these sites are not within the major airstreams, Mery et al. (1994) suggested that chloroform was not being delivered via the inspired air. For the low-solubility vapor, chloroform, absorption likely occurs in the lungs rather than the nose, and the solvent is then delivered to the critical tissue (e.g., olfactory mucosa) via the bloodstream. In this context it may be more appropriate for risk assessment purposes to consider the nose to be an extrarespiratory site and base dosimetric evaluations on physiologically based pharmacokinetic approaches similar to those in hepatic toxicity.

In summary, a variety of chemicals can produce nasal cytotoxic injury. Depending on the chemical, such injury can result from either systemic or inhalation exposure. Because chemical-induced nasal injury demonstrates characteristic distribution patterns within that site, mapping of the lesions is essential. Often the site-selective injury of inhaled chemicals depends on microdosimetric patterns within the nose. This is perhaps best exemplified by the three aldehydes: glutaraldehyde, formaldehyde, and acetaldehyde. All three produce nasal cytotoxicity with subsequent regenerative cell

proliferation and likely work through analogous mechanisms. Based on the solubility and reactivity properties, the sites of injury correlates with the predicted sites of deposition within the nose for these compounds. Glutaraldehyde, the most reactive and water soluble, produces injury in the anterior squamous epithelium. The soluble, but less reactive, formaldehyde produces injury in the respiratory epithelium, and the less soluble and less reactive acetaldehyde produces injury in the posterior, olfactory epithelium. In the case of acetaldehyde, biotransformation via olfactory metabolic pathways may also be important. As for any nasal toxicant, a complete understanding of the nasal toxicities of these aldehydes depends on the integration of information on site-selective injury with knowledge of the regional dosimetric patterns within the nose.

REFERENCES

Abraham, M.H., Kumarsingh, R., Cometto-Muniz, J.E., and Cain, W.S., An algorithm for nasal pungency thresholds in man, *Arch. Toxicol.,* 72, 227–232, 1998.

Alarie, Y., Sensory irritation by airborne chemicals*, Crit. Rev. Toxicol.,* 3, 299–363, 1973.

Alarie, Y., Bioassay for evaluating the potency of airborne sensory irritants and predicting acceptable levels of exposure in man, *Food Cosmet. Toxicol.,* 19, 623–626, 1981.

Alarie, Y., Schaper, M., Nielsen, G.D., and Abraham, M.H., Structure-activity relationships of volatile organic chemicals as sensory irritants, *Arch. Toxicol.,* 72, 125–140, 1998.

Andersen, M.E. and Jarabek, A.M., Nasal tissue dosimetry—issues and approaches for "category 1" gases, *Inhalation Toxicol.,* 13, 415–435, 2001.

Andersen, M.E., Sarangapani, R., Frederick, C.B., and Kimbell, J.S., Dosimetric adjustment factors for methyl methacrylagte derived steady-state analysis of a physiologically based clearance-extraction model, *Inhalation Toxicol.,* 11, 899–906, 1999.

Baraniuk, J.N., Neural control of the upper respiratory tract, in *Neuropeptides in Respiratory Medicine,* Kaliner, M.A., Barnes, P.J., Kunkel, G.H.H., and Baraniuk, J.N., Eds., Marcel Dekker, New York, 1994, pp. 79–123.

Barnes, P.J., Role of neural mechanisms in airway defense, in *Environmental Impact on the Airways,* Cretien, J. and Dusser, D., Eds., Marcel Dekker, New York, 1996, pp. 93–121.

Barnes, P.J., Baraniuk, J.N., and Belvisi, M.G., Neuropeptides in the respiratory tract, Part I, *Am. Rev. Respir. Dis.,* 144, 1187–1198, 1991a.

Barnes, P.J., Baraniuk, J.N., and Belvisi, M.G., Neuropeptides in the respiratory tract, Part II, *Am. Rev. Respir. Dis.,* 144, 1391–1399, 1991b.

Bascom, R., Kulle, T., Kagey-Sobotka, A., and Proud, D., Upper respiratory tract environmental tobacco smoke sensitivity, *Am. Rev. Respir. Dis.,* 143, 1304–1311, 1991.

Belinsky, S.A., White, C.M., Boucheron, J.A., Richardson, F.C., Swenberg, J.A., and Anderson, M., Accumulation and persistence of DNA adducts in respiratory tissue of rats following multiple administrations of the tobacco specific carcinogen 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone, *Cancer Res.,* 46, 1280–1284, 1986.

Bogdanffy, M.S., Vinyl acetate-induced intracellular acidification: implications for risk assessment, *Toxicol Sci.,* 66, 320–326, 2002.

Bogdanffy, M.S. and Keller, D.A., Metabolism of xenobiotics by the respiratory tract, in *Toxicology of the Lung,* 3rd ed., Gardner, D.E., Crapo, J.D., and McClellan, R.O., Eds., Taylor & Francis, Washington, DC, 1999, pp. 85–123.

Bogdanffy, M.S., Manning, L.A., and Sarangapani, R.H., High-affinity nasal extraction of vinyl acetate vapor is carboxylesterase dependent, *Inhalation Toxicol.,* 11, 927–941, 1999.

Bogdanffy, M.S., Morgan, P.H., Starr, T.B., and Morgan, K.T., Binding of formaldehyde to human and rat nasal mucus and bovine serum albumin, *Toxicol. Lett.,* 38, 145–154, 1987.

Bogdanffy, M.S. and Sarangapani, R., Physiologically-based kinetic modeling of vapours toxic to the respiratory tract, *Toxicol. Lett.,* 138, 103–117, 2003.

Bogdanffy, M.S., Sarangapani, R., Plowchalk, D.R., Jarabek, A., and Andersen, M.E., A biologically based risk assessment for vinyl acetate-induced cancer and noncancer inhalation toxicity, *Toxicol. Sci.,* 51, 19–35, 1999.

Bojsen-Moller, F. and Fahrenkrug, J., Nasal swell-bodies and cyclic changes in the air passage of the rat and rabbit nose, *J. Anat.,* 110, 25–37, 1971.

Bos, P.M., Zwart, A., Reuzel, P.G., and Bragt, P.C., Evaluation of the sensory irritation test for the assessment of occupational health risk, *Crit. Rev. Toxicol.,* 21, 423–450, 1991.

Brenneman, K.A., Wong, B.A., Buccellato, M.A., Costa, E.R., Gross, E.A., and Dorman, D.C., Direct olfactory transport of inhaled manganese ($^{54}MnCl_2$) to the rat brain: toxicokinetic investigations in a unilateral nasal occlusion model, *Toxicol. Appl. Pharmacol.*, 69, 238–248, 2000.

Brittebo, E.G., Eriksson, C., Feil, V., Bakke, J., and Brandt, I., Toxicity of 2,6-dichlorothiobenzamide (chlorthiamid) and 2,6-dichlorobenzamide in the olfactory nasal mucosa of mice, *Fundam. Appl. Toxicol.,* 17, 91–102, 1991.

Calderon-Garciduenas, L., Osorno Velazquez, A., Bravo Alvarez, H., Delgado Chavez, R., and Barrios Marquez, R., Histopathologic changes of the nasal mucosa in southwest metropolitan Mexico City inhabitants, *Am. J. Pathol.,* 140, 225–232, 1992.

Calderon-Garciduenas, L., Rodriquea Alcaraz, A., Garcia, R., Ramirez, L., and Barragan, G., Nasal inflammatory responses in children exposed to a polluted urban atmosphere, *J. Toxicol. Environ. Health,* 45, 427–437, 1995.

Carr, M.J. and Undem, B.J., Inflammation-induced plasticity of the afferent innervation of the airways, *Environ. Health Perspect.,* 109 (Suppl. 4), 567–571, 2001.

Casanova, M., Morgan, K.T., Gross, E.A., Moss, O.R., and Heck, H.d'A., DNA-protein cross-links and cell replication at specific sites in the nose of F344 rats exposed subchronically to formaldehyde, *Fundam. Appl. Toxicol.,* 23, 525–536, 1994.

Casanova, M., Morgan, K.T., Steinhagen, W.H., Everitt, J.I., Popp, J.A., and Heck, H.d'A., Covalent binding of inhaled formaldehyde to DNA in the respiratory tract of rhesus monkeys: pharmacokinetics, rat-to-monkey interspecies scaling, and extrapolation to man, *Fundam. Appl. Toxicol.,* 17, 409–428, 1991.

Cho, H.Y., Hotchkiss, J.A., and Harkema, J.R., Inflammatory and epithelial responses during the development of ozone-induced mucous cell metaplasia in the nasal epithelium of rats, *Toxicol. Sci.,* 51, 135–145, 1999.

Cometto-Muniz, J.E. and Cain, W.S., Trigeminal and olfactory sensitivity: comparison of modalities and methods of measurement, *Int. Arch. Occup. Environ. Health,* 71, 105–110, 1998.

Conolly, R.B., Kimbell, J.S., Janszen, D., Schlosser, P.M., Kalisak, D., Preston, J., and Miller, F.J., Biologically motivated computational modeling of formaldehyde carcinogenicity in the F344 rat, *Toxicol, Sci.,* 75, 423–447, 2003.

Cruzan, G., Carlson, G.P., Johnaon, K.A., Andrews, L.S., Banton, M.I., Bevan, C., and Cushman, J.R., Styrene respiratory tract toxicity and mouse lung tumors are mediated by CYP2F-generated metabolites, *Regul. Toxicol. Pharmacol.,* 35, 308–319, 2002.

Dahl, A.R., Contemporary issues in toxicology: dose concepts for inhaled vapors and gases, *Toxicol. Appl. Pharmacol.,* 103, 185–197, 1990.

Dahl, A.R. and Hadley, W.M., Nasal cavity enzymes involved in xenobiotic metabolism: effects on the toxicity of inhalants, *Crit. Rev. Toxicol.,* 21, 345–372, 1991.

Dalton, P., Evaluating the human response to sensory irritation: implications for setting occupational exposure limits, *Am., Ind. Hyg. Assoc. J.,* 62, 723–729, 2001.

Dawes, J.D.K., The course of the nasal airstreams, *J. Laryngol. Otol.,* 49, 583–593, 1952.

Dawes, J.D.K. and Prichard, M., Studies of the vascular arrangement of the nose, *J. Anat.,* 87, 311–322, 1953.

Ding, X. and Kaminsky, L.S., Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts, *Annu. Rev. Pharmacol. Toxicol.,* 43, 149–173, 2003.

Feron, V.J., Arts, J.H., Kuper, C.F., Slootweg, P.J., and Woutersen, R.A., Health risks associated with inhaled nasal toxicants, *Crit. Rev. Toxicol.,* 31, 313–347, 2001.

Fischman, J., Were dinos cold-blooded after all? The nose knows, *Science,* 270, 735–736, 1995.

Fisher, E.W., Scadding, G.K., and Lung, V.J., The role of acoustic rhinometry in studying the nasal cycle, *Rhinology,* 31, 57–61, 1993.

Frank, N.R., Yoder, R.D., Brain, J.D., and Yokoyama, E., SO_2 (^{35}S labeled) absorption by the nose and mouth under conditions of varying concentration and flow, *Arch. Environ. Health*, 18, 315–322, 1969.

Frederick, C.B., Bush, M.L., Lomax, L.G., Black, K.A., Finch, L., Kimbell, J.S., Morgan, K.T., Subramaniam, R.P., Morris, J.B., and Ultman, J.S., Application of a hybrid computational fluid dynamics and physiologically based inhalation model for interspecies dosimetry extrapolation of acidic vapors in the upper airways, *Toxico. Appl. Pharmacol.,* 152, 211–231, 1998.

Frederick, C.B., Lomax, L.G., Black, K.A., Finch, L., Scribner, H.E., Kimbell, J.S., Morgan, K.T., Subramaniam, R.P., and Morris, J.B., Use of a hybrid computational fluid dynamics and physiologically based inhalation model for interspecies dosimetry comparisons of ester vapors, *Toxicol. Appl. Pharmacol.,* 183, 23–40, 2002.

Genter, M.B., Deamer, N.J., Cao, Y., and Levi, P.E., Effects of P450 inhibition and induction on the olfactory toxicity of beta,beta'-iminodipropionitrile (IDPN) in the rat, *J. Biochem. Toxicol.,* 9, 31–39, 1994.

Genter, M.B., Llorens, J., O'Callaghan, J.P., Peele, D.B., Morgan, K.T., and Crofton, K.M., Olfactory toxicity of β,β′−iminodipropionitrile in the rat, *J. Pharmacol. Exp. Ther.,* 263, 1432–1439, 1992.

Gerde, P., Cheng, Y.S., and Medinsky, M.A., In vivo deposition of ultrafine aerosols in the nasal airway of the rat, *Fundam. Appl. Toxicol.,* 16, 330–336, 1991.

Gross, E.A., Mellick, P.W., Kari, F.W., Miller, F.J., and Morgan, K.T., Histopathology and cell replication responses in the respiratory tract of rats and mice exposed by inhalation to glutaraldehyde for up to 13 weeks, *Fundam. Appl. Toxicol.,* 23, 348–362, 1994.

Gross, E.A. and Morgan, K.T., Architecture of nasal passages and larynx, in *Comparative Biology of the Normal Lung,* Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992, pp. 7–25.

Hahn, I., Scherer, P.W., and Mozell, M.M., Velocity profiles measured for airflow through a large-scale model of the human nasal cavity, *J. Appl. Physiol.,* 75, 2273–2287, 1993.

Hanna, L.M. and Scherer, P.W., Regional control of local airway heat and water vapor loses, *J. Appl. Physiol*., 61, 624–632, 1986.

Hardisty, J.F., Garman, R.H., Harkema, J.R., Lomax, L.G., and Morgan, K.T., Histopathology of nasal olfactory mucosa from selected inhalation toxicity studies conducted with volatile chemicals, *Toxicol. Pathol.,* 6, 618–627, 1999.

Harkema, J.R., Epithelial cells of the nasal passages, in *Comparative Biology of the Normal Lung,* Parent, R.A., Ed., CRC Press, Boca Raton, FL, pp. 27–36, 1992.

Harkema, J.R., Comparative structure, function, and toxicity of the nasal airways, in *Toxicology of the Lung,* 3rd ed., Gardner, D.E., Crapo, J.D., and McClellan, R.O., Eds., Taylor & Francis, Washington, DC, 1999, pp. 55–82.

Harkema, J.R., Hotchkiss, J.A., and Griffith, W.E., Mucous cell metaplasia in rat nasal epithelium after a 20-month exposure to ozone: a morphometric study of epithelial cell differentiation, *Am. J. Respir. Cell Mol. Biol.,* 16, 521–530, 1997.

Harkema, J.R., Plopper, C.G., Hyde, D.M., St. George, J.A., and Dungworth, D.L., Effects of an ambient level of ozone on primate nasal epithelial mucosubstances. Quantitative histochemistry, *Am. J. Pathol.,* 127, 90–96, 1987a.

Harkema, J.R., Plopper, C.G., Hyde, D.M., St. George, J.A., Wilson, D.W., and Dungworth, D.L., Response of the macaque nasal epithelium to ambient levels of ozone. A morphologic and morphometric study of the transitional and respiratory epithelium, *Am. J. Pathol*., 128, 29–44, 1987b.

Heck, H.d'A., Casanova, M., and Starr, T.B., Formaldehyde toxicity—new understanding, *Crit. Rev. Toxicol.,* 20, 397–426, 1990.

Hotchkiss, J.A., Hilaski, R., Cho, H., Regan, K., Spencer, P., Slack, K., and Harkema, J.R., Fluticasone propionate attenuates ozone-induced rhinitis and mucous cell metaplasia in rat nasal airway epithelium, *Am. J. Respir. Cell Mol. Biol.,* 18, 91–99, 1998.

Hu, S.C., Ben-Jebria, A., and Ultman, J.S., Longitudinal distribution of ozone absorption in the lung: effects of respiratory flow, *J. Appl. Physiol.,* 77, 574–583, 1994.

Huang, Z.L., Ong, K.L., Goh, S.Y., Liew, H.L., Yeoh, K.H., and Wang, de Y., Assessment of nasal cycle by acoustic rhinometry and rhinomanometry, *Otolaryngol. Head Neck Surg.,* 128, 510–516, 2003.

Hubal, E.A., Schlosser, P.M., Conolly, R.B., and Kimbell, J.S., Comparison of inhaled formaldehyde dosimetry predictions with DNA-protein cross-link measurements in the rat nasal passages, *Toxicol. Appl. Pharmacol.,* 143, 47–55, 1997.

Hunter, D.D., Satterfield, B.E., Huang, J., Fedan, J.S., and Dey, R.D., Toluene diisocyanate enhances substance P in sensory neurons innervating the nasal mucosa, *Am. J. Respir. Crit. Care Med.,* 161, 543–549, 2000.

Hurtt, M.E., Thomas, D.A., Working, P.K., Monticello, T.M., and Morgan, K.T., Degeneration and regeneration of the olfactory epithelium following inhalation exposure to methyl bromide: Pathology, cell kinetics, and olfactory function, *Toxicol. Appl. Pharmacol.,* 94, 311–328, 1988.

Janey, B., Gallup, M., Tsuda, T., and Basbaum, C., Mucin gene expression in rat airways following infection and irritation, *Biochem. Biophys. Res. Commun.,* 181, 1–8, 1991.

Jarabek, A.M., Inhalation RfC methodology: dosimetric adjustments and dose-response estimation of noncancer toxicity in the upper respiratory tract, in *Nasal Toxicity and Dosimetry of Inhaled Xenobiotics, Implications for Human Health,* Miller, F.J., Ed., Taylor & Francis, Washington, DC, 1995, pp. 310–325.

Jeffrey, E.H. and Haschek ,W.M., Protection by dimethylsulfide against acetaminophen-induced hepatic, but not respiratory toxicity in the mouse, *Toxicol. Appl. Pharmacol.,* 93, 452–461, 1988.

Keyhani, K., Schrerer, P.W., and Mozell, M.M., Numerical simulation of airflow in the human nasal cavity, *J. Biomed. Eng.,* 117, 429–442, 1995.

Kimbell, J.S., Gross, E.A., Joyner, D.R., Godo, M.N., and Morgan, K.T., Application of computational fluid dynamics to regional dosimetry on inhaled chemicals in the upper respiratory tract of the rat, *Toxicol. Appl. Pharmacol.,* 121, 253–263, 1993.

Kimbell, J.S., Gross, E.A., Richardson, R.B., Conolly, R.B., and Morgan, K.T., Correlation of regional formaldehyde flux predictions with the distribution of formaldehyde-induced squamous metaplasia in F344 rat nasal passages, *Mutat. Res.,* 380, 143–154, 1997.

Kimbell, J.S., Subramaniam, R.P., Gross, E.A., Schlosser, P.M., and Morgan, K.T., Dosimetry modeling of inhaled formaldehyde: comparisons of local flux predictions in the rat, monkey and human nasal passages, *Toxicol. Sci.,* 64, 100–110, 2001.

Lofberg, B., Brittebo, E.B., and Tjalve, H., Localization and binding of N'-nitrosonornicotine metabolites in the nasal region and in some other tissues of Sprague-Dawley rats, *Cancer Res.,* 42, 2877–2883, 1982.

McBride, D.E., Koenig, J.Q., Luchtel, D.L., Williams, P.V., and Henderson, W.R., Inflammatory effects of ozone in the upper airways of subjects with asthma, *Am. J. Respir. Crit. Care Med.,* 149, 1192–1197, 1994.

Medinsky, M.A. and Bond, J.A., Sites and mechanisms for uptake of gases and vapors in the respiratory tract, *Toxicology,* 160, 165–172, 2001.

Medinsky, M.A., Bond, J.A., Schlosser, P.M., and Morris, J.B., Mechanisms and models for respiratory-tract uptake of volatile organic chemicals, in *Toxicology of the Lung,* 3rd ed., Gardner, D.E., Crapo, J.D., and McClellan, R.O., Eds., Taylor & Francis, Washington, DC, 1999, pp. 483–512.

Mery, S., Larson, J.L., Butterworth, B.E., Wolf, D.C., Harden, R., and Morgan, K.T., Nasal toxicity of chloroform in male F-344 rats and female B6C3F1 mice following 1-week inhalation exposure, *Toxicol. Appl. Pharmacol.,* 125, 214–227, 1994.

Miller, F.J., Dosimetry of particles in laboratory animals and man, in *Toxicology of the Lung,* 3rd ed., Gardner, D.E., Crapo, J.D., and McClellan, R.O., Eds., Taylor & Francis, Washington, DC, 1999. pp. 513–555.

Miller, F.J., McNeal, C.A., Kirtz, J.M., Gardner, D.E., Coffin, D.L., and Menzel, D.B., Nasopharyngeal removal of ozone in rabbits and guinea pigs, *Toxicology,* 14, 273–281, 1979.

Miller, R.R., Ayres, J.A., Jersey, G.C., and McKenna, M.J., Inhalation toxicity of acrylic acid, *Fundam. Appl. Toxicol.,* 1, 271–277, 1981.

Monticello, T.M., Miller, F.J., and Morgan, K.T., Regional increases in rat respiratory epithelial cell proliferation following acute and subacute inhalation of formaldehyde, *Toxicol. Appl. Pharmacol.,* 111, 409–421, 1991.

Morgan, K.T., A brief review of formaldehyde carcinogenesis in relation to rat nasal pathology and human health risk assessment, *Toxicol. Pathol.,* 25, 291–307, 1977.

Morgan, K.T., Nasal dosimetry, lesion distribution, and the toxicologic pathologist: a brief review, in *Nasal Toxicity and Dosimetry of Inhaled Xenobiotics, Implications for Human Health,* Miller, Ed., Taylor & Francis, Washington, DC, 1995, pp. 41–57.

Morgan, K.T., Jiang, X.Z., Patterson, D.L., and Gross, E.A., The nasal mucociliary apparatus: Correlation of structure and function in the rat, *Am. Rev. Respir. Dis.,* 130, 275–281, 1984.

Morgan, K.T. and Monticello, T.M., Airflow, mucociliary clearance, and lesion distribution in the nasal passages of experimental animals, in *Nasal Carcinogenesis in Rodents: Relevance to Human Health Risk,* Pudoc Wageningen, Wageningen, Netherlands, 1989, pp. 36–41.

Morgan, K.T. and Monticello, T.M. Airflow, gas deposition, and lesion distribution in the nasal passages, *Environ. Health Perspect.,* 85, 209–218, 1990.

Morris, J.B., First-pass metabolism of inspired ethyl acetate in the upper respiratory tracts of the F344 rat and syrian hamster, *Toxicol. Appl. Pharmacol.,* 102, 331–345, 1990.
Morris, J.B., Upper respiratory tract metabolism of inspired alcohol dehydrogenase and mixed function oxidase substrate vapors under defined airflow conditions, *Inhalation Toxicol.,* 5, 203–221, 1993.
Morris, J.B., In vivo measurements of uptake, in *Nasal Toxicity and Dosimetry of Inhaled Xenobiotics, Implications for Human Health,* Miller, F.J., Ed., Taylor & Francis, Washington, DC, 1995, pp. 99–111.
Morris, J.B., Uptake of acetaldehyde and aldehyde dehydrogenase levels in the upper respiratory tracts of the mouse, rat, hamster and guinea pig, *Fundam. Appl. Toxicol.,* 35, 91–100, 1997.
Morris, J.B., A method for measuring upper respiratory tract vapor uptake and its applicability to quantitative inhalation risk assessment, *Inhalation Toxicol.,* 11, 101–123, 1999.
Morris, J.B., Overview of upper respiratory tract vapor uptake studies, *Inhalation Toxicol.,* 13, 335–345, 2001.
Morris, J.B., Sensory-nerve mediated nasal vasodilatory response to inspired ethyl acrylate, *Inhalation Toxicol.,* 14, 585–597, 2002.
Morris, J.B. and Blanchard, K.T., Upper respiratory tract deposition of inspired acetaldehyde, *Toxicol. Appl. Pharmacol.,* 114, 140–146, 1992.
Morris, J.B. and Frederick, C.B., Upper respiratory tract uptake of acrylate ester and acid vapors, *Inhalation Toxicol.,* 7, 557–574, 1995.
Morris, J.B., Hassett, D.N., and Blanchard, K.T., A physiologically based pharmacokinetic model for nasal uptake and metabolism of non-reactive vapors, *Toxicol. Appl. Pharmacol.,* 123, 120–129, 1993.
Morris, J.B. and Smith, F.A., Regional deposition and absorption of inhaled hydrogen fluoride in the rat, *Toxicol. Appl. Pharmacol*., 62, 81–89, 1982.
Morris, J.B., Symanowicz, P.T., Olsen, J.E., Thrall, R.S., Cloutier, M.M., and Hubbard, A.K., Immediate sensory-nerve mediated respiratory responses to irritants in healthy and allergic airway diseased mice, *J. Appl. Physiol.,* 94, 1563–1571, 2003.
Moulin, F.J., Brenneman, K.A., Kimbell, J.S., and Dorman, D.C., Predicted regional flux of hydrogen sulfide correlates with distribution of nasal olfactory lesions in rats, *Toxicol. Sci.,* 66, 7–17, 2002.
Nielsen, G., Mechanisms of activation of the sensory irritant receptor by airborne chemicals, *Crit. Rev. Toxicol.,* 21, 183–208, 1991.
Nodelman, V. and Ultman, J.S., Longitudinal distribution of chlorine absorption in human airways: comparison of nasal and oral quiet breathing, *J. Appl. Physiol.,* 86, 1984–1993, 1999.
Piedimonte, G., Tachykinin peptides, receptors, and peptidases in airway disease, *Exp. Lung Res.,* 21, 809–834, 1995.
Proctor, D.F., The upper airways. I. Nasal physiology and defense of the lungs, *Am. Rev. Respir. Dis.,* 115, 97–129, 1977.
Proctor, D.F. and Chang, J.C.F., Comparative anatomy and physiology of the nasal cavity, in *Nasal Tumors in Animals and Man,* Vol. 1, Reznik, G. and Stinson, S.F., Eds., CRC Press, Boca Raton, FL, 1983, pp. 1–33.
Raabe, O.G., Al-Bayati, M.A., Teague, S.V., and Rasolt, A., Regional deposition of inhaled monodisperse coarse and fine aerosol particles in small laboratory animals, *Ann. Occup. Hyg.,* 32 (Suppl. 1), 53–63, 1988.
Reed, C.J., Drug metabolism in the nasal cavity: relevance to toxicology, *Drug Metab. Rev.,* 25, 173–205, 1993.
Reid, L., Animal models in clinical disease, in *Respiratory Tract Mucus,* Reid, L.M., and Jeffrey, P. Eds., Ciba Foundation Symposium 54 (new series), Elsevier, Amsterdam, 1978, pp. 297–307.
Rosenholtz, J.J., Carson, T.R., Weeks, M.H., Wilinski, F., Ford, D.F., and Oberst, F.W., A toxicopathologic study in animals after brief single exposures to hydrogen fluoride, *Am. Ind. Hyg. Assoc. J.,* 24, 253–261, 1963.
St. Clair, M.B.G., Gross, E.A., and Morgan, K.T., Pathology and cell proliferation induced by intra-nasal instillation of aldehydes in the rat: comparison of glutaraldehyde and formaldehyde, *Toxicol. Pathol.,* 18, 353–361, 1990.
Sanico, A.M., Koliatsos, V.E., Stanisz, A.M., Bienenstock, J., and Togias, A., Neural hyperresponsiveness and nerve growth factor in allergic rhinitis, *Int. Arch. Allergy Immunol.,* 118, 154–158, 1999.
Schaper, M., Development of a database for sensory irritants and its use in establishing occupational exposure limits, *Am. Ind. Hyg. Assoc. J.,* 54, 488–544, 1993.
Schlesinger, R.B., Comparative deposition of inhaled aerosols in experimental animals and humans: a review, *J. Toxicol. Environ. Health,* 15, 197–214, 1985.
Schreider, J.P., nasal airway anatomy and inhalation deposition in experimental animals and people, in *Nasal Tumors in Animals and Man,* Vol. III, Reznik, G. and Stinson, S.F., Eds., CRC Press, Boca Raton, FL, 1983, pp. 1–26.

Shusterman, D., Toxicology of nasal irritants, *Curr. Allergy Asthma Rep.,* 3, 258–265, 2003.

Shusterman, D., Balmes, J., Avila, P.C., Murphy, M.A., and Matovinovic, E., Chlorine inhalation produces nasal congestion in allergic rhinitics without mast cell degranualation, *Eur. Respir. J.*, 21, 652–657, 2003.

Shusterman, D.J., Tarun, A., Murphy, M.A., and Morris, J.B., Seasonal allergic rhinitic and normal subjects respond differentially to nasal provocation with acetic acid vapor, *Inhal. Toxicol.,* 17, 147–152, 2005.

Speizer, F.E. and Frank, N.R., The uptake and release of SO_2 by the human nose, *Arch. Environ. Health,* 12, 725–728, 1966.

Stanek, J.J., and Morris, J.B., The effect of inhibition of aldehyde dehydrogenase on nasal uptake of inspired acetaldehyde, *Toxicol. Sci.,* 49, 225–231, 1999.

Stanek, J., Symanowicz, P.T., Olsen, J.E., Gianutsos, G., and Morris, J.B., Sensory nerve mediated nasal vasodilatory response to inspired acetaldehyde and acetic acid vapors, *Inhalation Toxicol.,* 13, 807–822, 2001.

Subramaniam, R., Richardson, R.B., Morgan, K.T., Kepler, G., Kimbell, J.S., and Guilmette, R.A., Computational fluid dynamics simulations of inspiratory airflow in the human nose and nasopharynx, *Inhalation Toxicol.,* 10, 91–120, 1998.

Tai, C.F. and Baraniuk, J.N., Upper airway neurogenic mechanisms, *Curr. Opin. Allergy Clin. Immunol.,* 2, 11–19, 2002.

Thornton-Manning, J.R. and Dahl, A.R., Metabolic capacity of nasal tissue interspecies comparisons of xenobiotic-metabolizing enzymes, *Mutat. Res.,* 380, 43–59, 1997.

Thrall, K.D., Schwartz, R.E., Weitz, K.K., Soelberg, J.J., Foureman, G.L., Prah, J.D., and Timchalk, C., A real-time method to evaluate the nasal deposition and clearance of acetone in the human volunteers, *Inhalation Toxicol.,* 15, 523–538, 2003.

Undem, B.J., Kajekar, R., Hunter, D.D., and Myers, A.C., Neural integration and allergic disease, *J. Allergy Clin. Immunol.,* 106 (5 Suppl), S213–S220, 2000.

U.S. Environmental Protection Agency, Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry. EPA/600/8–90/066F. Office of Research and Development, National Center for Environmental Assessment, Washington, DC, 1994.

van Birgelen, A.P., Chou, B.J., Renne, R.A., Grumbein, S.L., Roycroft, J.H., Hailey, J.R., and Bucher, J.R., Effects of glutaraldehyde in a 2-year inhalation study in rats and mice, *Toxicol. Sci.,* 55, 195–205, 2000.

Widdicombe, J.G., Nasal pathophysiology, *Respir. Med.,* 84 (Suppl A), 3–10, 1990.

Woutersen, R.A., Appelman, L.M., Van Garderen-Hoetmer, A., and Feron, V.J., Inhalation toxicity of acetaldehyde in rats. III. Carcinogenicity study, *Toxicology,* 41, 213–231, 1986.

17 Effects of Inhaled Toxicants on the Nose and Nasal Function

Pamela Dalton and Richard Opiekum

CONTENTS

17.1 OVERVIEW

Historically, within the fields of toxicology, occupational medicine, and industrial hygiene, the nose and nasal function have garnered little attention as targets for toxic agents, in particular, when compared with other organs and organ systems. The nose has been viewed predominantly as a respiratory organ, functioning to filter and humidify inspired air and to detect chemicals via odor. Until recently, little emphasis has been given to its importance as a portal of entry for environmental toxicants and its role in vapor and particulate capture and removal. Furthermore, systemic effects from nasal uptake have only recently been considered in the context of exogenous exposures to metals and viruses. In this chapter we aim to clarify the importance and functionality of the nose in humans and to summarize the latest knowledge about the adverse effects associated with exposure

to occupational and environmental toxicants on the nose and nasal function, including olfactory dysfunction, sensory irritation, irritant rhinitis, sinusitis, septal perforation, and neoplasms. Because the majority of data on nasal toxicology from occupational and environmental toxicants derives from subchronic and chronic exposure studies in animals we will necessarily review the evidence from those but will primarily focus on the documented adverse effects of exposure in humans. We will also discuss the anatomical and metabolic factors that complicate the extrapolation of data from animal exposure assays to human risk assessment.

17.2 BACKGROUND: ANATOMY AND PATHOPHYSIOLOGY

17.2.1 Physiological Functions of the Upper Airway

In both humans and experimental animals, the nose serves as a filter, humidifier, and thermoregulator of inspired air; the upper airways also incorporate multiple mechanisms for removing volatiles and particulates from inspired airstreams—through mucociliary clearance, vascular uptake and diffusion, and extensive metabolic capacity to remove and detoxify soluble particulates and vapors. All these functions are subsumed in the role of the nose as gatekeeper and protector of the lower airways. For example, the ability of the nose to heat and humidify large volumes of inspired air is critical to protection of the lower airways. The nose also functions as a particle filter. Filtration of large particles occurs through their impaction on the nasal turbinates where they are trapped in the mucus layer and subsequently cleared. In contrast, finer particles ($<0.10\,\mu m$), which are often not captured by this method, can bypass this region and reach the lower respiratory tract. Many, if not most, water-soluble irritant vapors, such as ammonia and chlorine, are taken up in the anterior portion of the nasal-pharyngeal mucous membrane layer, thereby protecting the lower respiratory tract from exposure (Hornung and Mozell, 1977). The efficacy by which the nose acts to "scrub" vapors from the incoming airstream is a function of both the physical properties of the chemical and the anatomical structure of an individual's nose. Last but not least, the nose is also an important sensory organ, containing receptors that are capable of detecting a wide variety of airborne chemicals through the perception of odor and, at higher concentrations, the perception of nasal irritation or pungency (Dalton, 2002).

17.2.2 Anatomy

The framework of the nose is composed of a combination of bone and cartilage that is covered internally by an innervated mucous membrane layer containing blood vessels and receptors (Gray, 1974). The nasal cavity can be divided into three parts: the nasal vestibule, the respiratory region, and the olfactory region. The septum, consisting of a bony plate and a cartilaginous wall, divides the nasal cavity into two symmetrical halves. On average, in humans, the total surface area of both nasal cavities is approximately 150 cm^2 and the total volume is about 15 ml. The anterior portion of the nasal cavity opens in the nostril and the posterior part opens into the rhinopharynx. Volatile chemicals can be inhaled into the nasal cavity orthonasally through the nostril (or nares) or can enter retronasally from the mouth or during swallowing (thus enabling the significant contribution of olfactory perception to food flavor). These odor plumes are broken up by convolutions in the tissue, called turbinates (see Figure 17.1). Actual exposure to odorants depends on patterns of airflow through a geometrically complex structure, and only recently have models been developed that allow quantitative analysis of flow patterns. (Early attempts to determine flow patterns tried lining the noses of human subjects with litmus paper and having the subjects inhale ammonia fumes [Paulsen, 1882], but this technique proved unreliable. It was replaced by the development of physical models of the nasal cavity. Although early models were typically cast directly from the noses of human cadavers, modern anatomical casts have been developed from three-dimensional information provided by computed tomography [CT] scans.)

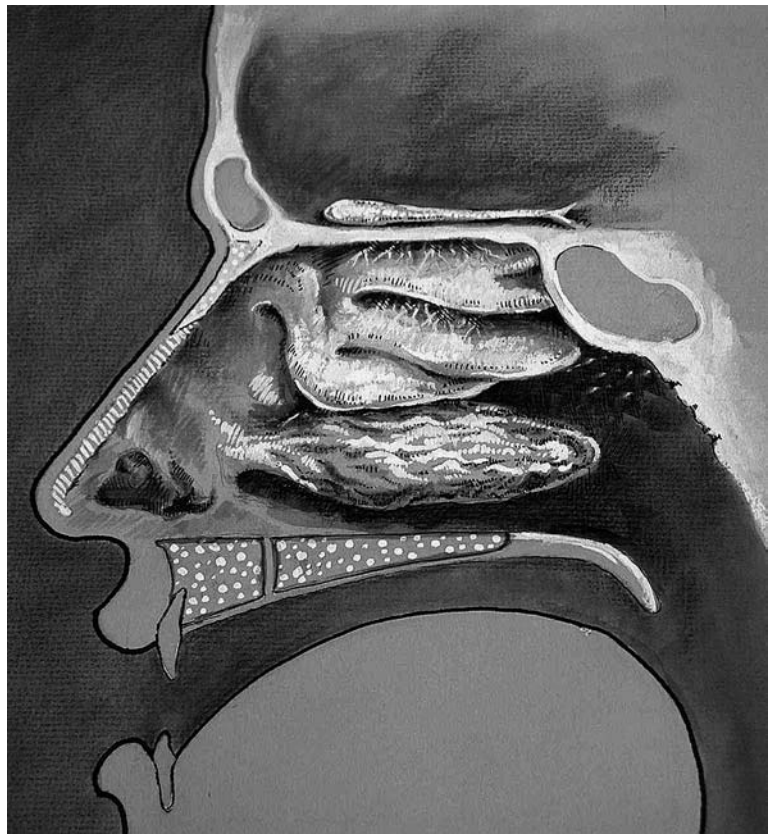


FIGURE 17.1 Sagittal view of the human nasal passages showing the nasal turbinates and the location of the olfactory epithelium.

The outermost openings of the nose, the nares, lead directly into the nasal vestibule, with the narrowest region of this opening being referred to as the nasal valve area. This segment of the upper airway is the most limiting, resulting in up to a 50% airflow reduction in certain ethnic groups (Cole, 1993). This portion of the nasal chamber is lined with keratinizing squamous epithelium containing sebaceous glands, sweat glands, and vibrissae (Clerico, 2003).

In both rodents and primates, the nasal passages contain four distinct types of epithelia: the stratified squamous epithelium that lines the nasal vestibule and the floor of the ventral meatus in the anterior part of the nose; the nonciliated, transitional epithelium that lies between the squamous epithelium and the respiratory epithelium; the ciliated, respiratory epithelium that lines the rest of the nasal cavity anterior and ventral to the olfactory epithelium; and the olfactory neuroepithelium. However, the distribution of these epithelia, their abundance, their susceptibility to injury, and their metabolic capacity vary considerably between rodents and humans (Harkema, 1999). For example, the olfactory epithelium (OE) lines approximately 50% and 37% of the nasal cavity surface area in F344 rats and B6C3F mice, respectively, whereas a mere 3% of the nasal cavity surface area is devoted to OE in humans.

The primary functions of the respiratory epithelium are to humidify, filter, and warm the incoming air during inspiration. The respiratory epithelium contains goblet cells in the vicinity of the inferior turbinate and basal cells underlying the mucosa, as well as both ciliated and nonciliated columnar cells (Clerico, 2003). In humans, the respiratory epithelium covers much of the upper two thirds of the nasal passages, except for those locations containing olfactory epithelium. The primary function of the olfactory epithelium is to detect odorants in the inspired air. The olfactory epithelium is located on both sides of the upper portion of the nasal cavity and the olfactory cleft

and, in humans, occupies a total area of about 3–5 cm^2, as compared with 20–200 cm^2 in dogs. Recent anatomical (Feron et al., 1998) and functional (Restrepo et al., 1993) studies have confirmed the existence of olfactory epithelium extending onto the middle turbinate and septum. Acute injury to the olfactory epithlium can result in loss of olfactory cilia, degeneration of epithelial cells, atrophy, necrosis, or ulceration. Inflammation may occur in both olfactory and respiratory epithelium and subacute to chronic lesions, but such damage often resolves through replacement of olfactory epithelium with ciliated respiratory epithlium or squamous metaplasia. If the progenitor basal cells remain intact and capable of proliferation, however, olfactory epithelium (at least in animals given chronic exposure to vapors) can regenerate (Schwob, 2002).

In addition to cilia and mucus, the nasal cavity is highly vascularized (Sorokin, 1988), which provides a substantial clearance mechanism for many inhaled gases and vapors. Blood is supplied to the nasal cavity from the carotid arteries. Because of repeated branching, the internal carotid artery connects with the ophthalmic artery, which further branches to the anterior and posterior ethmoid arteries. These arteries continue through the ethmoid bone where they branch into the meningeal and nasal branches that supply the dura matter and the nasal cavity, respectively (Clerico, 2003). The external carotid artery delivers blood into the nasal cavity through the facial and internal maxillary arteries. These arteries branch several times to supply the nasal cavity and septum at their terminus. Blood flow throughout the mucosa is regulated by an intricate series of resistance and capacitance vessels (Harkema, 1999). The resistance vessels are composed of small arteries, arterioles, and arteriovenous anatomoses, whereas capacitance vessels are innervated vessels that are used to increase nasal resistance and alter airflow and are where most of the blood volume is found and where regional blood volume is regulated (Harkema, 1999).

17.2.2.1 Sensory Innervation

The nasal cavity is innervated by the olfactory nerve (cranial nerve I) whose primary function is odorant detection and the ophthalmic and maxillary branches of the trigeminal nerve (cranial nerve 5) (Clerico, 2003), which provide information about pain, temperature changes, itch, and changes in airflow sensation. (Harkema, 1999).

The olfactory epithelium contains cells of three types: olfactory receptor neurons (ORNs), their precursors (basal cells), and sustentacular cells (serving glialike, supportive functions). The receptor cells (ORNs) are located beneath a watery, mucus layer in this epithelium; on one end of each receptor, projections of the hairlike, olfactory cilia extend down into the watery layer covering the membrane. The receptor sites for odorant molecules are on the cilia and as such, the cilia are the structures involved in the initial stages of olfactory signal transduction (Lowe and Gold, 1991). Odor information is transmitted via the bundles of axons that form the olfactory nerve (cranial nerve I), which extend from the olfactory receptor cells in the olfactory epithlium through the cribriform plate to synapse, unbranched, within the olfactory bulb, a small structure in the base of the forebrain where receptor input is integrated. The potential for some metals to reach the brain via the olfactory nerve (CN I) can produce impairment in olfactory ability via damage to central olfactory structures such as the olfactory bulb (Sunderman, 2001).

In addition to the olfactory receptors, the nasal cavity is innervated by unmyelinated, free nerve endings of the maxillary and ethmoid branches of the trigeminal nerve, which are capable of detecting chemically and mechanically stimulated sensations of pungency, irritation, warmth, and coolness. Formerly referred to as the "common chemical sense," the cutaneous mucosal sensation elicited by chemical stimulation is now known as "chemesthesis" (Green et al., 1990). The appeal of this term is that it does not imply that chemical sensitivity is a separate sensory modality but rather includes chemical sensitivity as a general property of the cutaneous senses, along with thermal and mechanical sensitivity. The primary function of chemesthesis in the nose is presumed to involve detection and avoidance of caustic or reactive chemicals in airway mucosal tissue and the lungs (Green et al., 1990). Nevertheless, it should be recognized that the appreciation of spicy foods, carbonated beverages, and cooling vapors (i.e., menthol) largely rely on the sensations of chemesthesis.

Recently, it has been shown that the terminal branches of the trigeminal nerve possess nociceptive neurons populated with assorted nociceptive ion channels (Tai and Baraniuk, 2002). The entire nasal mucosa is innervated with fibers of the sympathetic and parasympathetic nervous system. When these fibers are stimulated by sharp changes in temperature or with an irritating chemical vapor, they are responsible for altering blood flow and initiating congestion or decongestion of the nose. Activation of trigeminal fibers in the nasal passages via irritating vapors, particles, or dusts can initiate a variety of reflexes, leading to sneezing, rhinorrhea, nasal congestion, cough, and bronchospasm.

Although the nose, nasopharynx, and larynx are often described as separate sites of origin for respiratory tract reflexes and sensations, the boundaries of sensory innervation between these areas are rather diffuse (see Figure 17.2) (Widdicombe, 1986b). This redundancy or overlap complicates our ability to predict the irritancy of a chemical from the activity or response of a single afferent pathway, because many, if not most, vapor-phase stimuli will act upon several sites and on several afferent pathways in the upper respiratory tract. Even though chemical solubility, together with flow rate, determines the dominant patterns of chemical deposition in the upper airways, most inhaled irritant vapors or dusts have the potential of contacting multiple sites of mucosal tissue and thereby directly eliciting sensory irritation via the trigeminal, glossopharyngeal, or vagal nerves. Reflex responses to inhaled irritants can be elicited indirectly as well. For example, stimulation of the

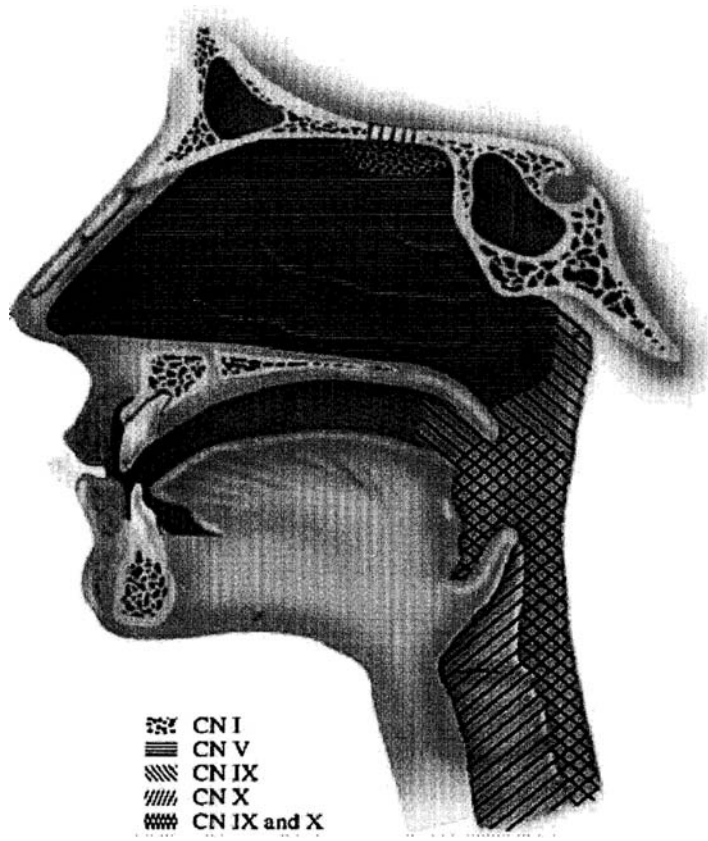


FIGURE 17.2 Representation of the regions within the nasal passages innervated by each of several cranial nerves. The cross-hatched area represents regions of overlap between CN IX and X. CN I may extend further down onto the middle turbinate than depicted here. CN I, olfactory nerve; CN V, trigeminal nerve; CN IX, glossopharyngeal nerve; CN X, vagal nerve. (Adapted with permission from Doty et al., 2004.)

trigeminal fibers in the nose can elicit reflexes from the nasopharynx, whereas nasal mucus that reaches the pharynx or larynx can elicit cough, even in the absence of direct stimulation by irritant vapors (Widdicombe, 1986a).

17.2.2.2 Mucosa and Mucociliary System

The inner surface of the nose beyond the nasal valve area starting anterior to the inferior turbinate is bathed in a thick, watery mucous film composed of approximately 96% water and 4% glycoprotein (Widdicombe and Wells, 1982). This material serves as a defense mechanism, trapping inspired particulate matter and vapors and allowing them to be held for removal by nose blowing or allowing them to be carried by the cilia to the nasopharynx where the mucus can be swallowed and ultimately removed. Secretory cells (e.g., Goblet cells) in the epithelium and in the subepithelial seromucinous glands within the lamina propria are responsible for mucus (mucin) production (Harkema, 1999). Vesicles from the Golgi apparatus secrete mucus in droplets between 1 and 2 μm in diameter (Quraishi et al., 1998). In healthy adults, the median goblet cell density consists of 5,700–11,000 cells per ml^2 throughout the nasal cavity, with lowest cell density on the septum and turbinates (Quraishi et al., 1998). Mucus within the nose is actually a biphasic sheet with a gel layer consisting mostly of glycoproteins with a depth between 0.5 and 2.0 μm and an aqueous layer between 7 and 10 μm deep (Quraishi et al., 1998). Cilia are located in this aqueous layer and beat with a forceful and rapid effective stroke flowed by a slower recovery stroke. The mucociliary system is a two-part system relying on mucus flow and secretion and ciliary beat (Quraishi et al., 1998). Throughout a 24-h period, mucus, composed of glycoproteins, ions, and water, is produced at a rate between 0.5 and $1ml/cm^2$ (Quraishi et al., 1998). The clearance of inhaled substances via mucociliary transport depends on the interaction between ciliary beat efficiency and mucus composition (Passali et al., 1999). Alterations in mucus composition or viscosity or the integrity of the cilia can impair the clearance of inhaled volatiles and particles from the nasal passages, thereby increasing the residence time of the toxicant and the effective dose to the nasal epithelium.

17.3 INHALATION EXPOSURE OF THE NOSE AND UPPER AIRWAYS: AIRFLOW AND DEPOSITION

As air enters the nasal passages and passes toward the first major segment of the respiratory system it is warmed and takes up water vapor. For nose breathers, adjustment of inspired air temperature begins at the nostrils, allowing temperature of an ambient airstream to reach between 28 and 37°C by the time the air traverses the nasopharynx. According to model studies, airflow at the entrance to the nostrils is laminar and continues as such until the end of the vestibular portion of the nose is reached (Brain et al., 1977). At this point a constriction with the smallest cross-sectional area of the nose, the nasal valve area, is encountered, resulting in the largest airflow velocity of any region of the tracheobronchial tree. After air passes through this region there is an abrupt increase in cross-sectional area, because of the presence of turbinates, and a large decrease in air velocity giving rise to turbulent flow. At the rear of this nasal chamber, air from parallel nasal passages combines and continues toward the nasopharynx. In the nasopharynx, the cross-sectional area decreases, increasing the air velocity slightly. As the tracheobronchial tree progresses, the velocity of airflow steadily declines. As air penetrates even deeper toward the lungs the cross-sectional area of the airways increases greatly from 2.5 cm^2 in the trachea to nearly 11,800 cm^2 in the alveoli (Ganong, 1991).

The laws of physics govern the transport of gases and particles entrained in the inhaled airstream; transport mechanisms include deposition, absorption, uptake, distribution metabolism, and elimination or clearance. Airway disposition will vary as a function of the inhaled agent for each region in the respiratory tract and across species. Along with the morphology of the nasal passages and other host characteristics (e.g., degree of airway humidity, airway diameters, and disease state) exposure to an inhaled volatile or particle is primarily determined by physicochemical properties

of inhaled volatiles and particles that influence the deposition of the material in the nasal passages, both in amount and location (Brain et al., 1977) and ultimately, the toxic effect.

17.3.1 Gas or Vapor Properties

The dominant processes affecting gas/vapor transport in the nose similarly govern transport in the respiratory tract as a whole, namely convection, diffusion, absorption, dissolution, and chemical reactions. The movement of a gas in the nasal tract is induced by pressure gradients and is termed convection. Diffusion is superimposed on convection due to local concentration gradients, whereas absorption removes gases from the lumen and affects the concentration gradients. Chemical reactions can be important drivers of the toxicity of a material, because they can increase absorption by acting as a sink to drive the concentration gradient. Properties such as water or lipid solubility and reactivity influence the interaction of gases with the respiratory tract and ultimately determine the degree of uptake.

Inhaled chemicals delivered as a gas or vapor to the upper respiratory tract can be cleared by any of four primary processes: desorption and elimination into exhaled air, entrainment into the mucus flow and elimination via the gastrointestinal (GI) tract, capillary uptake and transport to other parts of the body via the bloodstream, and local enzymatic or chemical degradation in the nasal tissue. From a toxicological perspective, it is also worth noting that some materials (e.g., metals, such as cadmium or mercury) can be transported from the nasal passage via the main olfactory nerve (CN I) and access the brain where central damage can occur.

17.3.2 Particle Properties

Nearly 100% of all particles greater than 10 μm in aerodynamic diameter will be deposited high in the respiratory tract, with many impacting within the nares. From inhaled particles between 2 and 5 μm, less than 10% are deposited in the tracheobronchial region and between 20 and 30% are deposited in the pulmonary region (Perera and Ahmed, 1979). Minimal deposition of particles between 0.3 and 1.0 μm occurs in the upper respiratory tract. The route of entry into the system also affects the total number of particles entering the lung. During normal conditions, the nasal route collects more particles than the oral route because of the presence of the vibrissae and the complex convolutions of the turbinates (Yu et al., 1981). Respiration rate also plays a pivotal role. The external flow in oral breathing is similar to nose breathing except that air velocity during mouth breathing is lower (Brain et al., 1977). Tidal volume, functional residual capacity, and breath holding will affect particle behavior as well (Hofmann, 1987).

17.4 UPPER AIRWAY HEALTH EFFECTS ASSOCIATED WITH INHALED NASAL TOXICANTS

Both experimental and epidemiological studies have documented a variety of effects that exposure to an inhaled occupational or environmental toxicant can have on the function and integrity of the nose and nasal function. This section will briefly review the adverse outcomes that have been associated with various classes of nasal toxicants in humans.

17.4.1 Olfactory Dysfunction

The nose is the site of olfactory sensation and, in this capacity, serves as one of the dominant windows of the brain on the external world. Among humans, impairment of the ability to smell can have serious consequences for the detection of many olfactory warning signals (e.g., smoke, spoiled food, and gas leaks) and can produce a significant impact on nutritional status, eating satisfaction, and many other issues related to quality of life. Awareness of the potential for olfactory loss from occupational

exposure to volatiles has been noted in the medical literature for more than 100 years (Mackenzie, 1884). However, until recently, the incidence of morbid or lethal outcomes from occupational exposures, such as lung disease or cancer, likely overshadowed concerns about the impact of occupational exposure on olfactory function.

Impairment of olfactory function due to acute or chronic exposure to airborne toxicants can be temporary, long-lasting, or permanent (Doty et al., 1991; Shusterman, 2003) and of different levels of severity. Although a total loss of olfactory sensitivity, *anosmia,* is a relatively rare outcome after chronic low-level occupational exposure, *hyposmia,* manifested as varying gradations of sensitivity loss to multiple odorants, is a far more common sequela and can render an individual highly susceptible to environmental dangers and produce substantial decrements in their quality of life. Additionally, the distortion of odorant quality or *dysosmia,* is a disturbingly common outcome after chemical damage and regeneration of the olfactory epithelium in both humans and animal models (Amoore, 1986; Schwob et al., 1995) and perhaps, more than other forms of olfactory dysfunction, has been characterized as an "extremely disconcerting experience for patients" (Cowart and Rawson, 2001).

The olfactory mucosa of rodents appears to be highly susceptible to damage from inhaled chemicals (see Table 17.1). Although it has been difficult to demonstrate complete loss of olfactory ability in animals after experimental chemical ablation of the olfactory epithelium by inhalation of compounds such as methyl bromide or zinc sulfate (Slotnick et al., 2000; Youngentob et al., 1997), there are several reasons why such findings might underestimate the impact on humans from comparable exposures. First, the majority of functional olfactory assessments employed in animal studies are fairly gross evaluations, capable only of discriminating total loss from some minor level of preserved olfactory function; studies that have used more sophisticated assays have indeed found significant functional deficits and persistent quality distortions following chemical damage to the epithelium (Schwob et al., 1995). Second, the olfactory epithelium in humans occupies a much smaller proportion of the total nasal passages than in the most widely used animal models (e.g., rats, mice) and, therefore, similar levels of chemical damage may compromise human olfactory function to a much greater degree. Finally, the protective and repair processes that occur after chemical exposure may differ substantially between animals and humans and the evidence shows that these regenerative changes themselves may have the greatest detrimental impact on chemosensory function (Feron et al., 2001). Thus, the available data from both human and animal studies yield ample evidence that chemosensory functions of the nose are compromised after chronic exposure to inhaled occupational chemicals (Morgan, 1994).

17.4.2 Sensory Irritation

Sensory irritation of the upper airways (also known as chemesthesis) is an acute and reversible condition resulting from exposure to workplace or household chemicals or cleaning products and volatile organic compounds used in building materials or furnishings and cigarette smoke (Cometto-Muniz and Cain, 1992; Hodgson, 1992, 1998). Chemesthesis can also result from exposure to mold spores and fungi that either directly irritate the mucous membranes or release irritating volatile organic compounds (Hendry and Cole, 1993). This sensation is brought about by nerve endings from branches of the trigeminal nerve, which innervate the epithelium of the nasal mucosa, eyes, and regions of the oral cavity. The anterior portion of the nose and walls contralateral to the septum are also innervated by the lateral and medial branches of the ethmoid nerve (Doty and Cometto-Muniz, 2003).

Volatile chemicals contained in the airstream during inhalation can act directly on the tissue of the mucosa or can stimulate receptors indirectly by acting on specific ion channels (e.g., H^+) and releasing specific endogenous chemicals (McClesky and Gold, 1999). In addition, these receptors can also be activated by excessive heat (Caterina et al., 1997). Once these receptors are activated, several physiological responses may occur and include changes in respiration rate, increased nasal secretion, feeling of pain, itch, cooling or burning, or alteration in odor perception (Doty and Cometto-Muniz, 2003).

Table 17.1 Effects of Toxic Agents on Nasal Mucosa of Common Laboratory Rodents

Compound, Concentration	Duration	Effects on Histology and Function	References
Acetaldehyde, 400–5,000 ppm	6 h/d, 5 d/wk for 1–28 mo	Degeneration, metaplasia, loss of Bowman's glands and nerve bundles, adenomas, squamous cell carcinoma	Appelman et al., 1982; Woutersen et al., 1984, 1986; Buckley et al., 1984
Acrelein, 17 ppm	6 h/d, for 5 d 13 wk	Hypertrophy, hyperplasia, erosion, ulceration, necrosis inflammation	Buckley et al., 1984
Acrylic acid, 5–75 ppm	6 h/d, 5 d/wk for 13 wk	Degeneration, replacement with respiratory epithelium, inflammation, hyperplasia of Bowman's glands	Miller et al., 1981
Benomyl, 50–200 mg/m^3	6 h/d, 6 d/wk	Degeneration	Warheit et al., 1983
Bromobenzene, 25 µmol/kg i.p.	[5 min–3 d]	Degeneration of olfactory epithelium and Bowman's glands	Brittebo et al., 1990
Cadmium, 250–500 µg/m^3	5 h/d, 5 d/wk for 20 wk	Little change	Sun et al., 1996
Chlorine gas, 0.4–11 ppm	6 h/d, 5 d/wk for 16 wk	Degeneration, septal perforations, intracellular deposits of eosinophilic material, mucus cell hypertrophy	Wolf et al., 1995
Chloroform, 300 ppm	6 h/d for 7 wk	Degeneration of Bowman's glands, cell proliferation in periosteum and bone	Mery et al., 1994
Chloropicrin, 8 ppm	6 h/d, for 5 wk	Hypertrophy, hyperplasia, ulceration, necrosis, inflammation	Buckley et al., 1984
Coumarin, 50 mg/kg i.p.	[48 h]	Necrosis, cell loss, and basal cell metaplasia in the olfactory mucosa	Gu et al., 1997
Chlorthiamid, 6–50 mg/kg i.p.	[8 h–7 d]	Degeneration of olfactory epithelium and Bowman's glands, replacement with respiratory epithelium fibrosis in lamina propria	Brittebo et al., 1991
Dibasic esters, 20–900 mg/m^3	4 h/d for 7–13 wk	Degeneration, sustentacular cells injured initially, cell proliferation	Keenan et al., 1990; Bogdanffy and Frame, 1994
1,2-Dibromo-3-chloropropane, 5–60 ppm	6 h 5 d/wk for 13 wk	Degeneration, metaplasia, hyperplasia	Reznik et al., 1980
1,2-Dibromoethane, 3–75 ppm	6 h 5 d/wk for 13 wk	Degeneration, metaplasia, hyperplasia	Reznik et al., 1980
Dichlobenil, 12–50 mg/g i.p.	[8 h–7 d]	Degeneration of olfactory epithelium, and Bowman's glands	Brandt et al., 1990
1,3-Dichloropropene, 30–150 ppm	6 h/d, 5 d/wk for 6–24 mo	Degeneration and/or metaplasia	Stott et al., 1988; Lomax et al., 1989
Dimethylamine, 10–511 ppm	6 h/d, 5 d/wk for 6–12 mo	Degeneration, loss of nerve bundles, hypertrophy of Bowman's glands	Buckley et al., 1984, 1985
1,4-Dithiane, 105–420 mg/kg i.p.	[90 d]	Anisotrophic crystals in giant cells (undetermined chemical composition)	Schieferstein et al., 1988

(*Continued*)

Table 17.1 Effects of Toxic Agents on Nasal Mucosa of Common Laboratory Rodents (Continued)

Compound, Concentration	Duration	Effects on Histology and Function	References
Epichlorohydrin, 687 ppm	6 h/d for 5 d	Ulceration, necrosis	Buckley et al., 1984
Ferrocene, 3–30 mg/m^3	6 h/d, 5 d/wk for 13 wk	Iron accumulation, necrotizing inflammation, metaplasia	Nikula et al., 1993
Formaldehyde, 0.25–15 ppm	6 h/d, 5, d/wk for 4 mo	Decreased number of bipolar cells, increased number of basal cells, degeneration of nerve bundles, reduced odor discrimination	Apfelbach et al., 1991, 1992
Furfural, 250–400 ppm	7 h/d, 5 d/wk for 52 wk	Disorientation of sensory cells, degeneration of Bowman's glands, cyct-like structures in lamina propria	Feron and Krysse, 1978
Furfural alcohol, 2–250 ppm	13 wk	Squamous and respiratory metaplasia of olfactory epithelium, inflammation, hyaline droplets, squamous metaplasia of ducts	Miller et al., 1991
Hexamethylene diisocyanate, 0.005–0.175 ppm	6 h/d, 5 d/wk for 12 mo	Degeneration, mucus hyperplasia	Foureman et al., 1994
ß, ß'- /Iminodipropionitrile, 200–400 mg/kg i.p.	[6 h–56 d]	Degeneration of axon bundles, increase of glial fibrillary acidic protein	Genter et al., 1992
Methyl bromide, 200 ppm	4 h/d, 4 d/wk	Degeneration, decreased carnosine, behavioral deficits	Hastings et al., 1991
3-Methylfuran, 148–322 µmol/l	1 h	Degeneration, more severe in rats than hamsters	Morse et al., 1984
3-Methylindole, 100–400 mg/kg i.p.	[7–90 d]	Degeneration, fibrous adhesions, osseous remodeling, Bowman's gland hypertrophy, behavioral deficits	Turk et al., 1987; Peele et al., 1991
Methyl isocyanate, 10–30 ppm	2 h	Degeneration of the respiratory and olfactory epithelium	Uraih et al., 1987
Napthalene, 400–1,600 mg/kg i.p.	[24 h]	Cytotoxicity (mice and hamsters), necrosis (rats)	Plopper et al., 1992
Nickel subsulfide, 0.11–1.8 mg/m^3	6 h/d, 5 d/wk for 13 wk	Atrophy	Dunnick et al., 1989
Nickel sulfate, 3.5–635 mg/m^3	6 h/d, 12–16 wk	Atrophy, degeneration, decrease in carnosine consecutive	Evans et al., 1995
N-Nitrosodimethylamine, 20–80 mg/kg i.p.	[6 h–30 d]	Degeneration of olfactory epithelium and Bowman's glands	Rangga-Tabbu and Sleight, 1992
N-Nitrosopyrrolidine, 30–100 mg/kg i.p.	[6 h–30 d]	Degeneration of olfactory epithelium and Bowman's glands	Rangga-Tabbu and Sleight, 1992
Propylene glycol monomethyl ether acetate, 3,000 ppm	2 wk	Slight to moderate degeneration of olfactory epithelium	Miller et al., 1984
Propylene oxide, 0–525 ppm	4 wk	Degeneration of the olfactory epithelim	Eldridge et al., 1995

Table 17.1 Effects of Toxic Agents on Nasal Mucosa of Common Laboratory Rodents (Continued)

Compound, Concentration	Duration	Effects on Histology and Function	References
Pyridine, 5–444 ppm	6 h–4 d	Degeneration of olfactory epithelim	Nikula and Lewis, 1994
RP 73401, 1 mg/kg/d	1 h–5 d	Degeneration of olfactory epithelium and Bowman's glands	Pino et al., 1999
Sulfur dioxide, 10–117 ppm	72 h, or 6 h/d, 5 d	Necrosis, edema, destruction, hyperplasia, hypertrophy	Giddens and Fairchild, 1972; Buckley et al., 1984
Sulfuryl fluoride, 0–600 ppm	6 h/d, 5 d for 2 wk	Inflammation	Eisenbrandt and Nitschke, 1989
Tetramethoxysilane, 1–45 ppm	6 h/d, 5, d for 28 d	Ulceration, inflammation, and necrosis of epithelium	Kolesar et al., 1989
2,4-Toluene diisocyanate, 0.4 ppm	6 h/d, 5 d	Ulceration, necrosis, inflammation, degeneration	Buckley et al., 1984
3-Trifluoromethyl pyridine, 0.1–329 ppm	6 h/d for 10–90 d	Degeneration, reduced Bowman's activity	Gaskell et al., 1988

Source: Adapted with permission from Hastings and Miller (2003).

Sensory irritation of the upper respiratory airways can be thought of as the first line of defense for protecting the upper and lower airways from toxic agents. Both animal and human studies have documented a sequence of reflex responses to the detection of a sensory irritant that include (1) removal of the organism from the site of exposure, (2) changes in breathing rate or volume to minimize the dose of the irritant, and (3) release of detoxifying enzymes—e.g., cytochrome P-450s, glutathione, and others, that are present in high concentrations in the nasal passages of many animal species and humans. Although sensory irritation can be elicited by many compounds that are not reactive and which, even in chronic exposures, do not damage the nasal epithelium or structures, this is not always the case. For example, formaldeldehyde, a potent sensory irritant, can be highly cytotoxic at higher doses to nasal mucosa and, in chronic and subchronic inhalation exposures, has been a nasal carcinogen in rodents. Another well-known trigeminal irritant, capsaicinoids (*Capsicum annum*), the lipid-soluble, oily extract of chili peppers used in a variety of nonlethal deterrent applications (e.g., pepper spray) has been shown to produce acute inflammation and dose-related damage to nasal cells in rodent inhalation studies (Reilly et al., 2003). Thus, although sensory irritation is a benign and transient, neural response to inhaled irritants, the avoidance response it elicits serves to protect the organism from damage after exposure to certain inhaled agents.

17.4.3 Rhinitis

Rhinitis is a heterogeneous inflammatory response of the nasal mucosa (Dykewicz et al., 1998), diagnosed primarily on the basis of etiology (allergic, nonallergic, infectious) and characterized by specific nasal symptomatology (e.g., rhinorrhea, sneezing, itching, and postnasal drainage) (Druce, 1998). Rhinosinusitis is an inflammatory response in the nasal and sinus mucosa, fluids, and underlying tissues and is also diagnosed on the basis of specific symptomatology. Rhinitis and rhinosinusitis can be differentiated into two classes, allergic and irritant. Although both classes are characterized by rhinorrhea, congestion of the nasal passages by vasodilation of the mucous membrane and sneezing,

allergic rhinitis is typically accompanied by excessive histamine release. Irritant or occupational rhinitis, on the other hand, is a nasal disorder induced by exposure to chemicals such as ozone, nickel, chrome, solvents, or tobacco smoke (Kulle and Cooper, 1975; Shusterman, 2003; Slavin, 2003). Of those members of the U.S. workforce with daily occupational exposure to chemicals or particulates, it has been estimated that as many as 40% report symptoms of chronic nasal inflammation or rhinitis that are known precursors of olfactory loss and potential damage to epithelial tissue.

17.4.4 Nasal Polyps

Nasal polyps are edematous growths in the lateral walls of the nose that represent the end stage of chronic nasal inflammation (Bernstein, 2001). Irritant-induced polypoid development begins with the stimulation of epithelial cells by a variety of agents that metabolically or physically alter or injure the surface epithelium. Once the epithelium is injured, a cascade of inflammatory changes occurs, including up-regulation of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-8 (IL-8), movement of eosinophils into the lamina propria of the polyp, the influx of water and albumin causing edema, and ultimately transformation of the normal respiratory epithelium into marked mucous cell hyperplasia, basal cell hyperplasia, or squamous metaplasia (Di Lorenzo et al., 2001). The resulting edematous growth can alter nasal airflow significantly, leading to congestion, blockages, and impaired olfactory function. Chronic nasal inflammation may also contribute significantly to chemically induced carcinogenesis (Woutersen et al., 1987, 1989).

17.4.5 Septal Perforations

Exposure to irritants or reactive inhaled agents that induce changes in the mucosal lining of the nose can also alter or destroy the cartilage and bone that support the nasal and sinus cavities. The cartilage of the anterior septum is highly susceptible to this type of secondary damage, because it is dependent on nourishment from the mucosal perichondrial membranes and its mucosal covering is a target site for the deposition of many inhaled agents. The deprivation of mucosal membrane will result in the necrosis and dissolution of the septal cartilage, leaving perforations such as those observed in the noses of chronic cocaine abusers and in chromium workers (Baruthio, 1992; Dingle, 1992).

17.4.6 Nasal Carcinogenesis

A variety of inhaled xenobiotics have been shown to induce upper respiratory tract tumors (primarily nasal lesions) in rodents (Table 17.2). These nasal lesions include both nonneoplastic (e.g., inflammation, epithelial cell necrosis, epithelial hyperplasia/metaplasia) and neoplastic (e.g., squamous cell carcinoma) changes. Ward et al. (1993) summarized the results of rodent inhalation studies from 19 chemicals sponsored by the National Toxicology Program. Five were found to be nasal carcinogens and induced inflammatory and proliferative nasal lesions, whereas the other fourteen produced similar lesions but not tumor formation. Toxicant-induced lesions in laboratory animals generally exhibit characteristic, site-specific distribution patterns. For example, lesions following formaldehyde exposure tend to be confined to the respiratory and transitional epithelium of the anterior nose, but lesions that occur following exposure to methyl bromide are confined to the olfactory epithelium. Site specificity has been observed for each of the four principal epithelial types lining rodent nasal airways, namely squamous (Gross et al., 1994), transitional (Harkema and Hotchkiss, 1994), respiratory (Morgan, 1986), and olfactory (Genter et al., 1992), and is a function of both regional tissue susceptibility to the particular agent as well as local dosimetry. Despite the ability of many inhaled chemicals to elicit proliferative changes leading to carcinogenesis in rodent models (Monticello and Morgan, 1997), epidemiological evidence from occupational cohorts has not revealed such an association in humans for most chemicals studied. An exception to that, however, occurs for exposure to select chemical mixtures (e.g., formaldehyde and wood dust) which appears to increase the risk of nasal tumors in humans (Feron et al., 2001).

Table 17.2 Inhaled Chemicals Inducing Nasal Tumors in Laboratory Rodents

Compound	Species	Reference
Acetaldehyde	Rat, hamster	Woutersen et al., 1986
Acrylonitrile	Rat	Gold et al., 1991
Allylglycidylether	Rat, mouse	Haseman and Hailey, 1997
Benzene	Rat	Gold et al., 1991
Bix(chloromethyl)ether	Rat	Leong et al., 1981
1,2-Dibromo-3-chloropropane	Rat, mouse	Gold et al., 1991
1,2-Dibromoethane	Rat, mouse	Gold et al., 1991
Dimethylcarbamoyl chloride	Rat	Sellakumar et al., 1989
Dimethyl sulfate	Rat	Sehlogel et al., 1970
Epichlorohydrin	Rat	Laskin et al., 1980
1,2–Epoxybutane	Rat	Huff et al., 1991
Ethylene dibromide	Rat	NCI, 1981
Formaldehyde	Rat	Gold et al., 1991
Hexamethylphosphoramide	Rat	Lee and Trochimovicz, 1982
Hydrazine	Rat, hamster	Vernot et al., 1985
N-Nitrosodimethylamine	Rat	Klein et al., 1990
1-Nitroso-4-methylpiperazine	Rat	Klein et al., 1999
Phenylglycidylether	Rat	Lee et al., 1993
Propylene oxide	Rat, mouse	Renne et al., 1986
PR 73401 (phosphodiesterase inhibitor)	Rat	Pino et al., 1999
2,3,4-Trichlorobutene-1	Rat	Feron et al., 1990
Vinyl acetate	Rat	Bogdanffy et al., 1994

Source: Adapted with permission from Feron et al. (2001).

17.5 INHALED AGENTS AND NASAL TOXICITY

Table 17.3 lists a variety of inhaled agents and the associated adverse nasal effects in humans following acute or chronic occupational exposures. Prior compilations of adverse effects of inhaled metals, vapors, or dusts on nasal complaints and disorders (including olfactory impairment) were primarily based on single-case studies or clinical reports and less often on epidemiological investigations with appropriately matched control groups (Amoore, 1986; Schiffman and Nagle, 1992). In contrast, here we have included both clinical reports and effects that have been documented in epidemiological studies conducted in the workplace. Although we recognize the value of single-case studies to highlight potential agents of concern, the scarcity of well-documented effects on nasal toxicity from inhaled chemicals in humans coupled with the problems associated with cross-species extrapolation complicates the interpretation of many clinical reports and highlights the need for continued investigation of nasal toxicity in occupational cohorts.

17.5.1 Metals

Several metals have been investigated for their ability to cause nasal dysfunction. Occupational exposure to inhalation of metal dusts or aerosols can lead to loss of olfactory acuity, atrophy of the nasal mucosa, mucosal ulcers, perforated nasal septum, and sinonasal cancer (Sunderman, 2001).

Nickel is often considered the classic example of a compound with nasal toxicity, ranging from relatively innocuous effects, such as rhinorrhea, to more detrimental outcomes, such as anosmia, polypoid disease, or squamous cell carcinoma (Boysen et al., 1984; Seilkop and Oller, 2003). However, exposure to nickel in occupational settings frequently occurs in combination with other putative nasal toxicants, such as cadmium or chromium, thus complicating the ability to establish clear dose–response outcomes in humans. For this reason, a review of epidemiological studies of nickel-exposed workers specifically excluded cohorts that were not free of other known or suspected carcinogenic metals (i.e., chromium and cadmium) (Seilkop and Oller, 2003). The review provided evidence that occupational exposure to nickel dust was associated with an extraordinarily high incidence of nasal sinus cancer (which is rare in the general population) with a strong association between level of risk and duration of employment in refinery operations with the highest levels of airborne nickel compounds. In general, the nasal neoplasms of nickel refinery workers involve the turbinates and the ethmoid sinuses, are locally aggressive, and metastasize widely, thus leading to poor prognosis. Rhinoscopic examination of refinery workers has revealed hyperplastic rhinitis of the middle turbinates, with advanced polypoid disease. Biopsies of the nasal mucosa revealed dysplastic and preneoplastic lesions and evidence of keratinization, suggesting that prolonged assault by nickel fumes leads to alterations that render the epithelium more resistant to further damage. The effect of nickel in rodents is somewhat different; 2-year bioassays conducted by the National Toxicology Program (NTP) revealed distinctly different carcinogenic risks for three nickel compounds, with nickel subsulfide showing substantially greater potential for promoting tumor incidence than nickel oxide and nickel sulfate hexahydrate eliciting no carcinogenic activity whatsoever (NTP, 1996a, 1996b, 1996c).

Nasal health effects from exposure to cadmium have also been documented. As early as 1948, Friberg reported that 37% of workers in an alkaline battery plant in Sweden experienced anosmia (Friberg, 1948), a finding that was later supported by studies in Germany (Baader, 1952), the United Kingdom (Adams and Crabtree, 1961), and Poland (Sulkowski et al., 2000), with deficits found in 27–65% of workers with high exposures to cadmium and nickel hydroxide. Although most of the investigators attributed the olfactory deficits to cadmium, the mixed exposures found in those worksites preclude determining whether the impairment was due to cadmium, nickel, or their combination.

Chromium exposure often occurs in combination with nickel and other metals, in particular, in the manufacture of steel alloys. Occupational exposure to chromium has long been associated with perforations of the nasal septum, with accompanying rhinitis, although this seldom resulted in reports of olfactory deficits. Evaluating workers who had a minimum of 7 years exposure in a chromate-production factory revealed that 51% had nasal septal perforations and 54% exhibited increased odor detection thresholds to five different compounds (Watanabe and Fukuchi, 2000). By comparison, however, with workers having exposure to nickel or cadmium compounds, sinonasal cancers were relatively uncommon in workers exposed to chromium, with only 22 documented cases of nasal cancer in chromate-exposed workers worldwide as of 1992 (Dingle, 1992).

17.5.2 Gases and Vapors

The toxicity of vapor interactions with biological receptors has historically been proposed to occur via two primary mechanisms, physical or chemical, leading to these volatiles being classified as nonreactive or reactive, respectively (Abraham et al., 1994). More recently, Alarie et al. evaluated 145 volatile organic chemicals, thus classified to determine what properties could be used to predict their respiratory sensory irritant potency. Although both categories of vapors caused sensory irritation in the upper respiratory tract, the irritancy of nonreactive chemicals could be estimated by using a variety of physicochemical properties (e.g., solubility, lipophilicity). In contrast, the irritant potential of reactive chemicals could best be estimated by using five different mechanisms of chemical reactivity, and doing so led to potency estimates that were higher than what would be predicted from physicochemical parameters alone (Alarie et al., 1996). This classification has also proven useful for

Table 17.3 Effects of Inhaled Chemicals, Metals, and Dusts on Nasal Symptoms in Humans

Category	Symptom	Reference
Metals		
Arsenic (III)	Mucosal alteration, septal peforation, cancer	Dunlap, 1921; Sunderman, 2001
Cadmium	Rhinorrhea, decreased mucociliary function	Friberg, 1950; Leopold, 1994; Schiffman et al., 1992; Dan's and Dorman, 1998
	Anosmia or hyposmia	
Cadmium compounds	Anosmia or hyposmia	Amoore, 1986
Chromate salts	Anosmia or hyposmia	Amoore, 1986
Chromium and chromium salts	Anosmia or hyposmia	Amdur et al., 1991; Schiffman et al., 1992; National Library of Medicine, 1995b; Sunderman, 2001
Chromium II	Perforation of nasal septum	Amdur et al., 1991; Schiffman et al., 1992; National Library of Medicine, 1995b; Sunderman, 2001
Chromium (IV)	Nasal irritation	Amdur et al., 1991; Schiffman et al., 1992; National Library of Medicine, 1995b; Sunderman, 2001
Iron carboxyl	Unspecified olfactory deficit	Murphy et al., 2003
Lead	Anosmia or hyposmia	Schiffman et al., 1992
Mercury	Anosmia or hyposmia	Schiffman et al., 1992
Nickel	Rhinitis, polyps, carcinoma, congestion, decreased mucociliary function, benign and malignant histopathological change	Leopold, 1994
	Anosmia or hyposmia	Schiffman et al., 1992
	Squamous cell carcinoma of nasal cavity	Sunderman, 2001
Nickel hydroxide	Anosmia or hyposmia	Amoore, 1986; Schiffman et al., 1992
Vanadium pentoxide	Increase in NAL PMNs, MPO, IL-8	Hauser et al., 1995
Zinc	Anosmia or hyposmia	Schiffman et al., 1992

(*Continued*)

Table 17.3 Effects of Inhaled Chemicals, Metals, and Dusts on Nasal Symptoms in Humans (Continued)

Category	Symptom	Reference
Zinc chromate	Anosmia or hyposmia	Amoore, 1986; Schiffman et al., 1992
Zinc sulfate	Temporary hyposmia	Amoore, 1986
Nonmetals/Inorganics		
Ammonia	Anosmia or hyposmia	Amoore, 1986
	Upper respiratory tract irritation	Amdur et al., 1991
Arsenic	Nasal irritation	Parmeggiani, 1983
Calcium chromate	Nasal tissue breakdown	Sittig, 1991
Carbon disulfide	Anosmia or hyposmia	Amoore, 1986
Carbon monoxide	Anosmia or hyposmia	Amoore, 1986
Chlorine	Anosmia or hyposmia	Amoore, 1986
	Rhinitis	Leroyer et al., 1999
Chlorine dioxide	Nasal discharge	Clayton and Clayton, 1981
Diesel fuel	Nasal irritation, benign histopathological change	Leopold, 1994
Fluorides	Anosmia or hyposmia	Amoore, 1986
Hydrazine	Anosmia or hyposmia	Amoore, 1986
Hydrogen cyanide	Perforation of nasal septum	National Library of Medicine, 1995a
Hydrogen selenide	Anosmia or hyposmia	Schiffman et al., 1992
Hydrogen sulfide	Temporary hyposmia	Amoore, 1986
Methyl bromide	Dysosmia	Morgan, 1994
n-Propanol	Sensory irritation	Repka-Ramirez and Baraniuk, 2002
Nitrogen oxides	Anosmia or hyposmia	Amoore, 1986
Ozone	Nasal irritation, benign histopathological change	Leopold, 1994
	Increase in NAL tryptase and albumin	Graham and Koren, 2004
Phosphorous oxychloride	Prolonged anosmia	MacIntyre, 1951
Reactive dyes	Rhinitis	Nilsson et al., 1993
Silicone dioxide	Unspecified olfactory deficit	Murphy et al., 2003
Styrene	Neoplasms	Kogevinas et al., 1994
Sulfur dioxide	Nasal irritation, rhinorrhea, xenobiotic metabolism	Leopold, 1994
	Permanent hyposmia	Amoore, 1986
Sulfur oxides	Anosmia or hyposmia	Amoore, 1986
Sulfuric acid	Temporary hyposmia	Amoore, 1986

Table 17.3 Effects of Inhaled Chemicals, Metals, and Dusts on Nasal Symptoms in Humans (Continued)

Category	Symptom	Reference
Tetrasodium pyrophosphate	Nasal irritation	American Conference of Governmental Industrial Hygienists, 1991; Sittig, 1991
Organics		
Acetate	Anosmia or hyposmia	Schiffman et al., 1992
Acetic anhydride	Ulceration of nasal mucosa	Proctor et al., 1988
Acetone	Temporary/permanent hyposmia/ anosmia	Amoore, 1986
	Sensory irritation	Morgott, 2001
Acetophenone	Anosmia or hyposmia	Amoore, 1986
Acrylates	Anosmia or hyposmia	Schworte et al., 1989
	Increase in NAL ECP	Granstrand et al., 1998
Alkylbenzene	Sensory irritation	Collaghan-Rose et al., 2001
Ammonia	Sensory irritation, increased nasal resistance	McLean et al., 1979; Sundblad et al., 2004
Benzene	Permanent hyposmia or anosmia; sensory irritation	Amoore, 1986; Collaghan-Rose et al., 2001
Benzol	Unspecified olfactory deficit	Murphy et al., 2003
Butyl acetate	Unspecified olfactory deficit	Murphy et al., 2003
Chloromethanes	Anosmia or hyposmia	Schiffman et al., 1992
Cyclohexanone	Temporary hyposmia	Amoore, 1986
Cyanoacrylates	Rhinitis	KopFerschmit-Kubler et al., 1996
Dimethylphthalate	Nasal irritation	Sittig, 1991
Dipropylene glycol methyl ether	Nasal irritation	Clayton et al., 1981
Ethyl acetate	Unspecified olfactory deficit	Murphy et al., 2003
Ethyl benzene	Nasal irritation	Proctor et al., 1988; American Conference of Governmental Industrial Hygienists, 1991
Ethylenediamine	Nasal irritation	Proctor et al., 1988
Ethyl ether	Nasal irritation	Proctor et al., 1988; American Conference of Governmental Industrial Hygienists, 1991

(*Continued*)

Table 17.3 Effects of Inhaled Chemicals, Metals, and Dusts on Nasal Symptoms in Humans (Continued)

Category	Symptom	Reference
Ethyl formate	Nasal irritation	Proctor et al., 1988
Ethyl silicate	Nasal irritation	Proctor et al., 1988
n-Ethylmorpholine	Nasal irritation	American Conference of Governmental Industrial Hygienists, 1991
Formaldehyde	Nasal irritation, rhinorrhea, benign histopathological change	Leopold, 1994
	Temporary hyposmia	Amoore, 1986
	Rhinorrhea and crusting	Edling et al., 1988
	Nasal irritation	Baradana and Montanaro, 1991
	Increase in NAL total protein, albumin and eosinophils	Pazdralc et al., 1993
	Nasal lesions, cancer	Morgan, 1994
	Rhinorrhea, crusting, cilia loss, goblet cell hyperplasia, squamous cell metaplasia, dysplasia	Edling et al., 1988
	Nasal irritation	Cain et al., 1986
Isopropyl acetate	Nasal irritation	National Library of Medicine, 1992
Isopropylamine	Nasal irritation	Hathaway et al., 1991
Menthol	Anosmia or hyposmia	Amoore, 1986
Methylethyl ketone	Sensory irritation; increased mucociliary transit time	Hansen et al., 1992; Muttray et al., 2002
Methyl tetrahy drophthalic anhydride	Increase in NAL tryptase	Nielsen et al., 1994
	Nasal irritation	Yokota et al., 1999
Pentachlorophenol	Anosmia or hyposmia	Amoore, 1986
Petroleum	Anosmia or hyposmia	Ählstrom et al., 1986
Solvent mixtures	Anosmia, hyposmla, parosmia	Ryan et al., 1988; Schwartz et al., 19906
tert-Butyl acetate	Nasal irritation	American Conference of Govermental Hygienists, 1993
tert-Butyl alcohol	Nasal irritation	Clayton et al., c 1981
Tetranitromethane	Nasal irritation	Horn, 1954
Tetryl	Nasal irritation	American Conference of Govermental Hygienists, 1993
Tetrahydrofuran	Hyposmia, phantosmia	Emmett, 1976
Trichloroethane	Increase in NAL IL-1β, IL-6, IL-8	Muttray et al., 1999
Trichloroethylene	Anosmia or hyposmia	Amoore, 1986

Table 17.3 Effects of Inhaled Chemicals, Metals, and Dusts on Nasal Symptoms in Humans (Continued)

Category	Symptom	Reference
Vinyl toluene	Nasal irritation	Clayton et al., 1981; American Conference of Govermental Hygienists, 1993
Dusts		
Cement	Unspecified olfactory deficit	Murphy et al., 2003
Chalk	Anosmia or hyposmia	Amoore, 1986
Coke	Unspecified olfactory deficit	Murphy et al., 2003
Grain	Increase in NAL MPOS, hyposmia	Ahman et al., 2001
Hardwood	Anosmia or hyposmia	Schiffman et al., 1992
Lime	Anosmia or hyposmia	Amoore, 1986; Schiffman et al., 1992
Potash	Unspecified olfactory deficit	Murphy et al., 2003
Tobacco	Hyposmia	Frye et al., 1990
Wood dust	Rhinorrhea, rhinitis, nasal obstruction, decreased mucociliary function, xenobiotic metabolism, benign and malignant histopathological change	Leopold, 1994
	Increase in NAL IL-6	Toren et al., 1996

NAL, nasal lavage; PMN, polymorphonuclear lymphocyte; ECP, eosinophil cationic protein; MPO, myeloperoxidase; IL, interleukin.

generating predictions of the deposition, clearance, metabolism, and toxic impact of gases in the nasal passages (Morris et al., 1993) and will be used to organize the discussion of effects that follows.

17.5.2.1 Nonreactive Volatiles

There are numerous case studies to document that acute and accidental exposures to high concentrations of many nonreactive volatile organics can have an adverse affect on nasal health and function. However, the vast majority of occupational exposures occur at far lower concentrations over much longer periods and the long-term impact on the health and function of the human nose is relatively unknown. Animal studies, primarily conducted on rodents, provide some evidence that chronic exposure to a number of nonreactive gases and solvents can damage the olfactory epithelium and its supporting structures when exposure occurs at sufficiently high concentrations (Refer to Table 17.1).

Because of their ubiquitous presence in many occupational environments, the nasal toxicity of solvents has probably been investigated more than any other class of inhaled agents. Occupational exposure to solvents or solvent mixtures appears to be associated with (1) some degree of impairment of olfactory sensitivity Sandmark, et al., 1989; Emmett, 1976; (Schwartz et al., 1990) and (2) nasal irritation as measured with psychophysical techniques Ählstrom et al., 1986 Seeber et al.,

1992; (Dalton et al., 1997) or assays of inflammatory biomakers (Mann et al., 2002). Although solvent effects on olfactory function have been shown to be transient and reversible following cessation of exposure (Emmett, 1976; Ähstrom et al., 1986), the ability to draw any firm conclusions regarding the long-term effects of solvent exposure on nasal health and function are limited by a few studies which have been conduced.

17.5.2.2 Reactive Volatiles

Ample animal evidence provide that inhalation exposure to reactive gases and vapors can cause a variety of toxic effects in all nasal regions, but the degree and the dominant site of injury will be determined by either the dose to the tissue, the cellular susceptibility, or both. Gases that are highly water soluble and highly reactive will be rapidly extracted from airstreams and thus cause a predictable pattern of lesions based on sites of the highest local concentrations (Kimbell and Morgan, 1991). In rodents, acute and chronic exposure to a variety of reactive compounds (e.g., diethylamine, glutaraldehyde, formaldehyde, chlorine) can induce (1) lesions and structural damage to the squamous epithelium lining the nasal vestibule (e.g., erosion or ulceration with or without inflammation); (2) inflammatory, necrotic, or hyperplastic and metaplastic reactions in the transitional epithelium; (3) inflammation, deciliation, and degeneration in the respiratory epithlium; and (4) inflammation coupled with loss of olfactory cilia, sensory cells, atrophy, or necrosis in the olfactory epithelium and Bowman's glands with ultimate replacement of OE by respiratory epithelium. Although many reactive compounds such as acrolein, furfural, dimethylamine, chlorine and ethyl acrylate are cytotoxic to nasal mucosa and can induce hyperproliferative changes, they do not typically lead to the formation of nasal tumors in rodents (Feron et al., 2001a). Thus, whether an inhaled toxicant that is capable of inducing proliferative changes in nasal tissue will lead to tumor formation depends on several factors, including the parent compound, its metabolism in nasal tissue, the local tissue dose, and tissue-specific differences in sensitivity.

However, robust data for similar effects in humans is lacking. Although all reactive compounds at sufficiently high doses appear to elicit intranasal sensory irritation, their ability to produce inflammation, necrosis, or metaplastic changes in the respiratory or sensory epithelium or structural damage to the human nasal passages may differ dramatically from the effects seen in rodents due to anatomical influences on airflow-driven dosimetry and metabolic differences in the underlying tissue.

Several epidemiological studies addressing the effects of exposure to toxicants on olfaction have produced mixed results. Schwartz et al. (1989) evaluated the olfactory function of 731 workers exposed to a variety of acrylates and methacrylates using a 40-item smell identification test. Workers having the highest level of cumulative exposures showed the greatest deficit in olfactory function, although the impairment did not appear to be clinically significant. Among workers exposed to styrene vapors in the reinforced-plastics industry, Dalton et al. (2003) found that, contrary to findings in the animal literature, there was no evidence of any long-term alteration in olfaction due to styrene exposure. Exposed workers did, however, exhibit a specific loss in sensitivity to styrene itself, which has been observed in other chemically exposed populations and likely represents a transient, reversible form of sensory adaptation (Ähstrom et al., 1986; Wysocki et al., 1997; Smeets and Dalton, 2001).

17.5.3 Dusts and Particulates

In comparison with research on exposure to metals and volatiles, relatively few studies have evaluated the toxicity of particulates and dusts in human nasal health. Much of the evidence for adverse effects on nasal health and function in animal assays, moreover, has examined the health impact of metal dusts (i.e., nickel, cadmium) (Sunderman, 2001) or mixed exposures (i.e., wood dust and formaldehyde) (Vaughan et al., 2000; Gosselin et al., 2003), and considerable uncertainty exists as to whether the physical or chemical interactions produce the greatest impact.

Both fine and coarse particles can enter the nasal cavity and can induce nasal irritation (Schiffman et al., 2001). Epidemiological studies of particulate exposure have reported associations between

concentrations of ambient particulate matter and nasal effects. Not only can the particles themselves produce irritation via physical mechanisms, but also particles can act as carriers and may concentrate odorants such as organic acids and ammonia on their surfaces (Schiffman et al., 2001). Recent studies evaluating exposure to calcium carbonate dust on various assays of human nasal function have found a dose-dependent decrease in nasal patency and mucociliary clearance as well as increases in symptoms such as dryness and perceived obstruction (Riechelmann et al., 2003).

17.6 MECHANISMS OF RHINOTOXICITY

Inhaled volatiles or dusts can affect olfactory function *indirectly,* by inducing inflammation in the nasal passages and impeding airflow to the OE, which has been termed a conductive loss (Zhao et al., 2003). The peripheral olfactory epithelium is also at *direct* risk from exposure to certain classes of inhaled toxicants (e.g., reactive chemicals such as chlorine or formaldehyde). The responses of olfactory epithelium to toxic insult include degeneration, necrosis, atrophy, hyperplasia, metaplasia, and neoplasia. Necessarily, the type of alteration is a function of the mechanism of action of the toxicant. Nonspecific necrosis of cells in OE may be a result of reactive irritants such as chlorine or sulfur dioxide, whereas cell-specific toxicity (e.g., sustentacular or sensory cells) may occur after exposure to toxicants such as dibasic esters and methyl bromide (Genter et al., 1992). It is also important to recognize that certain changes in the OE (i.e., regeneration of OE to respiratory epithelium) may reflect an adaptation that enhances the resistance of the epithelial barrier to further toxic insult, while reducing the sensory acuity of the organism.

Mechanisms of carcinogenesis in the nasal cavity of humans have not been well established for many vapors, metals, or dusts. However, substantial evidence demonstrates that persistent tissue damage followed by sustained cell proliferation (repair or hyperplasia) in both humans and experimental animals may be a precursor to tumor formation in other organs and organ systems and may play a significant role in the pathogenesis of nasal toxicity as well (Grasso et al., 1991; Preston-Martin et al., 1993).

17.7 CROSS-SPECIES PHYSIOLOGICAL AND BIOCHEMICAL DIFFERENCES

Although a substantial body of data on nasal toxicity from inhaled agents has been established in animal toxicological studies, it is of great importance to take into account the anatomic and metabolic differences among species that may preclude extrapolation from such studies to human risk effects. Perhaps in no other organ or organ system are anatomical differences among species, such as rodents and humans, of greater importance in controlling differences in regional toxicity of inhaled materials. Differences in gross anatomy, nasal epithelia, and the distribution and composition of mucous secretions have been comprehensively documented (Harkema, 1999) and all are important factors in determining the actual inhaled dose of any airborne agent. One of the most obvious and significant physiological differences between rodents and humans is that humans are oronasal breathers whereas rodents are obligate nose breathers. Species differences in clearance include differences in mucociliary flow, chemotactic attraction of macrophages, and the biochemistry of airway activation, detoxification, and tissue response (Bogdanffy and Jarabek, 1995). For example, interspecies comparison of metabolic capability in the upper respiratory tract has shown that cytochrome P-450 activities are less efficient in human nasal mucosa than in the nasal mucosa of rodents (Dahl and Hadley, 1991). Some of these differences have been characterized quantitatively, but many have not, and this presents uncertainty in the interspecies extrapolation of risk.

Although rodents in chronic and subchronic inhalation studies frequently develop nonneoplastic or neoplastic lesions in the nasal passages, the evidence for similar effects in humans is far from clear. For example, findings of extensive damage to the olfactory epithelium in mice and rats following chronic exposure to styrene vapor at levels comparable to occupational exposures prompted a human epidemiological investigation of olfactory function in a group of reinforced-plastics workers (Dalton

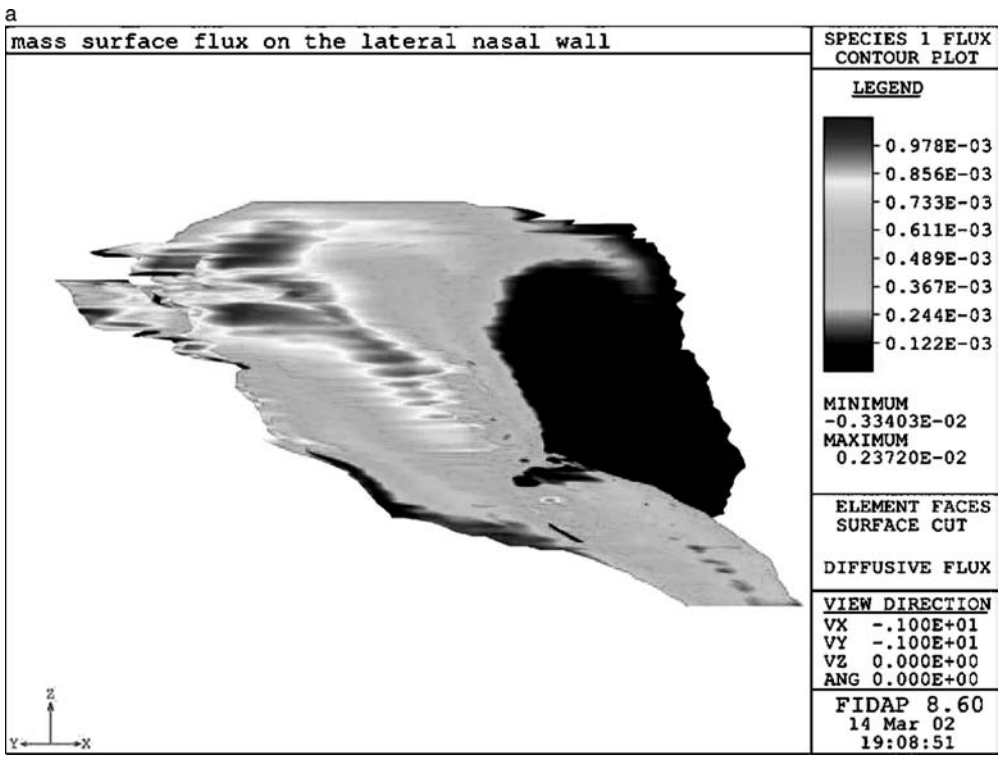


FIGURE 17.3 Comparison of airflow-driven dosimetry for acetone in the rat and human nose. Simulated pattern of chemical (amyl acetate) vapor mass surface (mucus) uptake flux on the lateral wall of the nasal cavity of rat (a) and human (b). In (a), the half (right) nasal airflow rate through a rat nose in the simulation is 250 ml/min. Ten contour levels are plotted, the unit in the label is g/cm^2. In (b), the total (both) nasal airflow rate through a human nose in the simulation is 300 ml/sec. Twenty contour levels are plotted, the unit in the label is kg/m^2, corresponding to 0.1 g/cm^2, so the level of the surface flux between the two species is roughly in the same range under resting breathing conditions. The uptake pattern however is quite different and would lead to obvious differences in tissue-specific dose to the olfactory region.

et al., 2003). Contrary to the findings from the animal literature, there was no evidence of any dose-related long-term alteration in olfactory function due to styrene exposure. This discrepancy could be due to several factors. For example, the cross-species differences in the distribution and location of olfactory epithelium and the airflow and deposition patterns in the nasal passages can greatly alter the dose at any site (see Figure 17.3). Thus, inhaled styrene vapor may deposit in the nasal passages of humans but, unlike in rodents, high levels of deposition do not occur in areas that subserve olfaction. Alternatively (or additionally), metabolic capacities in the nasal tissue of rodents and humans could play a role. In styrene, the primary metabolic pathway is the oxidation by cytochromes P-450 to two enantiomeric forms of styrene oxide (Bond, 1989). When this metabolic process is prevented by preexposure to the cytochrome P-450 enzyme inhibitor (5-phenyl-1-pentyne), the development of olfactory lesions in rodents after exposure to styrene does not occur (Green, 1999). This strongly suggests that the lesions found in rodent olfactory tissue are induced by the primary metabolite of styrene, styrene oxide, and not by exposure to styrene *per se*.

Evidence that this metabolite may not be present in human nasal epithelium exposed to styrene comes from a recent investigation that compared metabolic activity of styrene *in vitro* in rat, mouse, and human nasal respiratory tissue and found important differences in the metabolic activity of styrene across these species (Green et al., 2001). Specifically, rat and mouse nasal respiratory fractions

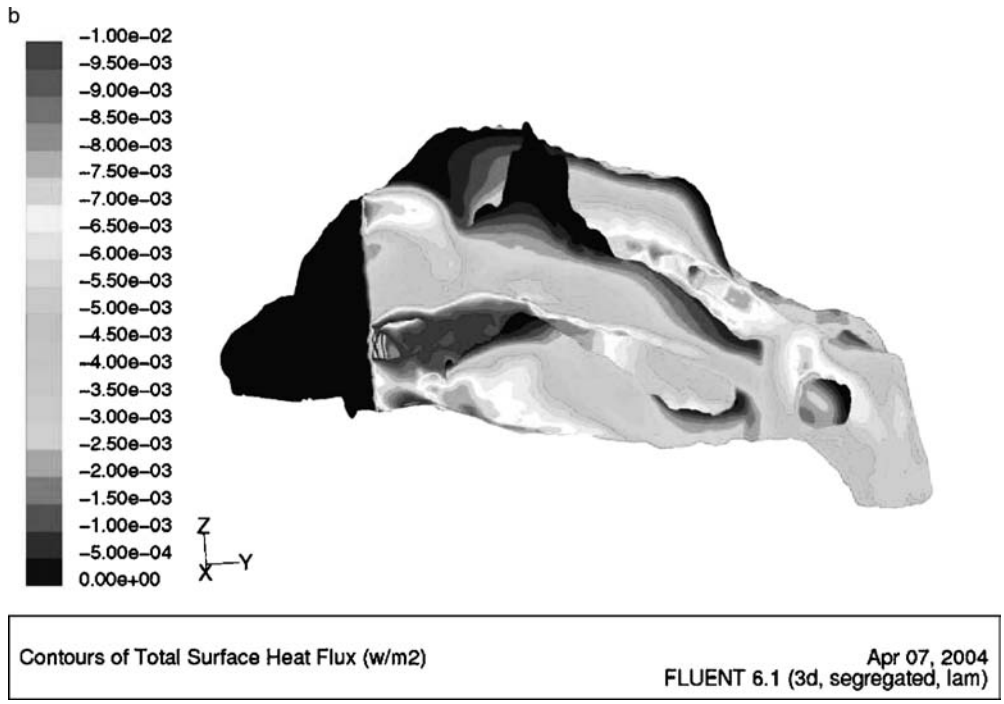


FIGURE 17.3 (Continued)

were found to contain high concentrations of the two cytochrome P-450 isoforms necessary for the conversion of styrene into styrene oxide, whereas human nasal fractions did not. Species differences in the nasal metabolism of other chemicals that produce rat nasal tumors have been recently reported as well (Green et al., 2000), suggesting that the relevance of nasal tumorigenesis studies in rodents for human risk prediction may need to be evaluated on a chemical-by-chemical basis. Both anatomical and metabolic differences could explain differences in the toxic effects of styrene on the olfactory epithelium in humans and rodents.

One approach to cross-species extrapolation is to employ a strategy based on anatomically correct geometric models of the nasal passages of the relevant species that are used to estimate airflow-driven dosimetry of various chemicals (Kimbell et al., 1993; Keyhani et al., 1997; Bush et al., 1998; Frederick et al., 1999). Coupling the results of such modeling with knowledge of site-specific lesions from rodent assays and information about tissue-specific metabolizing enzymes can greatly improve the accuracy of human risk assessment (Frederick et al., 2002).

17.8 SUMMARY

The toxicity of inhaled chemicals, particulates, and dusts on nasal passages and function has historically been neglected in occupational and environmental health fields. To be sure, most areas of the human nose that are likely to be targeted by inhaled toxicants are refractory to easy examination during routine physical examinations; endoscopic inspections capable of visualizing these areas require both special skills and equipment. Furthermore, functional assays of the nose such as evaluation of olfactory or irritant sensitivity have also been restricted to specialized clinics or research laboratories and are typically not routinely evaluated unless a problem is identified. Because of the often gradual nature of sensory loss, moreover, exposed individuals are likely not to recognize their impairment until the damage has progressed to an extreme degree.

Ethical issues that preclude experimental studies of nasal toxicity from inhaled chemicals in humans necessitate reliance on data from controlled animal assays. However, significant differences in nasal anatomy and metabolism lend uncertainty to our understanding of the relevance of nasal effects in rodents for human health. As Morgan has suggested, however, a unified strategy consisting of mapping of nasal lesions in rodents combined with computational modeling of airflow-driven dosimetry and knowledge of local metabolism can be used as a sound basis for extrapolation of animal data to human risk assessment. However, the availability of this approach should not forestall efforts to evaluate nasal health and function in occupationally exposed workers. This is important not only to validate interspecies extrapolation, but also to develop a substantive database of human nasal toxicity from environmental and occupational inhalants that can be used to promote better surveillance among occupational medicine practitioners and heightened attention to protection from adverse effects on the nose and nasal function in the field of toxicology and industrial hygiene.

REFERENCES

Abraham, M.H., Nielsen, G.D., and Alarie., Y., The Ferguson principle and an analysis of biological activity of gases and vapors, *J. Pharmacol. Sci,* 83, 680–688, 1994.

Adams, R.G. and Crabtree, N., Anosmia in alkaline battery workers. *Br. J. Ind. Med*., 18, 216–221, 1961.

Ählstrom, R., Berglund, B., Berglund, U., Lindvall, T., and Wennberg, A., Impaired odor perception in tank cleaners, *Scan. J. Work Environ. Health*, 12, 574–581, 1986.

Alarie, Y., Schaper, M., Nielsen, G.D., and Abraham, M.H., Structure-activity relationships of volatile organic chemicals as sensory irritants, *Arch.Toxicol*., 72, 125–140, 1998.

Amdur, M.O., Doull, J., and Klaassen, C.D., *Toxicology: The Basic Science of Poisons*, 4th ed., Pergamon Press, New York, 1991.

American Conference of Governmental Industrial Hygienists (ACGIH), *Documentation of the Threshold Limit Values and Biological Exposure Indices,* 6th ed., ACGIH, Cincinnati, OH, 1991.

American Conference of Governmental Industrial Hygienists (Ed.) (ACGIH), *Threshold Limit Values for Chemical Substances and Physical Agents and Biological Exposure Indices. 1993-1994*, ACGIH, Cincinnati, OH, 1993.

Amoore, J.E., Effects of chemical exposure on olfaction in humans, In *Toxicology of the Nasal Passages*, Barrow, C.S., Ed., Hemisphere Publishing Corporation, Washington, DC, 1986, pp. 155–190.

Apfelbach, R., Sensitivity to odors in Wistar rats is reduced after low-level formaldehyde gas exposure, *Naturwissenschaften*, 78, 221–223, 1991.

Apfelbach, R. Reibenspies, M., Schmidt, R., Weller, E., Binding, N., and Camman, K., Behavioral and structural modifications in the olfactory epithelium after low level formaldehyde gas exposure, in *Proceedings of the 3rd International Conference on Role of Formaldehyde in Biological Systems. Methylation and Demethylation Process,* Hungarian Biochemical Society, Sopron, Hungary, 1992, pp. 57–62.

Appleman, L.M., Woutersen, R.A., and Feron, V.J., Inhalation toxicity of acetaldehyde in rats. I. Acute and subacute studies, *Toxicology,* 23, 293–307, 1982.

Baader, E.W., Chronic cadmium poisoning, *Ind. Med. Surg*., 21, 427–430, 1952.

Baradana, E.J. and Montanaro, A., Formaldehyde: an analysis of its respiratory, cutaneous, and immunology effects, *Ann. Allergy*, 66, 441–452, 1991.

Baruthio, F., Toxic effects of chromium and its compounds, Biol. Trace Elem. Res., 32, 142–153, 1992.

Bernstein, J.M., The molecular biology of nasal polyposis, *Curr. Allergy Asthma Rep.,* 1, 262–267, 2001.

Bogdanffy, M.S., and Frame, S.R., Olfactory mucosal toxity. Integration of morphological and biochemical data in mechanism studies: dibase ester as an example, *Inhalation Toxicol.,* 6 (suupl.), 205–219. 1994.

Bogdanffy, M.S., and Jarabek., A.M., Understanding mechanism of inhaled toxicants: implications for replacing default factors with chemical-specific dates, Toxicol. Lett., 82-83, 919–932, 1995.

Bogdanffy, M.S., Dreef-van der Meulen, H.C., Beems, R.B., Feron, V.J., Cascieri, T.C., Tyler, T.R., Vinegar, M.B., and Ricard, R.W., Chronic toxity and oncogenicity inhalation study with vinyl acetate in the rate and the mouse, *Fundam. Appl. Toxicol.,* 23, 215–229, 1994.

Bond, J.A., Review of the toxicology of styrene, *CRC Crit. Rev. Toxicol.*, 19, 227–249, 1989.

Boysen, M., Solberg, L.A., Torjussen, W., Poppe, S., and Hogetveit, A.C., Histological changes, rhinoscopical findings and nickel concentration in plasma and urine in retired nickel workers, *Acta Otolaryngol.,* 97, 105–115, 1984.

Brain, J.D., Proctor, D.F., and Reid, L.M., *Respiratory Defense Mechanisms*, Marcel Dekker, New York, 1977.

Brandt, I., Brittebo, E.B., Feil,V.J., and Bakke, J.E., Irreversible binding and toxicity of the herbicide dichlobenil (2,6 dichlorobenzonitrile) in the olfactory mucosa of mice, *Toxicol. Appl. Pharmacol.* 103, 491–501, 1990.

Brittebo, E.B., Eriksson, C., and Brandt, I., Activation and toxicity of bromobenzene in nasal tissues in mice, *Arch. Toxicol.* **64**, 54,60, 1990.

Brittebo, E.B., Eriksson, C., Feil, V., Bakke, J., and Brandt, T., Toxicity of 2,6-dichlorothiobenzamide (chlorothiamid) and 2,6,–dicholorobenzamide in the olfactory nasal mucosa of mice, *Fundam. Appl. Toxicol.,* 17, 92–102, 1991.

Buckley, L.A., Jiang, X.Z., James, R.A., Morgan, K.T., and Barrow, C.S., Respiratory tract lesions induced by sensory irritants and the RD50 concentration, *Toxicol. Appl. Pharmacol.* **74**, 417–429, 1984.

Buckley, L.A., Morgan, K.T., Swenberg, J.A., James, R.A. Hamm, T.E.J., and Barrow, C.S., The toxicity of dimethylamine in F-344 rats and B6C3P1 mice following a 1-year inhalation exposure, *Fundam. Appl. Toxicol.,* 5, 341–352, 1985.

Bush, M.L., Frederick, C.B., Kimbell, J.S., and Ultman, J.S., A CFD-PBPK hybrid model for simulating gas and vapor uptake in the rat nose, *Toxicol. Appl. Pharmacol*., 150, 133–145, 1998.

Cain, W.S., See, L. C., and Tosun, T., Irritation and odor from formaldehyde: chamber studies, *Am. Soc. Heat. Refrig. Assoc. Eng.,*14, 126–137, 1986.

Callaghan-Rose, M., Nickola, T.J., and Voynow, J.A., Airway mucus obstruction: Mucin glycoproteins, MUC gene regulation and goblet cell hyperplasia, *Am. J. Respir. Cell Mol. Biol.,* 25, 533–537, 2001.

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., and Julius, D., The capsaicin receptor: a heat-activated ion channel in the pain pathway, *Nature*, 389, 816–824, 1997.

Clayton, G. and Clayton, F., *Patty's Industrial Hygiene and Toxicology*, 3rd ed., Wiley, New York, 1981.

Clerico, D.M. To, W.C., and Lanza, D.C. Anatomy of the human nasal passages, in *Handbook of Olfaction and Gustation*, Doty, R. L., Ed., Marcel Dekker, New York, 2003, pp. 1–16.

Cole, P., *Respiratory Role of the Upper Airways*, Mosby-Year Book, St. Louis, MO, 1993.

Cometto-Muniz, J.E. and Cain, W.S., Sensory irritation: relation to indoor air pollution, *Ann. N. Y. Acad. Sci.,* 641, 137–151, 1992.

Cowart, B.J. and Rawson, N.E., Olfaction, in *The Blackwell Handbook of Perception*, Goldstein, E.B., Ed., Blackwell Publishers, Oxford, 2001, pp. 567–600.

Dahl, A.R. and Hadley, W.M., Nasal cavity enzymes involved in xenobiotic metabolism: effects on the toxicity of inhalants, Scit Rev. Toxicol, 21, 345–372, 1991.

Dalton, P., Olfaction, in *Sensation and Perception*, Yantis, S., Ed., 2002, pp. 691–746.

Dalton, P., Lees, P.S.J., Cowart, B.J., Emmett, E.A., Dilks, D.D., Stefaniak, A., and Gould, M., Olfactory function in workers exposed to styrene in the reinforced-plastics industry, *Am. J. Ind. Med.*,, 44, 1–11, 2003.

Dalton, P., Wysocki, C.J., Brody, M.J., and Lawley, H.J., Perceived odor, irritation and health symptoms following short-term exposure to acetone, *Am. J. Ind. Med., 31*, 558–569, 1997.

Davis, J.M. and Dorman, D., Health risk assessments of manganese - differing perspectives: session VIII summary and research needs, *Neurotoxicology*, *19*, 488–489, 1998.

Di Lorenzo, G., Drago, A., Esposito, P.M., Candore, G., Colombo, A., Gervasi, F., Pacor, M.L., Purello, D.F., and Caruso, C., Measurement of inflammatory mediators of mast cells and eosinophils in native nasal lavage fluid in nasal polyposis, *Int. Arch. Allergy Immunol*., 125, 164–175, 2001.

Dingle, A.F., Nasal disease in chrome workers, *Clin. Otolaryngol.*, 17, 287–288. 1992.

Doty, R.L., Bartoshuk, L.M., and Snow, J.B., Jr., Causes of olfactory and gustatory disorders, in *Smell and Taste in Health and Disease*, Getchell, T.V., Doty, R.L., Bartoshuk, L.M., and Snow, J.B., Jr., Eds., Raven Press, New York, 1991, pp. 449–462.

Doty, R.L. and Cometto-Muniz, J.E., Trigeminal chemosensation, in *Handbook of Olfaction and Gustation*, Doty, R.L., Ed., Marcel Dekker, New York, 2003, pp. 981–999.

Druce, H.M., Allergic and nonallergic rhinitis, in Allergy Principles and Practice, Middleton, E.J., Reed, C.E., and Ellis, E.F., Eds., Mosby-Year Book, St. Louis, MO, 1998, pp. 1005–1016.

Dunlap, L.G., Perforations of the nasal septum due to inhalation of arsenous oxide, *JAMA,* 76, 568–569, 1921.

Dunnick, J.K., Elwell, M.R., Benson, J.M, Hobbs, C.H., Hahn, F.F., Haly, P.I, Cheng, Y.S., and Eldson, A.F., Lung toxicity after 13-week inhalation exposure to nickel oxide, nickel subsulfide, or nickel sulfate hexahydrate in F344/N rats and B6C3F1 mice, *Fundam. Appl. Toxicol.* 12, 584–594, 1989.

Dykewicz, M.S., Fineman, S., Skoner, D.P., Nicklas, R., Lee, R., Blessing-Moore, J., Li, J.T., Bernstein, I.L., Berger, W., Spector, S., and Schuller, D., Diagnosis and management of rhinitis: complete guidelines of the Joint Task Force on Practice Parameters in Allergy, Asthma and Immunology, American Academy of Allergy, Asthma, and Immunology, *Ann. Allergy Asthma Immunol*., 81 (5 Part 2), 478–518, 1998.

Edling, C., Hellquist, H., and Odkvist, L.M., Occupational exposure to formaldehyde and histopathological changes in the nasal mucosa, *Br. J. Ind. Med.* 45, 761–765, 1988.

Eisenbrandt, D.L. and Nistschke, K.D., Inhalation toxicity of sulfuryl fluoride in rats and rabbits, *Fundam. Appl. Toxicol.,* 12, 540–557, 1989.

Eldridge, S.R., Bogdanffy, M.S., Jokinen, M.P., and Andrews, L.S., Effects of propylene oxide on nasal epithelial cell proliferation in F344 rats, *Fundam. Appl. Toxicol.,* 27, 25–32, 1995.

Emmett, E.A., Parosmia and hyposmia induced by solvent exposure, *Br. J. Ind. Med., 33*, 196–198, 1976.

Evans, J.E., Miller, M.L., Andringa, A., and Hastings, L., Behavioral, histological and neurochemical effects of nickel (II) on the rat olfactory system, *Toxicol. Appl. Pharmacol.* 130, 209–220, 19.

Feron, F., Perry, C., McGrath, J.J., and Mackay-Sim, A., New techniques for biopsy and culture of human olfactory epithelial neurons, Arch. Otolaryngol. Head Neck Surg., 124, 861–866, 1998.

Feron, V.J., Arts, J.H., Kuper, C.F., Slootweg, P.J., and Woutersen, R.A., Health risks associated with inhaled nasal toxicants, *Crit. Rev. Toxicol*., 31, 313–347, 2001b.

Feron, V.J. and Knrysse, A., Effects of exposure to furfural vapour in hamsters simultaneously treated with benzo(alpha) pyreme or diethyllnitrosamine, *Toxicology*, 11, 127–144, 1978.

Feron, V.J., Woutersen, R.A., van Garderen-Hoetmer, A., and Dreef-van der Meulen, H.C., Upper respiratory tract tumours in Cpb-WU (Wistar random) rats, *Environ. Health Perspect*., 75, 305–315, 1990.

Foureman, G.L., Greenberg, M.M., Sangha, G.K., Stuart, B.P., Shiotsuka, R.N., and Thyssen, J.H., Evaluation of nasal tract lesions in derivation of the inhalation reference concentration for hexamethylene diisocyanate, *Inhalation Toxicol.,* 6 (Suppl), 341–355, 1994.

Frederick, C.B., Bush, M.L., Lomax, L.G., Black, K.A., Finch, L., Kimbell, J.S., Morgan, K.T., Subramaniam, R.P., Morris, J.B., and Ultman, J.S., Application of hybrid computational fluid dynamics and physiologically based inhalation model for interspecies dosimetry extrapolation of acidic vapors in the upper airways, *Toxicol. Appl. Pharmacol*., 152, 211–231, 1999.

Friberg, L., Proteinuria and kidney injury among workmen exposed to cadmium and nickel dust, *J. Ind. Hyg. Toxicol*., 30, 32–36, 1948.

Friberg, L., Health hazards in the manufacture of alkaline accumulators with special reference to chronic cadmium poisoning, *Acta Med Scand.,* 138, 1–124, 1950.

Ganong, W.F., *Review of Medical Physiology*, 15th ed., Appleton & Lange, Norwalk, CT, 1991.

Gaskell, B.A., Hext, P.M., Pigott, G.H. Hodge, M.C., and Tinston, D.J., Olfactory and hepatic changes following inhalation of 3-trifluoromethyl pyridine in rats, *Toxicology,* 50, 57–68, 1988.

Genter, M.B., Llorens, J., O'Callaghan, J.P., Peele, D.B., Morgan, K.T., and Crofton, K.M., Olfactory toxicity of beta, beta'-iminodipropionitrile in the rat, *J. Pharmacol. Exp. Ther.*, 263, 1432–1439, 1992.

Giddens, W.E. and Fairchild, G.A., Effects of sulfur dioxide on the nasal mucosa of mice, *Arch. Environ. Health,* 25, 166–173, 1972.

Gold, L.S., Slone, T.H., Manley, N.B., and Bernstein, L., Target organs in chronic biassays of 533 chemical carcinogens, *Environ. Health Perspec.,* 93, 233–246, 1991.

Gosselin, N.H., Brunet, .C., and Carrier, G., Comparative occupational exposures to formaldehyde released from inhaled wood product dusts versus that in vapor form, *Appl. Occup. Environ. Hyg.,* 18, 384–393, 2003.

Graham, D.E., and Koren, H.S., Biomarkers of inflammation in ozone-exposed humans. Comparison of the nasal and bronchoalvelor lavage, *Am. Rev. Respir. Dis.,* 142, 152–156, 2004.

Grandstrand, P., Nylander-French, L., and Holmstrom, M, Biomakers of nasal inflammation in wood-surface coating industry workers, *Am. J. Ind. Med.,* 33, 392–399, 1998.

Grasso, P., Sharratt, M., and Cohen, A.J., Role of persistent, non-genotoxic tissue damage in rodent cancer and relevance to humans, *Annu. Rev. Pharmacol. Toxicol.*, 31, 253–287, 1991.

Gray, H., *Gray's Anatomy*, 1901 ed., Running Press, Philadelphia, 1974.

Green, B.G., Mason, .R., and Kare, M.R., *Chemical Senses*, Vol. 2. *Irritation*, Marcel Dekker, New York, 1990.

Green, T., The Metabolism of Styrene by Rat, Mouse and Human Nasal Cytochrome P-450's, Cheshire, UK, CTL/R/1412, 1999.

Green, T., Lee, R., Moore, R.B., Ashby, J., Willis, G.A., Lund, V.J., and Clapp, M.J., Acetochlor-induced rat nasal tumors: further studies on the mode of action and relevance to humans, *Regul. Toxicol. Pharmacol*., 32, 127–133, 2000.

Green, T., Lee, R., Toghill, A., Meadowcroft, S., Lund, V., and Foster, J., The toxicity of styrene to the nasal epithelium of mice and rats: studies on the mode of action and relevance to humans, *Chem. Biol. Interact*., 137, 185–202, 2001.

Gross, E.A., Mellick, P.W., Kari, F.W., Miller, F.J., and Morgan, K.T., Histopathology and cell replication responses in the respiratory tract of rats and mice exposed by inhalation to glutaraldehyde for up to 13 weeks, *Fundam. Appl. Taxicol.,* 23, 348–362, 1994.

Gu, J., Walker, V.E., Lipinskas, T.W., Walker, D.M., and Ding, X., Intraperitoneal administration of coumarin causes tissue-selective depletion of cytochromes P450 and cytotoxicity in the olfactory mucosa, *Toxicol. Appl. Pharmacol.,* 146, 134–143, 1997.

Hansen, L.F., Knudsen, A., and Neilson, G.D., Sensory irritation effects of methyl ethyl ketone and its receptor activation mechanism, *Pharmacol. Toxicol.,* 71, 201–208, 1992.

Harkema, J.R., Comparative structure, function, and toxicity of the nasal airway, in *Toxicology of the Lung*, Gardiner, D.E., Crapo, J.D., and McClellann P.O., Eds. Taylor & Francis, London, 1999, pp. 55–83.

Harkema, J.R. and Hotchkiss, J.A., Ozone- and endotoxin-induced mucous cell metaplasias in rat airway epithelium: never animal models to study toxicant-induced epithelial transformation in airways, *Toxicol. Lett.,* 68, 251–263, 1993.

Haseman, J.K., and Hailey, J.R., An update of the national toxicology program database on nasal carcinogens, *Mutat. Res.,* 380, 3–11, 1997.

Hastings, L., Miller, M.L., Minnema, D., Evans, J., and Radike, M., Effects of methyl bromide on the rat olfactory system, *Chem. Senses*. 16, 43–55, 2002.

Hathaway, G.H., Proctor, N.H., Hughes, J.P., and Fischman, M.L., *Proctor and Hugh's Chemical Hazards of the Workplace,* 3rd ed., Van Nostrand Reinhold, New York, 1991.

Hauser, R., Elreedy, S., Hoppin, J.A., and Christiani, D.C., Upper airway response in workers exposed to fuel oil ash: nasal lavage analysis, *Occup. Environ.* Med., 52, 353–358, 1995.

Hendry, K.M. and Cole, E.C., A review of mycotoxins in indoor air, *J. Toxicol. Environ. Health*, 38, 183–198, 1993.

Hodgson, M., Field studies on the sick building syndrome, *Ann. N. Y. Acad. Sci*., 641, 21–36, 1992.

Hodgson, M., The sick building syndrome, *Occup. Med*., 10, 167–175, 1998.

Hofman, W., Ed., Deposition and Clearance of Aerosols in the Human Respiratory Tract, Second International Symposium, Salzbarg, Austria, September 18–20, 1986, Facultas Universitatsverlag, Vienna, Austria, 1987.

Horn, H.J., Inhalation toxicology of tetranitromethane, *AMA Archives Ind. Hyg. Occup. Med.,* 10, 213–222, 1954.

Hornung, D.E. and Mozell, M.M., Factors influencing the differential sorption of odorant molecules across the olfactory mucosa, *J. Gen. Physiol.,* 69, 343–361, 1977.

Huff, J., Cirvello, J., Haseman, J., and Bucher, J., Chemicals associated with site-specific neoplasia in 1394 long-term carcinogenesis experiments in laboratory rodents, *Environ. Health. Perspect.,* 93, 247–270, 1991.

Keenan, C.M., Kelly, D.P., and Bogdanffy, M.S., Degeneration and recovery of rat olfactory epithelium following inhalation of dibasic esters, *Fundam. Appl. Toxicol.,* 15, 381–393, 1990.

Keyhani, K., Scherer, P.W., and Mozell, M.M., A numerical model of nasal odorant transport for the analysis of human olfaction, *J. Theor. Biol*., 186, 279–301, 1997.

Kimbell, J.S., Gross, E.A., Joyner, D.R., Godo, M.N., and Morgan, K.T., Application of computational fluid dynamics to regional dosimetry of inhaled chemicals in the upper respiratory tract of the rat, *Toxicol. Appl. Pharmacol.,* 121, 253–263, 1993.

Kimbell, J.S. and Morgan, K.T., Airflow effects on regional disposition of particles and gases in the upper respiratory tract, *Radiat. Prot. Dosimetry*, 38, 213–219, 1991.

Klein, R.G., Janowsky, L., and Pool-Zobel, B.L., Long-term inhalation of low-dose N-nitrosodium-ethylamine (NDMA) in rats, *Proc. Am. Assoc. Cancer. Res.,* 31, 87, 1990.

Klein, R.G., Schmezer, P., Hermann, R., Waas, P., Spiegelhalder, B., and Bartsch, H., Strong nasal carcinogenicity and genotoxicity of 1-nitroso-4-methylpiperazine after low dose inhalation in rats, Carcinogenesis, 20, 1629–1631, 1999.

Kogevinas, M., Ferro, G., Anderson, A., Bellander, T., Biocca, M., Coggon, D., et al., Cancer mortality in a historical cohort study of workers exposed to styrene, *Scand. J. Work, Environ. Health,* 20, 251–261, 1994.

Kolesar, G.B., Siddiqui, W.H., Geil, R.G., Malczewski, R.M., and Hobbs, E.J., Subchronic inhalation toxicity of tetramethoxysilane in rats, *Fundam. Appl. Toxicol.,* 13, 285–295, 1989.

Kopferschmit-Kubler, M.C., Stenger, R., Blaumeiser, M., Eveilleau, C., Bessot, J.C., and Pauli, G., Asthma rhinitis and dermatitis after occupational exposure to cyanoacrylate glues, *Rev. Mal. Resp.,* 13, 305–307, 1996.

Kulle, T.J. and Cooper, G.P., Effects of formaldehyde and ozone on the trigeminal nasal sensory system, *Arch. Environ. Health*, 30, 237–243, 1975.

Laskin, S., Sllakumar, A.R., and Kuschner, M., Inhalation carcinogenicity of epichlorohydrin in noninbred Sprague-Dawley rats, *J. Nat. Cancer Inst.,* 65, 751–757, 1980.

Lee, K.P. Schneider, P.W., and Trochimowicz, H.J., Morphological expression of glandular differentiation in the epidermoid nasal carcinomas induced by phenylglycidylether inhalation, *Am. J. Pathol.,* 111, 140–148, 1983.

Lee, K.P., and Trochimowicz, H.J., Metapolastic changes of nasal respiratory epithelium in rats exposed to h examethylphosphoramide (HMPA) by inhalation, *Am. J. Pathol.,* 106, 8–19, 1982.

Leong, B.K.J, Kociba, R.J., and Jersey, G.C., A lifetime study of rats and mice exposed to vapours of bis(chloromethyl)ether, *Toxicol. Appl. Pharmacol.*, 58, 269–281, 1981.

Leopold, D.A., Nasal toxicity:end point of concern in humans, *Inhalation Toxicol.,* 6, 23–39, 1994.

Leroyer, C., Malo, J., Girard, D., Dufour, J., and Gautrin, D., Chronic rhinitis in workers at risk of reactive airways dysfunction syndrome due to exposure to chlorine, Occup. Environ. Med., 56, 334–338, 1999.

Lomax, L.G., Stott, W.T., Johnson, K.A., Calhoun, L.L, Yano, B.L. and Quast, J.F., The chronic toxicity and oncogenicity of inhaled technical-grade 1,3-dicholoropropene in rats and mice, *Fundam. Appl. Toxicol*., 12, 418–431, 1989.

Lowe, G. and Gold, G.H., The spatial distributions of odorant sensitivity and odorant-induced currents in salamander olfactory receptor cells, *J. Physiol.,* 442, 147–168, 1991.

Mackenzie, M., *A Manual of Diseases of the Throat and Nose,* Vol. II. *Diseases of the Oesophagus, Nose, and Naso-pharynx*. Wood, New York,1884.

Mann, W.J., Muttray, A., Schaefer, D., Klimek, L., Faas, M., and Konietzko, J., Exposure to 200 ppm of methanol increases the concentrations of interleukin-1 beta and interkeukin-8 in nasal secretions of healthy volunteers, *Ann. Otol. Rhinol. Laryngol., 111*, 633–638, 2002.

McLean, J.A., Mathews, K.P., Solomon, W.R., Brayton, P.R., and Bayne, N.K., Effect of ammonia on nasal resistance in atopic and nonatopic subjects, *Ann. Otol. Rhinol. Laryngol*., 88, 228–234, 1979.

McClesky, E.W. and Gold, M.S., Ion channels of nociception, *Ann. Rev. Physiol*., 61, 835–856, 1999.

Mery, S., Larson, J.L., Butterworth, B.E., Wolf, D.C., Harden, R, and Morgan, K.T., Nasal toxicity of chloroform in male F-344 rats and female B6C3F1 mice following a 1-week inhalation exposure, *Toxicol. Appl. Pharmacol*., 125, 214–227, 1994.

Miller, R.R., Ayres, J.A., Jersey, G.C., and Mckenna, M.J., Inhalation toxicity of acrylic acid, *Fundam. Appl. Toxicol*., 1, 271–277, 1981.

Miller, R.R., Herman, E.A., Young, J.T., Calhoun, L.L., and Kastl, P.E., Propylene glycol monomethyl ether acetate (PGMEA) metabolism, disposition, and short-term vapor inhalation toxicity studies, *Toxicol*. Appl. Pharmacol., 75, 521–530, 1984.

Miller, R.A., Mellick, P.W., Leach, C.L., Chou, B.J., Irwin, R.D., and Roycrott, J.H., Nasal toxicity in B6C3F1 mice inahaling furfuryl alcohol for 2 or 13 weeks, *Toxicologist*, 11, 669A, 1991.

Morgan, K.T., Nasal dosimetry, lesion distribution, and the toxicologic pathologist: a is brief review, *Inhalation Toxicol*., 6, 41–57, 1994.

Morgan, K.T., Jiang, X.Z., Starr, T.B., and Kerns, W.D., More precise localization of rasal tumors associated with chronic exposure of F-344 rats to formaldehyde gas, Toxicol. Appl. Pharmacol., 82, 264–274, 1986.

Morgott, D.A., Acetone, in *Patty's Industrial Hygiene and Toxicology*, 4th ed., Clayton, G.D. and Clayton, F.E. Eds., Wiley, New York, 1993, pp. 149–281.

Morris, J.B., Hassett, D.N., and Blanchard, K.T., A physiologically based pharmacokinetic model for nasal uptake and metabolism of nonreactive vapors, *Toxicol. Appl. Pharmacol*., 123, 120–129, 1993.

Morse, C.C., Boyd, M.R., and Witschi, H., The effect of 3-methylfuran inhalation exposure on the rat nasal cavity, *Toxicology*, 30, 195–204, 1984.

Murphy, C., Doty, R.L., and Duncan, H.J., Clinical disorders of olfaction, in *Handbook of Olfaction and Gustation,* 2nd ed., Doty, R.L. Ed., Marcel Dekker, New York, 2003, pp. 461–478.

Muttray, A., Jung, D., Klimek, L., and Kreiner, C., Effects of an external exposure to 200 ppm methyl ethyl ketone on nasal mucosa in healthy volunteers, *Int. Arch. Occup. Environ. Health*, 75, 197–200, 2002.

Muttray, A.M., Klimek, L., Faas, M., Schafer, D., Mann, W., and Konietzko, J., The exposure of healthy volunteers to 200 ppm 1,1,1-trichloroethane increases the concentration of proinflammatory cytokines in nasal secretions, *Int. Arch. Occup. Environ. Health*, 72, 485–488, 1999.

National Cancer Institute (1981) Carcinogenesis bioassay 1,2-dibromomethane (inhalation study) TR-210 (CAS 106-93-4) carcinogenesis testing program, DHHS Publication (NIH) 81-1766. National Cancer Institute, Bethesda, MD.

National Library of Medicine. *Hazardous Substances Data Bank*, Report Isopropyl Acetate, National Library of Medicine, Bethesda, MD, 1992.

National Library of Medicine. *Hazardous Substances Data Bank*, Report chromium II chloride, chromium II dioxide, chromium II oxalate. National Library of Medicine, Bethesda, MD, 1995b.

National Library of Medicine, *Hazardous Substances Data Bank,* Report hydrogen cyanide, National Library of Medicine, Bethesda, MD, 1995a.

Nielsen, J., Welinder, H., Bensryd, I., Andersson, P., and Skerfving, S., Symptoms and immunologic markers induced by exposure to methyltetrahydrophthalic anhydride, *Allergy, 49,* 28–286, 1994.

Nikula, K.J. and Lewis, J.L., Olfactory mucosal lesions in F344 rats following inhalation exposure to pyridine at threshold limit value concentrations, *Fundam. Appl. Toxicol*., 25, 510–517, 1994.

Nilsson R., Nordlinder, R., Wass, U., meding, B., and Being, L., Asthma, rhinitis, and dermatitis in workers exposed to reactive dyes, *Br. J. Ind. Med*., 50, 65–70, 1993.

NTP (National Toxicology Program), Toxicological and Carcinogenesis Studies of Nickel Oxide in F344/N Rats and B6C3F1 Mice, NIH Publication Series 96-3363, NTP TR 451, 1996a.

NTP (National Toxicology Program), Toxicological and Carcinogenesis Studies of Nickel Subsulfide in F344/N Rats and B6C3F1 Mice, NIH Publication Series 96-3369, NPT TR 453, 1996b.

NTP (National Toxicology Program), Toxicological and Carcinogenesis Studies of Nickel Sulfate Hexahydrate—in F344/N Rats and B6C3F1 Mice, NIH Publication Series 96–3370, NTP TR 454, 1996c.

Parmeggiani, L., *Encyclopedia of Occupational Health and Safety,* 3rd ed., International Labor Organisation, Geneva, 1983.

Passali, D., Mezzedimi, C., Passali, C., and Bellussi, L., Monitoring methods of nasal pathology, *Int. J. Pediatr. Otorhinolaryngol.*, 49 (Suppl 1), S199–S202, 1999.

Paulsen, E., Experimentalle Untersuchungen uber die stronmungen der luft in der nasenhohle, *Sitzung. K. Akad. Wissensch.,* 85, 348, 1882.

Pazdrak, K., Gorski P., Krakowiak, A., and Ruta, U., Changes in nasal lavage fluid due to formaldehyde inhalation, I*nt. Arch. Occup. Environ. Health, 64,* 515–519, 1993.

Peele, D.B., Alison, S.D., Bolon, B., Prah, J.D., Jensen, K.F., and Morgan, K.T., Functional deficits produced by 3-methylindole-induced olfactory mucosal damage revealed by a simple learning task, *Toxicol. Appl. Pharmacol*., 107, 191–202, 1991.

Perera, F.P. and Ahmed, A.K., *Respirable Particles: Impact of Airborne Fine Particulates on Health and the Environment*, Ballinger Publishing Co., Cambridge, MA, 1979.

Pino M.V., Valerio, M.G., Miller, G.K., Larson, J.L., Rosolia, D.L., Jayyosi, Z., Crouch, C.N., Trojanowski, J.Q., and Geiger, L.E., Toxicologic and carcinogenic effects of the type IV phosphodiesterase inhibitor RP 73401 on the nasal olfactory tissue in rats, *Toxicol. Pathol*., 27, 383–394, 1999.

Plopper, C.G., Suverkropp, C., Morin, D., Nishio, S., and Buckpitt, A., Relationship of cytochrone P-450 activity to Clara cell cytotoxicity. I. Histopathologic comparison of the respiratory tract of mice, rats and hamsters after parenterol administration of naphthalene, *J. Pharmacol. Exp. Ther*., 261, 353–363, 1992.

Preston-Martin, S., Pike, M.C., Ross R.K., and Henderson, B.E., Epidemiologic evidence for the increased cell proliferation model of carcinogenesis, *Environ. Health Perspect.*, 101 (Suppl 5), 137–138, 1993.

Proctor, N. H., Hughes, J. P., and Fischman, M.L., *Chemical Hazards of the Workplace,* J.B. Lippincott Company, Philadelphia, 1988.

Quraishi, M.S., Jones, N.S., and Mason, J., The rheology of nasal mucus: a review, *Clin. Otolaryngol.,* 23, 403–413, 1998.

Rangga-Tabbu, C., and Sleight, S.D., Sequential study in rats of nasal and hepatic lesions induced by N-nitrosodimethylamine and N-nitrosopyrrolidine, *Fundam. Appl. Toxicol*., 19, 147–156, 1992.

Reilly, C.A., Taylor, J.L., Lanza, D.L., Carr, B.A., Crouch, D.J., and Yost, G.S. Capsaicinoids cause in Flammation and epithelial cell death through activation of vanilloid receptors, Toxicol. Sci., 73, 170–181, 2003.

Renne, R.A., Giddens, W.E., Boorman, G.A., Kovatch, R., Haseman, J.E., and Clarke, W.J., Nasal cavity neoplasia in F344/N rats and (C57BL/6xC3H)F1 mice inhaling propylene oxide for up two years, *J. Natl. Cancer Inst*., 77, 573–582, 1986.

Repka-Ramirez, M.S., and Baraniuk, J.N., Histamine in health and disease, *Allergy,* 1–25, 2002.

Restrepo, D., Okada, Y., Teeter, J.H., Lowry, L.D., and Cowart, B., Human olfactory neurons respond to odor stimuli with an increase in cytoplasmic Ca^{++}, *Biophys. J.*, 64, 1961–1966, 1993.

Reznik, G., Stinson, S.F., and Ward, J.M., Respiratory pathology in rats and mice after inhalation of 1,2 dibromo-3-chloropropane or 1,2 dibromoethane for 13 weeks, *Arch. Toxicol.* 46, 233–240, 1980.

Riechelmann, H., Rettinger, G., Weschta, M., Keck, T., and Deutschle, T., Effects of low-toxicity particulate matter on human nasal function, *J. Occup. Environ. Med.*, 45, 54–60, 2003.

Sandmark, B., Broms, I., Lofgren, L., and Ohlson, C.G., Olfactory function in painters exposed to organic solvents, *Scand J. Work Environ. Health*, 11, 703–714, 1989.

Schieferstein, G.J., Sheldon, W.G. Cannell, S.A., and Reddy, G., Subchronic toxiocity study of 1,4- dithiane in the rat, *Fundam. Appl. Toxicol*. 11, 703–714, 1988.

Schiffman, S.S. and Nagle, H.T., Effect of environmental pollutants on taste and smell, *Otolaryngol. Head Neck Surg.,* 106, 693–700, 1992.

Schiffman, S.S., Walker, J.M., Dalton, P., Lorig, T.S., Raymer, J.H., Shusterman, D., and Williams, C.M., Potential health effects of odor from animal operations, wastewater treatment, and recycling of byproducts, *J. Agromed*., 7, 7–81, 2001.

Schlögel, F.A.,and Bannasch, P., Toxicity and cancerogenic properties of inhaled dimethyl sulfate, *Arch. Pharmacol*., 266, 441, 1970.

Schwartz, B.S., Bolla, K.I., Ford, D.P., Agnew, J., Rothman, N., and Bleeker, M.L., Solvent-associated decrements in olfactory function in paint manufacturing workers, *Am. J. Ind. Med*., 18, 697–706, 1990.

Schwartz, B.S., Doty, R.L., Monroe, C., Frye, R., and Barker, S., Olfactory function in chemical workers exposed to acrylate and methacrylate vapors, *Am. J. Public Hea*lth, 79, 613–618, 1989.

Schwob, J.E., Neural regeneration and the peripheral olfactory system, *Anat. Rec.,* 269, 33–49, 2002.

Schwob, J.E., Youngentob, S.L., and Mezza, R.C., Reconstitution of the rat olfactory epithelium after methyl bromide-induced lesion, *J. Comp. Neurol*., 359, 15–37, 1995.

Seeber, A., Kiesswetter, R.R., Vangala, M., Blaszkewicz, M., and Golka, K., Combined exposure to organic solvents: an experimental approach using acetone and ethyl acetate, *Appl. Psychol. Int. Rev.,* 41, 281–292, 1992.

Seilkop, S.K. and Oller, A.R., Respiratory cancer risks associated with low-level nickel exposure: an integrated assessment based on animal, epidemiological, and mechanistic data, *Regul. Toxicol. Pharmacol.,* 37, 173–190, 2003.

Sellakumar, A., Synder, C., Patil, G., and Burns, F., Inhalation Carcinogenisis of dimethylcarbamoyl chloride. A dose response effect and induction of naso-pharyngeal and nasal cavity tumors in rats, *Proc. Am. Assoc. Cancer Res*., 30, 140, 1989.

Shusterman, D., Toxicology of nasal irritants, *Curr. Allergy Asthma Rep.,* 3, 258–265, 2003.

Sittig, M., *Handbook of Toxic and Hazardous Chemicals*, 3rd ed.,. Noyes Publications, Park Ridge, NJ, 1991.

Slavin, R.G., Occupational rhinitis, *Ann. Allergy Asthma Immunol*., 90 (5 Suppl 2), 2–6, 2003.

Slotnick, B., Glover, P., and Bodyak, N., Does intranasal application of zinc sulfate produce anosmia in the rat? *Behav. Neurosci*., 114, 814–829, 2000.

Smeets, M.A. and Dalton, P., Perceived odor and irritation of isopropanol: a comparison between naive controls and occupationally exposed workers, *Int. Arch. Occup. Environ. Health*, 75, 541–548, 2001.

Sorokin, S.P., The respiratory system, in *Cell and Tissue Biology: A Textbook of Histology*, Weiss, L., Ed., Urban & Schwarzenberg, Baltimore, 1988, pp. 753–814.

Stott, W.T., Young J.T., Calhoun, L.L, and Battjes, J.E., Subchronic toxicity of inhaled technical grade, 1,3-dicholorpropene in rats and mice, *Fundam. Appl. Toxicol*., 11, 207–220, 1988.

Sulkowski, W.J., Rydzewski, B., and Miarzynska, M., Smell impairment in workers occupationally exposed to cadmium, *Acta Otolaryngol.,* 120, 316–318, 2000.

Sun, T.J., Miller, M.L., and Hastings, L., Effects of inhalation of cadmium on the rat olfactory system: behavior and morphology, *Neurotoxicol. Teratol*., 18, 89–98, 1996.

Sundblad, B.M., Larsson, B.M., Acevedo, F., Ernstgard, L., Johanson, G., Larsson, K., et al., Acute respiratory effects of exposure to ammonia on healthy persons, *Scand. J. Work Environ. Health, 30,* 313–321, 2004.

Sunderman, F.W., Jr., Nasal toxicity, carcinogenicity, and olfactory uptake of metals, *Ann. Clin. Lab. Sci.,* 31, 3–24, 2001.

Tai, C.F. and Baraniuk, J.N., Upper airway neurogenic mechanisms, *Curr. Opin. Allergy Clin. Immunol*., 2, 11–19. 2002.

Toren, K., Brisman, J., Hagberg, S., and Karlsson, G., Improved nasal clearance among pulp-mill workers after the reduction of time dust, *Scand. J. Work Environ. Health, 22,* 100–105, 1996.

Turk, M.A., Henk, W.G., and Flory, W., 3-Methylindole-induced nasasl mucosal damage in mice, *Vet. Pathol.*, 24, 400–403, 1987.

Uraih, L.C., Talley, F.A. Mitsumori, K., Gupta, B.N., Bucher, J.R., and Boorman, G.A., Ultrastructural changes in the nasal mucosa of Fischer 344 rats and B6c3F1 mice following an acute exposure to methyl isocyanate, *Environ. Health Perspect.*, 72, 77–88, 1987.

Vaughan, T.L., Stewart, P.A., Teschke, K., Lynch, C.F., Swanson, G.M., Lyon, J.L., and Berwick, M. Occupational exposure to formaldehyde and wood dust and nasopharyngeal carcinoma, *Occup. Environ. Med.*, 57, 376–384, 2000.

Vernot, E.H., MacEwen, J.D., Bruner, R.H., Bruner, R.H., Haun, C.C., Kinkead, E.R., Prentice, D.E., Hall, A. III, Schmidt, R.E., Eason, R.L., Hubbard, G.B., and Young, J.T., Long-term inhalation toxicity of hydrazine, *Fundam. Appl. Toxicol.*, 5, 1050–1064, 1985.

Ward, J.M., Uno, H., Kurata, Y., Weghorst, C.M., and Jang, J.J., Cell proliferation not associated with carcinogenesis in rodents and humans, *Environ. Health Perspect.*, 101 (Suppl 5), 125–135, 1993.

Warheit, D.B., Kelly, D.P., Carakostas, M.C., and Singer, A.W., A 90-day inhalation toxicity study with benomyl in rats, *Fundam. Appl. Toxicol.*, 12, 333–345, 1989.

Watanabe, S. and Fukuchi, Y., Occupational impairment of the olfactory sense of chromate producing workers, J. Ind. Health,, 23, 606–611, 2000.

Widdicombe, J.G., Reflexes from the upper respiratory tract, in *Handbook of Physiology,* Cherniak, N.S., and Widdicombe, J.G., Eds., American Physiological Society, Bethesda, MD, 1986a, pp. 363–394.

Widdicombe, J.G., Sensory innervation of the lungs and airways, in, *Progress in Brain Research*, Vol. 67, Cervero, F. and Morrison, B.V., Eds., Elsevier, London, 1986b, pp. 49–64.

Widdicombe, W.G. and Wells, U.M., Airway secretions, in *The Nose: Upper Airway Physiology and the Atmospheric Environment,* Proctor, D.F. and Andersen, I.B., Eds., Elsevier Biomedical Press, New York, 1982, pp. 215–244.

Wolf D.C., Morgan, K.T. Gross, E.A., Barrow, C., Moss, O.R., James, R.A., and Popp, J.A., Two-year inhalation exposure of female and male B6C3F1 mice and F344 rats to chlorine gas induces lesions confined to the nose, *Fundam. Appl. Toxicol.*, 24, 111–131, 1995.

Woutersen, R.A., Appelman, L.M., Feron, V.J., and Van der Heijden, C.A., Inhalation toxicity of acetaldehyde in rats. II. Carcinogenicity study: interim results after 15 months, *Toxicology,* 31, 123–133, 1984.

Woutersen, R.A., Appelman, L.M., Feron, V.J., and Van der Heijden, C.A., Inhalation toxicity of acetaldehyde in rats. II. Carcinogenicity study, *Toxicology,* 31, 213–231, 1984.

Woutersen, R.A., Appelman, L.M., van Garderen-Hoetmer, A., and Feron, V.J., Inhalation toxicity of acetaldehyde in rats. III. Carcinogenicity study, *Toxicology*, 41, 213–231, 1986.

Woutersen, R.A., Appelman, L.M., Wilmer, J.W., Falke, H.E., and Feron, V.J., Subchronic (13-week) inhalation toxicity study of formaldehyde in rats, *J. Appl. Toxicol.*, 7, 43–49, 1987.

Woutersen, R.A., Garderen-Hoetmer, A., Bruijntjes, J.P., Zwart, A., and Feron, V.J., Nasal tumours in rats after severe injury to the nasal mucosa and prolonged exposure to 10 ppm formaldehyde, *J. Appl. Toxicol.*, 9, 39–46, 1989.

Wysocki, C.J., Dalton, P., Brody, M.J., and Lawley, H.J., Acetone odor and irritation thresholds obtained from acetone-exposed factory workers and from control (occupationally non-exposed) subjects, *Am. Ind. Hyg. Assoc. J.*, 58, 704–712, 1997.

Yokota, K., Johyama, Y., Yamaguchi, K., Takeshita, T., and Morimoto, K., Exposure-response relationships in rhinitis and conjunctivitis caused by methyltetrahydrophthalic anhydride, *Int. Arch. Occup. Environ. Health, 72,* 14–18, 1999.

Youngentob, S.L., Schwob, J.E., Sheehe, P.E., and Youngentob, L.M., Odorant threshold following methyl bromide-induced lesions of the olfactory epithelium, *Physiol. Behav.*, 62, 1241–1252, 1997.

Yu, C.P., Diu, C.K., and Soong, T.T., Statistical analysis of aerosol deposition in nose and mouth, *Am. Ind. Hyg. Assoc. J.*, 42, 726–733, 1981.

Zhao, K., Scherer, P.W., Hajiloo, S.A., and Dalton, P. Effect of anatomy on human nasal airflow and odorant transport patterns: implications for olfaction, *Chem. Senses*, 29, 365–379, 2004.

18 Toxicokinetics: Deposition, Absorption, Distribution, and Excretion

Mario A. Inchiosa

CONTENTS

18.1 INTRODUCTION

The basic premise underlying this chapter is that the systemic toxic manifestations of a substance can often be correlated with the plasma concentration, organ concentration, or total body burden of that substance. It follows from this premise that valuable information can be gained by considering the factors that influence the concentration of an agent in body fluids and tissues following transient or chronic exposure. In this context, these factors include the deposition, absorption, distribution, and elimination of the agent after or during inhalation exposure.

18.2 DEPOSITION IN THE RESPIRATORY TRACT

Inhaled toxicants may be in the form of gases, vapors of volatile liquids, or aerosols. Aerosols may represent particulate matter or small liquid droplets. The deposition and subsequent removal of aerosols in the respiratory tract depend on physical forces and biological clearance mechanisms. These factors are also considered elsewhere in this volume and will only be summarized here. Particles having a diameter greater than 5 μm (5–30 μm) are principally deposited in the nasopharyngeal region by impaction (air velocity and turbulence are highest in this portion of the respiratory tract and decrease progressively toward the alveoli). Particles of 1–5 μm in diameter deposit along the tracheobronchial tract by sedimentation, with the smallest particles reaching the region approaching the alveoli. Particles less than 1 μm in diameter, which reach the alveoli, are deposited primarily by diffusion (Casarett, 1972).

18.3 CLEARANCE MECHANISMS

The clearance of substances deposited in the respiratory tract may take place by a combination of several mechanisms (Klassen, 1980). Clearance routes are illustrated in Figure 18.1. The mechanisms

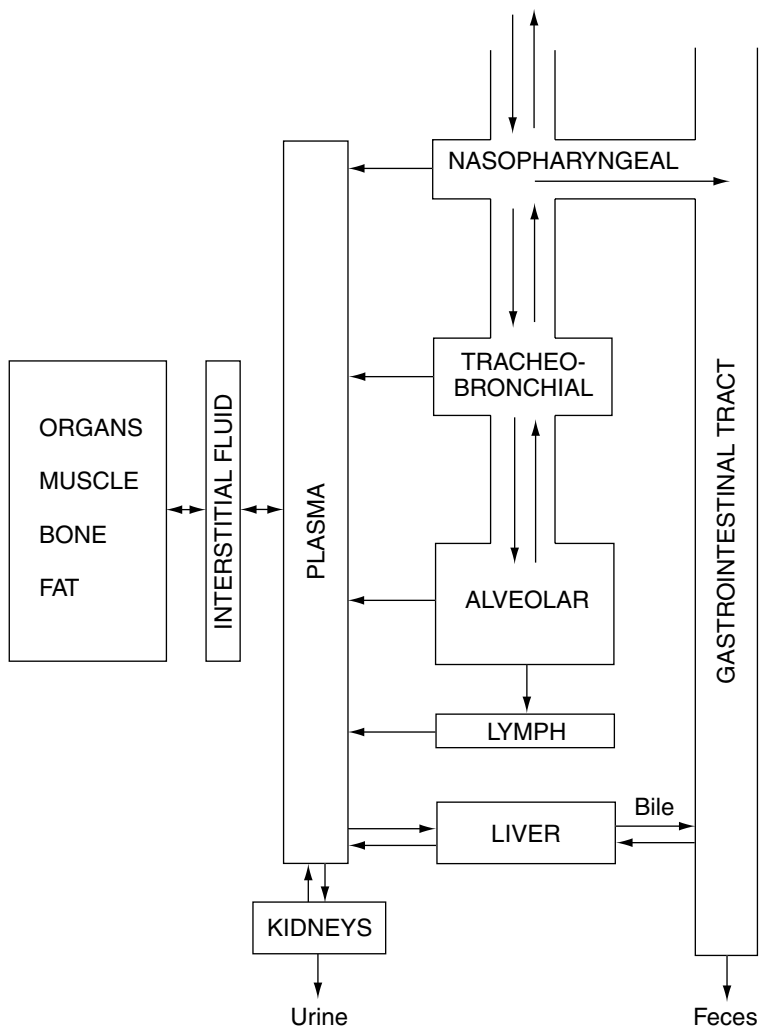


FIGURE 18.1 Schematic diagram of routes of deposition, absorption, distribution, and excretion of inhaled agents. (Modified from Klaassen, 1980.)

that may be involved for a particular material depend on the physical and physicochemical properties of the substance. Particles deposited in the nasopharyngeal region may be removed in conjunction with secretions as a result of coughing, sneezing, or being physically wiped away from the nasal orifices. Also, a portion of the material deposited in this region may be swallowed.

A primary clearance mechanism for particles deposited in the tracheobronchial region is the extremely efficient mucociliary escalator system in this region, which raises particles to where they may be expectorated or swallowed. Although the ultimate swallowing of inhaled substances represents a clearance mechanism from the standpoint of the lung, in the larger context of this chapter this constitutes another route for possible systemic absorption. These considerations will be addressed in further detail below.

The phagocytic activity of macrophages is another important mechanism for the clearance of particles deposited in the tracheobronchial tree and the alveoli. The lytic enzymes contributed by the macrophages may directly aid in the dissolution of particles resulting in products that can be absorbed directly into the circulation. In other instances, particles ingested into macrophages may pass with these cells into the lymphatic circulation. The lymphatic drainage of the alveoli also represents a direct clearance mechanism that can be utilized by macromolecules, aggregates of macromolecules, and particles of appropriate dimensions (Kilburn, 1977; Klaassen, 1980; Menzel and McClellan, 1980).

Finally, nonparticulate material, such as the solutes present in an aerosol, vapors of volatile liquids, and gases may be absorbed directly into the plasma along the entire surface of the respiratory tract. The experimental evidence demonstrates that the ease of absorption through the respiratory epithelium is directly related to the lipid solubility of a substance (Burton and Schanker, 1974a, 1974b).

18.4 ALVEOLAR ABSORPTION

Although absorption may take place over the entire respiratory tract, the largest surface area is presented by the alveolar epithelium. In the adult, this area is estimated to be approximately 35 m^2 during expiration and 100 m^2 in maximal inspiration (von Hayek, 1960; Weibel, 1963). The relatively small total volume of blood in the lungs (60–140 ml), which is spread over this large surface area, results in an exceedingly thin film of blood (the thickness of this film of blood is equal to the diameter of the pulmonary capillaries, which is approximately 8 μm), which optimizes the process of absorption from the alveolar space into the plasma (Guyton, 1981). The vascular organization at this level is illustrated in Figures 18.2 and 18.3, which schematically demonstrate the cellular layers constituting the respiratory membrane. The surface of the alveoli is covered with a surfactant layer (composed primarily of dipalmitoyl lecithin) and, apparently, also an aqueous layer between the surfactant and the alveolar squamous epithelium. These cells are only 0.1 to 0.2 μm thick (Menzel and McClellan, 1980). The basement membrane of the alveolar epithelium and the capillary basement membrane are separated by a thin interstitial space in some areas and fused in others. The capillary endothelial cell is the final layer of the respiratory membrane; the total distance between the alveolar space and plasma is estimated to be between 0.5 and 2.5 μm (Menzel and McClellan, 1980; Guyton, 1981).

18.4.1 Absorption of Gases

The rate of diffusion of gases across the respiratory membrane has been demonstrated to be related primarily to the diffusion coefficient of the gas in aqueous media (Hill, 1980). Because most gases of toxicological importance are adequately lipid soluble to readily pass through cell membranes of the alveolar epithelium and the capillary endothelium, cell water presents the major impediment to diffusion. Thus, the diffusion coefficient for a gas across the respiratory membrane can be approximated from its diffusion in water. This is usually determined from the following relationship (Hill, 1980; Guyton, 1981):

$$\text{Diffusion coefficient} = \frac{\text{solubility coefficient of gas in water}}{\sqrt{\text{molecular weight of gas}}} \tag{18.1}$$

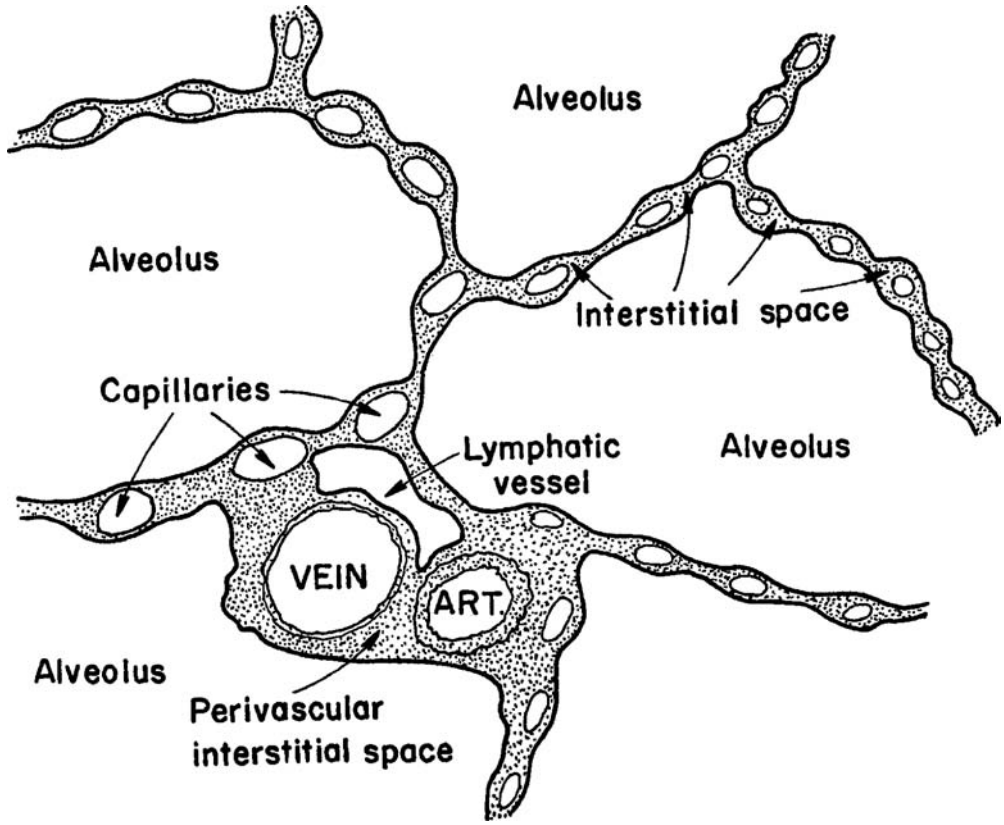


FIGURE 18.2 Schematic representation of a cross-section through the lung demonstrating alveolar walls and vascular organization. (From Guyton, 1981. Reproduced by permission of W.B. Saunders Company.)

The solubility coefficients for most gases are greater for blood than for water; this has been thoroughly investigated for anesthetic gases (Larson et al., 1962; Beattie, 2001). Because of large differences among anesthetic gases relative to blood–gas partition coefficients, the contribution of this factor to the rate of anesthesia induction, potency, and rate of emergence has been extensively investigated (Hill, 1980; Beattie, 2001) and will not be reviewed here.

The second factor that determines the diffusion rate of a gas is the concentration gradient between alveolar air and plasma. Although a toxic gas may be present in low concentrations in the inspired air, a substantial gradient may still serve as a driving force toward the plasma and tissues.

18.4.2 Absorption of Solutes

The absorption from the lung of solutes contained in aerosols is principally a process of simple diffusion. With only a few isolated exceptions, there has been no evidence for active or facilitated transport mechanisms for most compounds that have been studied (Burton and Schanker, 1974a, 1974b). Rates of diffusion were not saturable as the concentration of the instilled solute was increased; instead, the rates of diffusion appeared to be proportional to the concentration.

Although diffusion of a particular substance from the lung is proportional to the concentration gradient, a wide range in the permeability of the respiratory membrane exists for different compounds. The physicochemical properties of the solute determine its permeability, as follows.

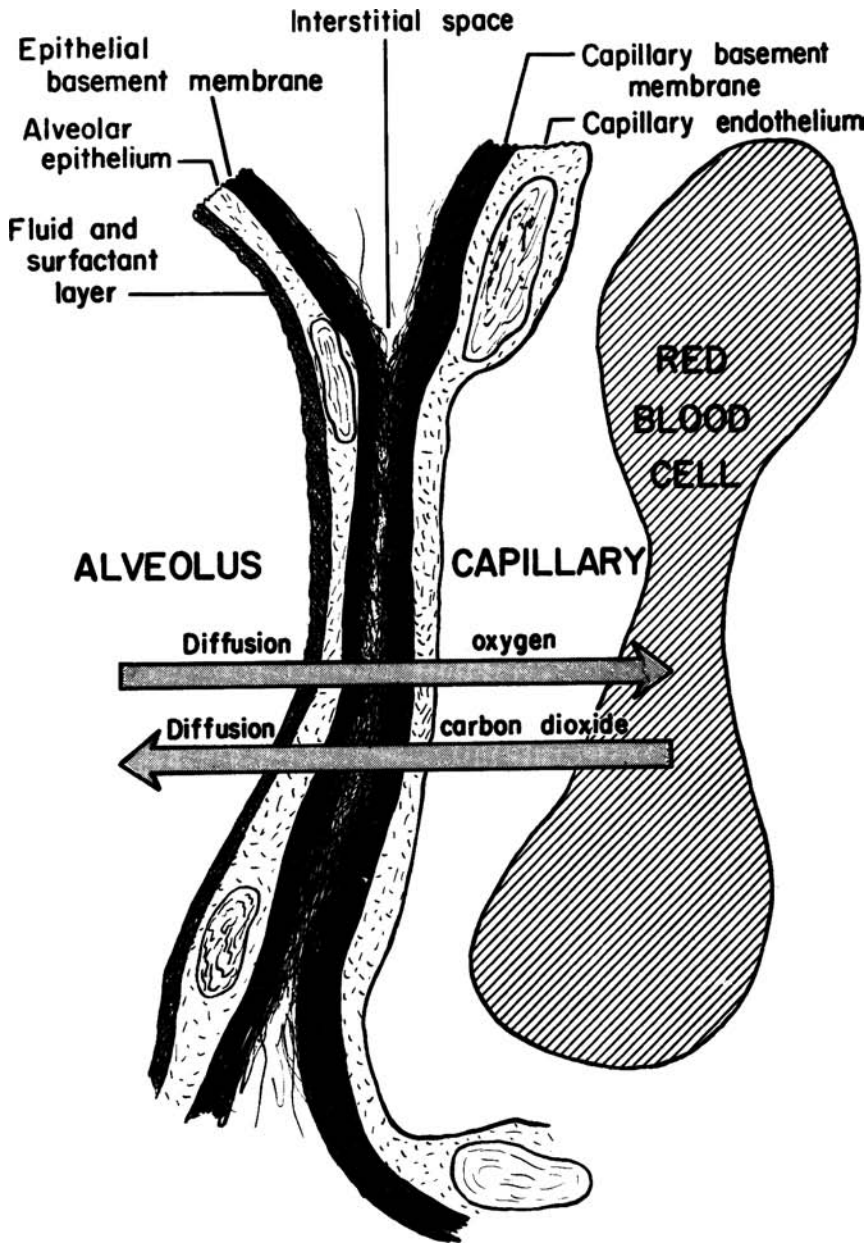


FIGURE 18.3 Schematic representation of components of the respiratory membrane. (From Guyton, 1981. Reproduced by permission of W.B. Saunders Company.)

18.4.2.1 Lipid-Insoluble Neutral Compounds

The cellular membranes of alveolar epithelial cells are the major barriers to the absorption of polar compounds. This barrier is represented by the bimolecular lipid layer of the cell membrane. Because the epithelial cells are tightly apposed, the major pores through which water-soluble molecules penetrate are of dimensions similar to those present in most cellular membranes. These pores are believed to be primarily associated with protein molecules, which pass through the cell membrane (Figure 18.4). Studies by Taylor and Gaar (1970) in the dog have demonstrated that the pores of the alveolar epithelium have a radius of 6–10 Å. Thus, water molecules, urea, and potassium ions pass

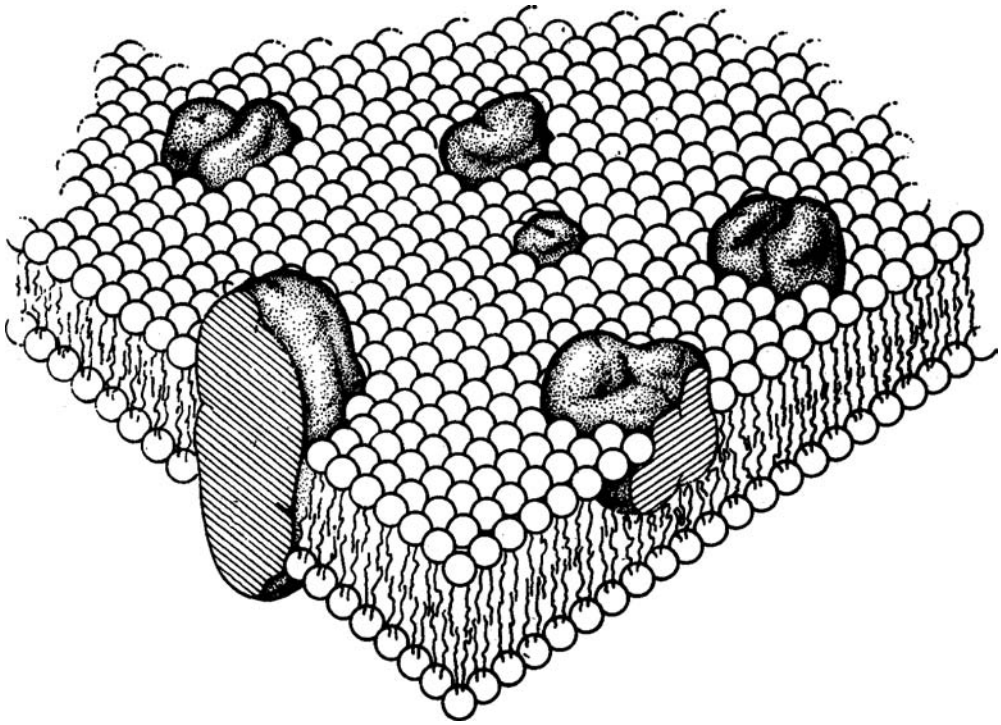


FIGURE 18.4 Fluid mosaic model of the cell membrane. Globular proteins are embedded within and traverse a bimolecular lipid matrix. (From Singer and Nicolson, 1972. Reproduced by permission of the American Association for the Advancement of Science.)

through with relative ease, whereas mono- and disaccharides are poorly permeable. This situation is markedly different from that at the level of pulmonary capillaries, where there are relatively large pores (40–58 Å equivalent radius) between the endothelial cells of the capillaries. Glucose, urea, sodium, and other water-soluble molecules of similar dimensions diffuse through these pores at rates 100 or more times as great as through the alveolar epithelium (Guyton, 1981).

18.4.2.2 Lipid-Soluble Compounds

The absorption of lipid-soluble molecules from the alveoli is primarily related to the lipid–aqueous partition coefficient of the compound. The relative rates of diffusion for a number of lipophilic compounds were predicted on the basis of the rank order of their chloroform–water partition coefficients (Burton and Schanker, 1974a, 1974b). Several antibiotics and steroids showed half-times of absorption ranging from 1.9 to 33 min. Paradoxically, compounds with very high partition coefficients, such as DDT and leptophos, showed slow rates of absorption ($t_{1/2}$ values of absorption of about 300 min) (Menzel and McClellan, 1980); this may be explained by the partitioning of molecules into membrane lipids, resulting in slow absorption from the lung.

18.4.2.3 Ionizable Compounds

Weak acid or weak base solutes deposited in the lung are absorbed into the systemic circulation in relation to their nonionized fractions in the lung and the intrinsic lipid solubility of the nonionized form. Only the nonionized molecules possess any degree of membrane permeability.

The degree of ionization of a particular compound can be determined from the pKa of the dissociating group and the pH of the alveolar fluid (the available evidence indicates a pH close to neutrality) (Mason et al., 1977). When a compound is in solution at a pH that is the same as its pKa, it exists in

TABLE 18.1 Ionization Ratio of Weak Electrolytes in Relation to pKa and pH

		[Ionized]:[Nonionized]	
		Weak Acids	**Weak Bases**
	pH − pKa		
	+4	10000:1	1:10000
	+3	1000:1	1:1000
(pH > pKa)	+2	100:1	1:100
	+1	10:1	1:10
(pH = pKa)	0	1:1	1:1
	−1	1:10	10:1
(pH < pKa)	−2	1:100	100:1
	−3	1:1000	1000:1
	−4	1:10000	10000:1

equal concentrations of the ionized and nonionized forms. The Henderson–Hasselbalch equations can be used to calculate the ratio of the ionized and nonionized fractions when the pH differs from the pKa, as follows (illustrated for a carboxyl group and a primary amine):

	Predominant form when pH < pKa	Predominant form when pH > pKa	Henderson–Hasselbalch equations

For acids: $R-COOH \rightleftharpoons R-COO^{-}H^{+}$ $\quad pH - pKa = \log \dfrac{[\text{ionized}]}{[\text{nonionized}]}$ (18.2)

For bases: $R-\overset{+}{N}H_3 \rightleftharpoons R-NH_2$ $\quad pH - pKa = \log \dfrac{[\text{nonionized}]}{[\text{ionized}]}$ (18.3)

Table 18.1 lists the ratios of ionized and nonionized forms of weak electrolytes for one-unit changes in pH in relation to pKa.

The implications of these relationships for the systemic absorption of inhaled solutes can be illustrated by the following examples (assuming that the pH of the interface between air and the epithelial membrane of the respiratory tract is 7). A weak acid with a pKa of 4 (99.9% ionized at pH 7) would be expected to be much more slowly absorbed upon inhalation than a weak base with pKa of 4 (0.1% ionized at pH 7). In considering the overall bioavailability of inhaled solutes, it is necessary to anticipate the systemic absorption of solute which may eventually be swallowed. In the example of the weak acid (pKa 4), the molecules that reach the stomach will establish a new equilibrium in gastric juice (pH approximately 2) with only 1% of the molecules in the ionized form. Absorption would be expected to be relatively rapid under these conditions. Because inhaled solute may be absorbed by two routes, which establish different ionization equilibria, it is frequently difficult to observe the relationship between pKa and absorption. However, the importance of this relationship has been established from systematic studies of the intestinal absorption of acids and bases with varied pKa values and in different pH environments (Hogben et al., 1959).

18.4.2.4 Effective Partition Coefficient

The interactions of the two prior topics (absorption of lipid-soluble and ionizable compounds) are embodied in the concept of "effective partition coefficient." This coefficient is defined as the product of the percent nonionized compound (at the pH under consideration) and the partition coefficient of

the nonionized form. In comparing effective partition coefficients of compounds, it is essential that the partition coefficients be determined in the same system (e.g., chloroform/water, olive oil/water, etc.). The rank order for the rate of penetration of a series of weak acids and bases into cerebrospinal fluid was shown to be the same as for their effective partition coefficients (data compiled by Goldstein et al., 1974). These principles would apply to the permeability of weak electrolytes through the lipid barrier presented by all cell membranes.

A practical note can be introduced here. The effective partition coefficient, also termed the overall partition coefficient (Bowman and Rand, 1980), can be determined directly by measuring the partition coefficient in a system in which the aqueous phase is buffered at the pH of consideration. For the lung, this pH may be considered to be in the neutral range (Mason et al., 1977). Only the fraction of molecules nonionized at this pH will partition into the organic phase and, therefore, an overall partition coefficient will be obtained. A series of compounds may be compared in this way as part of the evaluation of the relative ease of absorption by inhalation. An equation described for this relationship (Bowman and Rand, 1980) is rearranged to a more convenient form where P_o is the overall

$$P_0 = \frac{P_n}{\text{antilog}\,(\text{pH} - \text{pKa}) + 1} \tag{18.4}$$

(or "effective") partition coefficient and P_n is the intrinsic partition coefficient for the nonionized form. Equation (18.4) is in the form for calculations concerning acids; the term (pH − pKa) is reversed to (pKa − pH) in calculations for bases.

18.5 DISTRIBUTION IN THE BODY

Once a substance has been absorbed from the respiratory tract into the plasma (either directly or after being swallowed), it can be distributed more widely in the body. The extent of this distribution is largely determined by the same physicochemical factors discussed in relation to absorption. The major aqueous compartments of the body into which chemicals distribute are illustrated in Figure 18.5. The

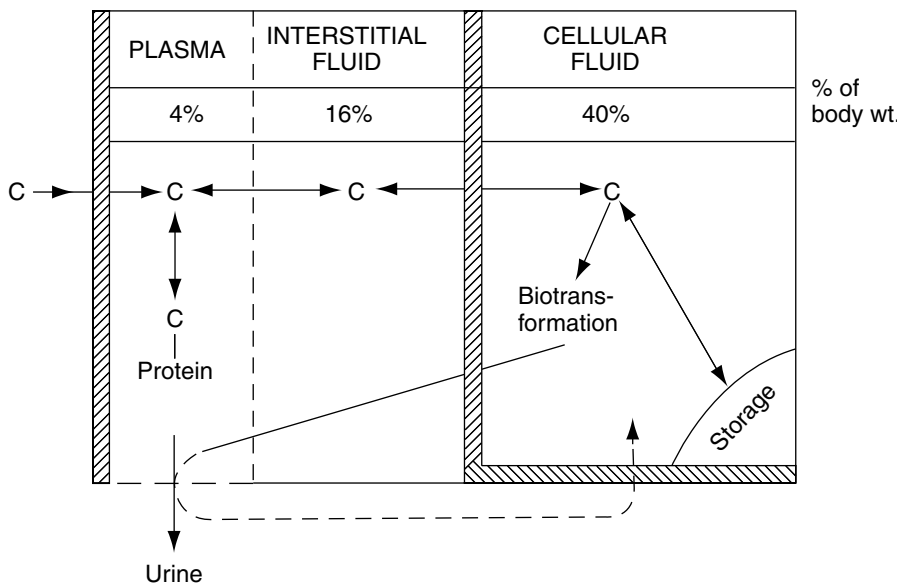


FIGURE 18.5 The major anatomical compartments that constitute body water, expressed as percentages of body weight. C represents chemical agent. (Modified from Goldstein et al., 1974.)

total volume of these compartments (about 60% of body weight) is commonly termed body water. For a 70-kg man, plasma volume is approximately 3 l, interstitial fluid is 11 l, and cell water is 28 l (a total of 42 l). As noted above, the specific properties of a compound determine the volume of body water into which it may distribute, i.e., the volume of distribution (V_d). This volume is more appropriately termed the apparent volume of distribution, as will be discussed later.

18.5.1 Plasma Protein Binding

Compounds absorbed into the plasma may be relatively restricted or retarded from wider distribution as a result of binding to plasma proteins. Most of this interaction takes place with albumin, which is the protein present in the highest concentration in plasma. The binding with plasma proteins predominantly takes the form of electrostatic interaction, where ionized compounds are bound to the counter ion carried by charged groups of the protein. The groups primarily involved in this binding are listed in Table 18.2. In the case of therapeutic drugs, this binding is viewed as being completely reversible. There is a wide variation among drugs in the characteristic percentage bound to plasma protein. It is common for a drug to exist in a ratio of 99% bound to 1% free in plasma. The fraction of bound drug is not filtered in the kidney glomeruli and is not readily available for metabolism in the liver. Binding to plasma protein is, as a rule, associated with a prolongation of the half-life of an agent in the body. If the binding to plasma protein represents an equilibrium between bound and free agent, as described above, this ratio is usually maintained over a wide range in the plasma concentration of the agent. Thus, as free molecules are filtered in the kidneys or metabolized, protein-bound compound dissociates to reestablish the equilibrium ratio. It is only at the extremes of total plasma concentration of an agent that marked differences may be seen in the equilibrium.

18.5.2 Diffusion into Interstitial Fluid

The movement of unbound agents across capillary membranes into the interstitial fluid (see Figure 18.5) is influenced by the same factors considered above in relation to the diffusion of compounds from pulmonary alveoli into plasma, i.e., the relative water or lipid solubility of an agent and the molecular dimensions of lipid-insoluble compounds. An important difference from the pulmonary membrane, however, is the substantially larger pores that exist in the capillary membrane through which water-soluble compounds can diffuse. The size of the capillary pores has been approximated from measurements of the capillary permeability of water-soluble molecules of varying molecular size. The relationship between molecular weight and capillary permeability is presented in Figure 18.6.

TABLE 18.2 Potential Sites for Binding of Ionized Compounds to Proteins

Amino Acid	Group	Number of residues per molecule
Aspartic and glutamic	$-COO^-$	101
Tyrosine	$-O^-$	18
Cysteine	$-S^-$	0.7
Terminal	$-COO^-$	1
Histidine	$-NH^+-$	17
Lysine	$-NH_3^+$	57
Arginine	$=NH_2^+$	22
Terminal	$-NH_3^+$	1

Source: From Goldstein et al., 1974. Reproduced by permission of John Wiley and Sons, Inc. Original analytical data from Tanford et al., 1955, by permission of the American Chemical Society.

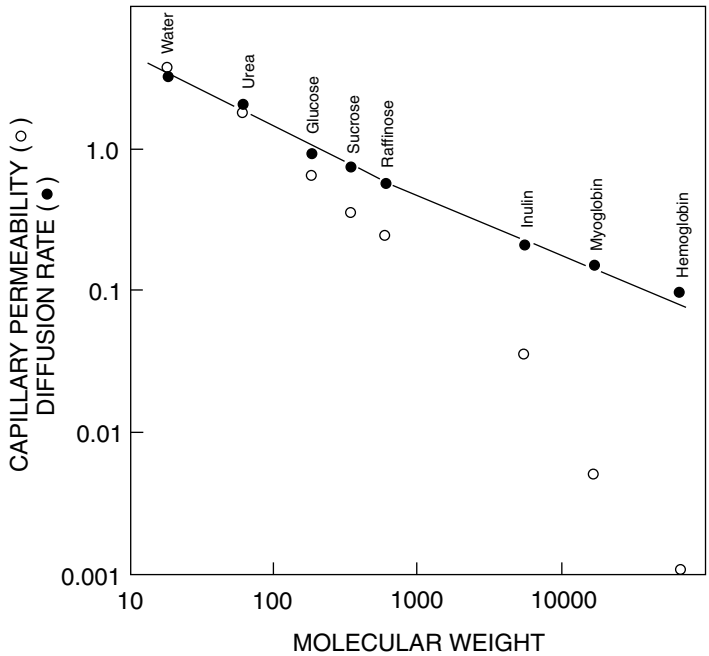


FIGURE 18.6 Relationship between diffusion rate and molecular weight for water-soluble molecules of widely different sizes. The filled circles represent the diffusion rates (cm^2/sec) of the molecules in water at 37°C. The open circles are the permeability coefficients (cm^3/sec/100 g) for passage of the same compounds across skeletal muscle capillaries. (Data from Pappenheimer et al., 1951; Renkin, 1964.)

This figure contrasts the change in diffusion in water as molecular weight increases, to the change in diffusion across the capillary membrane in muscle. A marked disparity occurs in the relationship between molecular weight and diffusion across capillaries when raffinose (mol wt 594) and inulin (mol wt 5,500) are compared. Raffinose diffuses relatively freely whereas inulin is much more restricted. From measurements such as these, it was estimated that the capillary pores had a radius of approximately 30 Å (Renkin, 1964). (Note that capillary pores in kidney glomeruli are considerably larger, because inulin is freely filtered from plasma into the kidney tubules.)

Lipid-soluble molecules will diffuse across capillary membranes in relation to their partition coefficients, as discussed above. It is not necessary for such molecules to utilize pores, because they can diffuse through membranes of capillary endothelial cells.

18.5.3 Diffusion into Cells

The further distribution of an agent from interstitial fluid into cellular water (see Figure 18.5) primarily depends on its lipid–water partition coefficient. The cellular membranes of organs present the same barrier to water-soluble molecules as described above for alveolar epithelial cells.

Protein binding must be considered in conjunction with the permeability of an agent for capillary and cellular membranes in anticipating the extent of distribution of a substance in the compartments of body water. These principles are illustrated in Figure 18.7.

As noted above, the extent to which an agent is distributed is termed its volume of distribution, V_d; more appropriately, it should be termed the apparent volume of distribution because estimates of this volume are usually only based on the concentration of an agent in plasma after the administration of a known dose. The experimental approach to estimating V_d will be discussed under Kinetic Considerations.

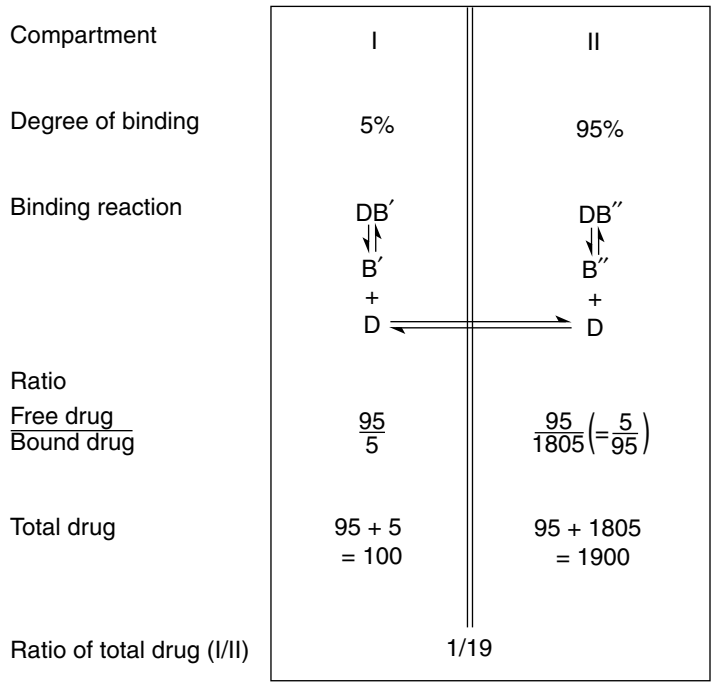


FIGURE 18.7 Influence of drug binding on the distribution of total drug between two compartments separated by a membrane. Only free drug (D) can diffuse across the membrane. The example illustrates the distribution at equilibrium. (From Bowman and Rand, 1980. Reproduced by permission of Blackwell Scientific Publications.)

18.5.4 Diffusion into Saliva

Many agents diffuse rapidly from plasma into saliva. Also, because a constant ratio exists between the plasma and saliva concentrations of many drugs, determinations in saliva have been applied to bioavailability evaluations, pharmacokinetic studies, and routine drug monitoring (Matin et al., 1974; Breckenridge et al., 1977; Parsons and Neims, 1978; Newton et al., 1981; Poland and Rubin, 1982). Saliva determinations have been used to study the pharmacokinetics of caffeine in rabbits (Inchiosa et al., 1981). Figure 18.8 shows the saliva concentration–time course following the intravenous bolus injection of caffeine to rabbits. The pharmacokinetic information gained from such data is discussed in Section 18.7. From other studies, the saliva/plasma ratio for caffeine in the rabbit has been found to be 0.78; this same ratio in humans is 0.74 (Newton et al., 1981). Matin et al. (1974) have combined the factors essential for predicting the saliva/plasma ratio in the following equation:

$$R = \frac{1 + 10^{(\text{pHs} - \text{pKa})}}{1 + 10^{(\text{pHp} - \text{pKa})}} \frac{\text{fp}}{\text{fs}} \tag{18.5}$$

where

R = saliva/plasma ratio
pHs = saliva pH
pHp = plasma pH
fp = fraction of drug unbound in plasma
fs = fraction of drug unbound in saliva.

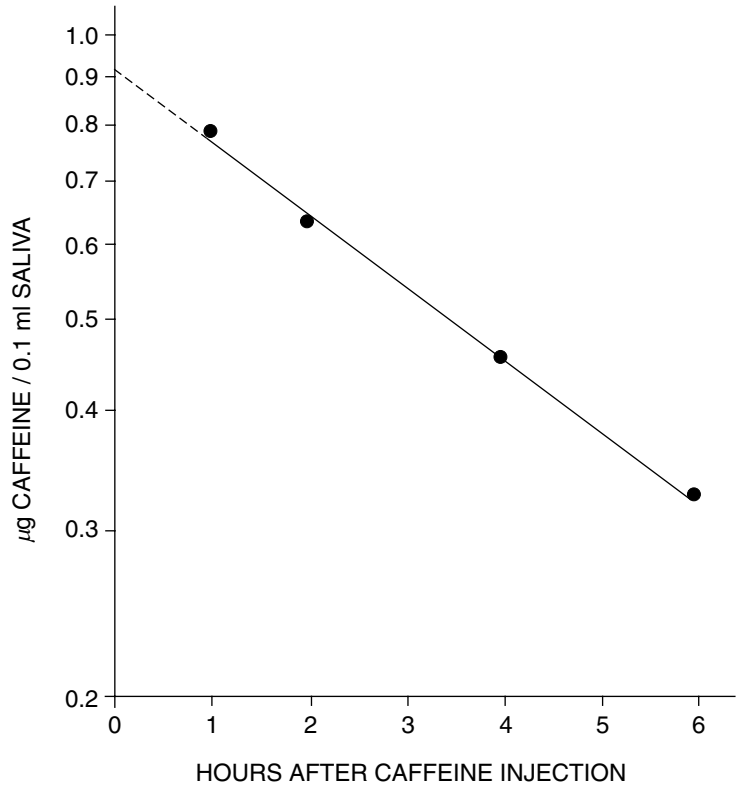


FIGURE 18.8 Semilogarithmic plot of caffeine concentration of saliva as a function of time after i.v. injection of a bolus dose (8 mg/kg) . The extrapolated saliva concentration at time 0 was 9.15 μg/ml. (From Inchiosa et al., 1981. Reproduced by permission of the New York Academy of Medicine.)

Equation (18.5) is the form for calculations concerning acids; in calculations for bases, the exponent pHs − pKa in the numerator and pHp − pKa in the denominator are reversed to pKa − pHs and pKa − pHp in the numerator and denominator, respectively.

The use of saliva measurements to estimate plasma concentrations is particularly convenient because the procedure is noninvasive and repeated samples may be obtained easily. Saliva measurements may be of value in humans for detecting and monitoring agents that have been absorbed systemically following their inhalation. Care may be necessary for some materials to ensure that the agent measured is in freshly secreted saliva and does not represent particles being raised from the respiratory tract.

18.6 EXCRETION

All secretions from the body represent potential routes for the elimination of toxicants following their systemic absorption (clearance of agents directly from the respiratory tract before systemic absorption was discussed above). The kidneys, liver, and biliary system are usually of prime importance in the elimination of toxicants from the body, with an important exception; substances with high vapor pressures at body temperature may be excreted primarily by the lungs (e.g., diethyl ether). Although toxicants may be secreted in sweat, tears, and milk (Stowe and Plaa, 1968), these usually represent minor routes of excretion.

18.6.1 Renal Excretion

The kidneys receive approximately 25% of the cardiac output, and of this volume, about 20% is filtered in the glomeruli. With values for the adult male, glomerular filtration is usually given the average value of 125 ml/min. Because the capillary pores in the glomeruli allow the passage of relatively large molecules, most toxicants that are not bound to plasma proteins would be readily filtered. Toxicant present in the unfiltered fraction of renal plasma may reach the tubules by passive diffusion or active transport. With respect to passive diffusion, consideration of the effective partition coefficient of the agent becomes of primary importance (discussed above). Organic acid and base secretory mechanisms exist in the kidney, which may actively contribute to the excretion of a toxicant. Because of the high-affinity characteristics of some active transport processes, even the fraction of compound bound to plasma protein becomes available for transport. The renal excretion of a toxicant may involve a combination of all three mechanisms (glomerular filtration, passive diffusion , and active transport). Agents filtered or secreted into the tubules may be passively reabsorbed into the plasma, based again on their lipophilic properties.

An attempt may be made to evaluate the contribution of the mechanisms above in the renal excretion of an agent. If renal clearance of the agent is distinctly below the glomerular filtration rate (usually determined by comparing the clearance of the toxicant to that of exogenously administered inulin or endogenous creatinine, both of which are only excreted by glomerular filtration), it would be assumed that the agent is substantially reabsorbed from the kidney tubules. A clearance rate greater than the glomerular filtration rate indicates a contribution of toxicant present in the unfiltered fraction of renal plasma flow (see, e.g. , Creasey, 1979, or Klaassen, 1980, for further discussion).

Piotrowski (1971) has suggested the use of the urine/plasma (U/P) concentration ratio as a rough indicator of renal excretory mechanisms. Because about 99% of the volume filtered in the glomerulus is reabsorbed from the tubules, a substance that is only freely filtered in the glomerulus and not reabsorbed becomes concentrated approximately 100-fold in urine (Figure 18.9). Inulin is a substance that would be expected to have a U/P ratio of 100. A U/P ratio greater than 100 suggests that secretory processes contribute to excretion; ratios less than 100 would be expected to reflect tubular reabsorption of the solute (Table 18.3). Foulkes (1977) discussed the qualifications in interpreting U/P ratios.

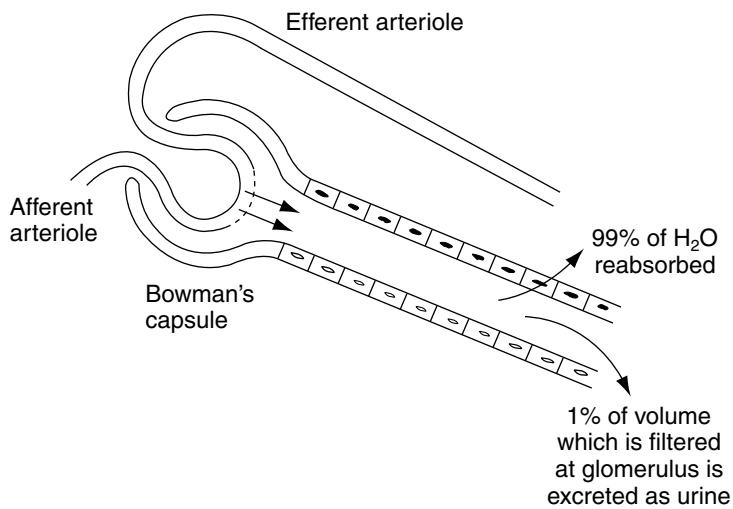


FIGURE 18.9 Schematic representation of the filtration of plasma at the kidney glomerulus and the extensive reabsorption of water from the kidney tubule. See text for discussion of urine/plasma ratios of toxicants.

TABLE 18.3 Urine-to-Plasma (U/P) Concentration Ratios for Selected Organic Compounds

Substance	U/P Ratio
Aliphatic hydrocarbons	0.07–0.09
Chlorinated hydrocarbons	0.1–1
Short-chain ketones	1–1.3
Short-chain aliphatic alcohols	1–1.3
Glycols, glycerol	3–5
Polyglycol ethers, mol wt 400–6,000	40–70
Dinitro-*o*-cresol	0.2
Trichloroacetic acid	3
Conjugated phenol	300

Source: From Foulkes, 1977. By permission of the American Physiological Society.

18.6.2 Contribution of Liver Metabolism to Excretion

The biotransformation of toxicants after inhalation exposure is also discussed in other chapters of this work. Most of this metabolism takes place in the liver, although the kidney, intestinal mucosa, and lung also possess significant capacity for xenobiotic metabolism. The discussion here will not address the consequences of xenobiotic metabolism in relation to detoxification or activation but rather the pharmacokinetic importance of liver metabolism.

A multiplicity of enzymes is present in the smooth endoplasmic reticulum (microsomal fraction) of the mammalian hepatocyte that functions to metabolize lipophilic agents to more polar substances, which are more readily excreted by the kidneys and in the bile. This metabolizing system is described by several synonyms: cytochrome P-450 oxidase, mixed function oxidase, mono oxygenase, and microsomal enzyme-oxidizing system (MEOS). Actually, the enzymic repertoire of these membranes includes oxidation, reduction, hydrolysis, and conjugation reactions of a wide diversity. It is beyond the scope of this discussion to detail these reactions. In general, the metabolic attack is classified in two steps: Phase I usually involves an oxidation, reduction, or hydrolytic reaction that increases water solubility and may also result in a group vulnerable for conjugation. Phase II involves synthetic reactions in which glucuronic acid, acetic acid, glycine, inorganic sulfate, glutathione, or ribose may be conjugated to the original agent or the product of a phase I reaction. The conjugated products are usually much more water soluble and less readily reabsorbed from the kidney tubules. Conjugated compounds may also be preferentially secreted into bile. Only the glucuronidation reactions are carried out by microsomal enzymes; the other conjugases are nonmicrosomal. Cytosolic enzymes and mitochondrial and plasma enzymes also may be important in the biotransformation of specific agents or classes of compounds. The reader is referred to comprehensive treatments of this subject (e.g., Lu and West, 1980; Nebert et al., 1981; Minchin and Boyd, 1983; Wilkinson, 2001).

Given the primary focus of this chapter, several comments are in order regarding the pharmacokinetic implications of xenobiotic metabolism. In most lipophilic agents, metabolism by enzymes in the liver and other tissues is probably the principal determinant of the half-life of the agent in the body. Another factor to be considered is the possible induction of liver microsomal enzyme capacity as a result of exposure to a number of agents (hundreds of agents have been recognized as microsomal enzyme inducers, e.g., ethyl alcohol, phenobarbital, 3,4-benzpyrene, DDT, polycyclic hydrocarbons in cigarette smoke, nicotine, etc.). Because microsomal induction often results in a general increase in the activity of many or all enzymes of the system, the usual consequence is a shortening of the

half-life of the agent that prompted the induction or of other agents which may be metabolized by microsomal enzymes. (It is not essential for a substance to be a substrate for microsomal enzymes to be an inducer.) Thus, in repeated or prolonged inhalation exposure, changes may occur in the pharmacokinetic behavior of an agent which may be attributable to induced metabolism.

18.6.3 Biliary Excretion

Excretion in bile may represent a primary or ancillary route of elimination for toxicants. Some compounds can be actively secreted by the liver into bile, resulting in high bile/plasma ratios. Specialized transport mechanisms are in the liver for secretion of organic acids, organic bases, and neutral organic compounds into bile. Apparently, the liver also has a mechanism for the secretion of heavy metals (lead is concentrated in bile) (Klaassen, 1976). Equilibrium binding of toxicants to plasma proteins does not limit their transport by the high-affinity biliary secretory mechanisms; this is similar to the characteristics of renal tubular secretion.

Factors that determine whether an agent will be excreted in bile are not well understood. One generalization, however, is that compounds with relatively low molecular weights (below 300) are preferentially excreted in urine, whereas larger compounds often demonstrate considerable biliary excretion. Data describing this phenomenon are presented in Figure 18.10. The threshold for substantial excretion of a compound into bile (10%) was found to be a molecular weight of approximately 325 in the rat (Hirom et al., 1972); the thresholds in the guinea pig and rabbit were 440 and 475, respectively. The metabolic conjugation of chemicals or their metabolites may be an important factor contributing to the extent of biliary secretion, because these reactions often

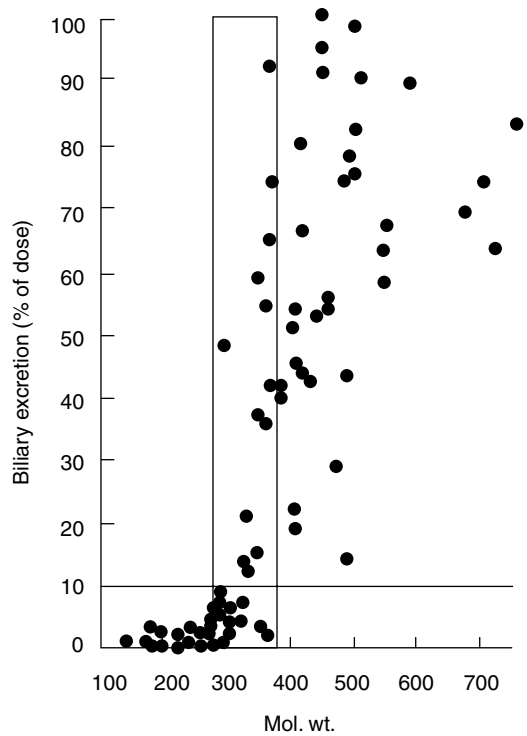


FIGURE 18.10 Relationship between extent of biliary excretion and molecular weight in the female rat. (From Hirom et al., 1972. Reproduced by permission of the Biochemical Society.)

result in an increase in molecular weight, which is sufficient to exceed the threshold for biliary excretion. The excretion of an agent or metabolite into the bile does not ensure its elimination from the body. If the compound possesses adequate lipid solubility at intestinal pH, it may be reabsorbed. Conjugated compounds may be hydrolyzed by the microflora of the intestine, resulting in the release of the original agent or metabolite, which may then be reabsorbed. These situations may result in an enterohepatic recirculation of the compound, prolonging its half-life in the body.

As an introduction to the next section, note that all the processes of elimination contribute to the plasma–disappearance curve following administration of (or inhalation exposure to) an agent. In the pharmacokinetic examples to be discussed, the plasma concentration–time course data refer to the original agent administered; the disappearance rates are the composite effect of all metabolic and excretory mechanisms.

18.7 KINETIC CONSIDERATIONS

18.7.1 Absorption

Excellent comprehensive treatments of pharmacokinetic principles are available (e.g., Gibaldi and Perrier, 1975; Wagner, 1975; Ritschel, 1976; Renwick, 1982; Schwinn and Shafer, 2000; Wilkinson, 2001). Relatively elementary models are presented in this section, and the discussion is developed toward practical applications to inhalation exposure. Nevertheless, the simple models discussed are frequently adequate for pharmacokinetic characterizations or first approximations.

Inhalation exposure can take the form of continuous exposure (24 h/d), as in a contaminated environment, part or all of a working day, or a single breath of acutely contaminated air (resulting from an accidental spill or release of a toxic gas).

It is convenient to consider the extreme situations in relation to the kinetics of absorption from the lung. The case in which a single breath (or a few breaths) represents the inhalation exposure can be simplified by assuming that the inhaled material is a solute that reaches the alveoli and is only cleared from the lung by its diffusion into the plasma. The kinetics of disappearance from the lung would be expected to typically take the form illustrated in Figure 18.11a. Figure 18.11b shows the same relationship on a semilogarithmic plot; as can be seen, this substance shows first-order kinetics of absorption, i.e., a constant percentage of the substance is absorbed per unit of time. In this theoretical example, the half-time ($t_{1/2}$) for absorption is 5 min, i.e., 50% is absorbed in the first 5 min and 50% of the remaining amount is absorbed in each subsequent 5-min period. Burton and Schanker (1974a) have presented data showing the first-order rates of absorption of several antibiotics from the lung (Figure 18.12). It was noted above that these authors have concluded that the relative lipid solubility of the compounds is the primary factor determining rate of absorption from the lung.

In the examples above (Figures 18.11 and 18.12), there was a brief period of deposition of an agent into the lungs followed by a first-order clearance of the substance; there was a constant percentage absorbed per unit of time, which represented decreasing absolute amounts absorbed per unit of time. Continuous inhalation exposure, which constantly renews the amount of agent present in the lung, is probably best viewed as an example of "zero-order" rate of administration; in such a case it would be expected that a constant absolute amount of the agent would be absorbed per unit of time. (Constant intravenous infusion is the classical example of zero-order administration.)

This chapter is primarily concerned with the pharmacokinetics of inhaled toxicants. In such situations a much higher concentration of the toxicant is usually in the inhaled atmosphere than in the plasma and tissues of the exposed individual. As long as a substantial concentration gradient exists across the alveolar membrane, the contribution of back-diffusion of the toxicant would be negligible (Goldstein et al., 1974). The kinetic examples presented in this section are based on this assumption.

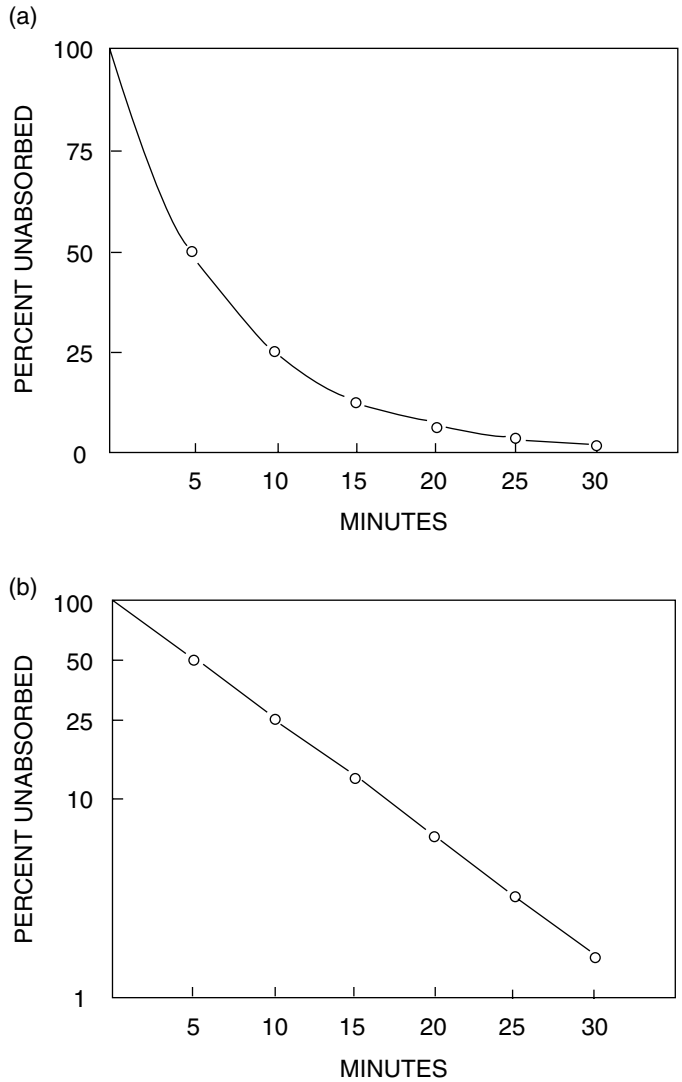


FIGURE 18.11 Theoretical example of first-order kinetics of absorption of a substance from the lung. (a) Rectilinear coordinates. (b) Semilogarithmic plot of the same data.

18.7.2 Distribution and Elimination

The apparent volume of distribution of a particular agent can be best estimated from plasma concentration data after an intravenous injection. Any other route of administration may not result in complete absorption.

18.7.2.1 One-Compartment Open Model

This discussion first considers the situation in which the body behaves as a single compartment in regard to the distribution of an agent (Figure 18.13). This is termed a one-compartment open model. The designation "open" refers to the fact that the system has input and output, i.e., it is not a closed system. A plasma concentration–time course following an intravenous bolus injection into

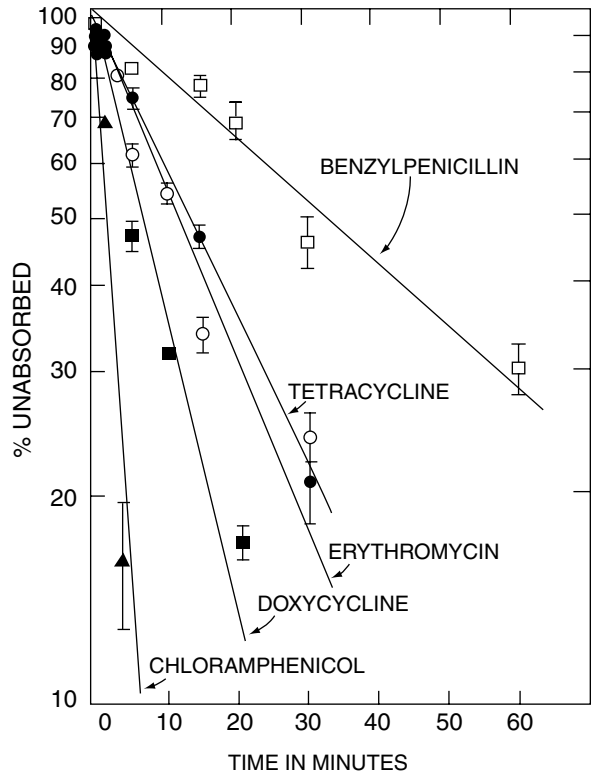


FIGURE 18.12 Semilogarithmic plots of the rates of absorption of several antibiotics from the rat lung. The compounds were administered intratracheally to anesthetized rats through a tight-fitting tracheal cannula. (From Burton and Schanker, 1974a, used with permission.)

a one-compartment system is illustrated schematically in Figure 18.14. This example shows that the plasma concentration decreases rapidly during the early minutes after the injection and by 30 min has entered a stage of first-order elimination from the plasma. Although this system may exhibit one-compartment behavior, an agent rarely distributes in only one anatomical compartment, i.e., the vascular space. The essential feature of the one-compartment model is that the agent diffuses so rapidly into its ultimate volume of distribution (which may include extracellular and cellular space as well) that the system virtually behaves as though it were a single compartment (see, e.g., Ritschel, 1976). Another criterion for a one-compartment model is that it also behaves as a virtual single compartment as the agent is progressively eliminated from the plasma (by kidney excretion, liver metabolism, etc.). That is, there should be essentially instantaneous reequilibration of the remaining agent among the anatomical compartments in which it is distributed.

18.7.2.1.1 Apparent Volume of Distribution (Bolus Intravenous Injection)

In a one-compartment system, the apparent volume of distribution of an agent can often be adequately estimated from plasma concentration data, as presented in Figure 18.14. Extrapolation of the first-order curve to the ordinate (time 0) provides an estimate of the theoretical plasma concentration that would have resulted if the agent was immediately distributed in its ultimate volume of distribution. In the example of Figure 18.14, 180 mg of the compound was injected intravenously as a bolus at time 0. The intercept on the ordinate obtained from the back-extrapolation of the

FIGURE 18.13 Diagram of one-compartment open model.

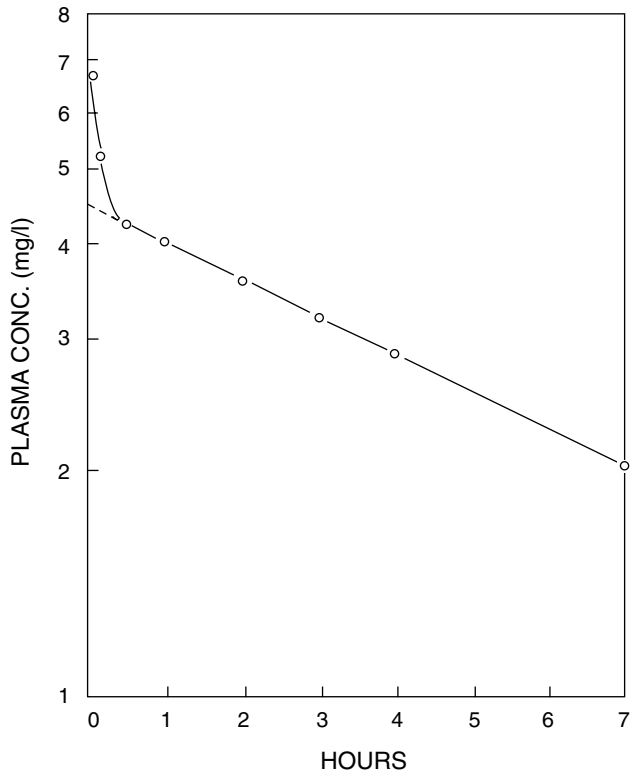


FIGURE 18.14 Theoretical example of a plasma concentration–time course following an i.v. bolus injection in a virtual one-compartment open system (see text for explanation); 180 mg of the compound was injected at time 0.

first-order disappearance rate is approximately 4.5 mg/l. The apparent volume of distribution is calculated from the following equation.

$$V_d = \frac{\text{Dose}}{\text{C}(0)}$$
$$V_d = \frac{180 \text{ mg}}{4.5 \text{ mg/l}} = 40.0 \text{ l} \tag{18.6}$$

In Figure 18.5, it was noted that total body water represents about 60% of body weight. For a 70-kg man, this would amount to 42 l. Thus, if the experiment illustrated in Figure 18.14 represented the data from a 70-kg individual, the result would approximate the one expected for an agent that is

uniformly distributed in body water. Similarly, there are drugs that do not enter cells because of their extremely low lipid solubility (e.g., neuromuscular blocking agents) and are restricted to a volume representing approximately 20% of body weight (the sum of plasma and interstitial fluid volumes, as depicted in Figure 18.5). Although many examples may be cited for agents that yield estimates of V_d which suggest distribution into certain anatomical compartments, numerous exceptions exist. The most notable examples occur when an agent is sequestered in body fat or muscle, resulting in a proportionately low plasma concentration and a large volume from the calculation in Equation (18.6). For this reason volumes of distribution are always considered apparent volumes. This does not influence the use of such volumes in the kinetic considerations to be discussed, however.

It is usually preferable to express the apparent V_d as 1/kg or ml/kg rather than the volume based on total body weight.

18.7.2.1.2 Elimination Rate Constant

The data in Figure 18.14 also allow an estimation of the half-time of elimination of the agent. Inspection reveals that the plasma concentration decreases by 50% over a period of 6 h. The elimination rate constant, k_{el}, can then be determined from Equation (18.7).

$$K_{el} = \frac{0.693}{t_{1/2}}$$
$$K_{el} = \frac{0.693}{6\ \text{h}} = 0.1155/\text{h, or } 11.55\%/\text{h} \tag{18.7}$$

where 0.693 is a constant derived from the first-order equation. In the case where $t_{1/2}$ is known, $k = \ln 2/t_{1/2} = 0.693/t_{1/2}$; see, e.g., Goldstein et al. (1974) for the derivation.

The common form of the equation for determining plasma concentration at a time after administration of a bolus of drug in a one-compartment model is presented in Equation (18.8):

$$\text{Plasma concentration at time "}t\text{"} = \frac{\text{dose}}{V_d} \times e^{-k_{el}t} \tag{18.8}$$

18.7.2.1.3 Apparent V_d Obtained from Area Under the Curve (AUC)

In many instances it is more precise to determine V_d utilizing the entire area under the plasma concentration–time curve (AUC (0–∞)). This approach usually requires the analysis of plasma concentration over a prolonged period.

The AUC is determined from the plasma concentration–time course plotted on rectilinear graph paper. The data from Figure 18.14 are presented with linear coordinates in Figure 18.15 and extended for a total of 25 h of observation. Any appropriate method may be used for determining this area. A common approach is to divide the area into a series of narrow, vertical trapezoids, determine their individual areas, and sum them. Only the estimation of the area from the last data point to ∞ remains. This is determined by dividing the value for the last plasma concentration data point (C_{tz}) by the rate of elimination (k_{el}). The solution of C_{tz}/k_{el} yields the AUC (tz – ∞); this is added to the AUC (0 – tz) determined from the trapezoid calculations to obtain the total area (AUC (0 – ∞)) The apparent V_d is then obtained from the relationship:

$$V_d = \frac{\text{dose}}{\text{AUC}\ (0-\infty) \cdot K_{el}} \tag{18.9}$$

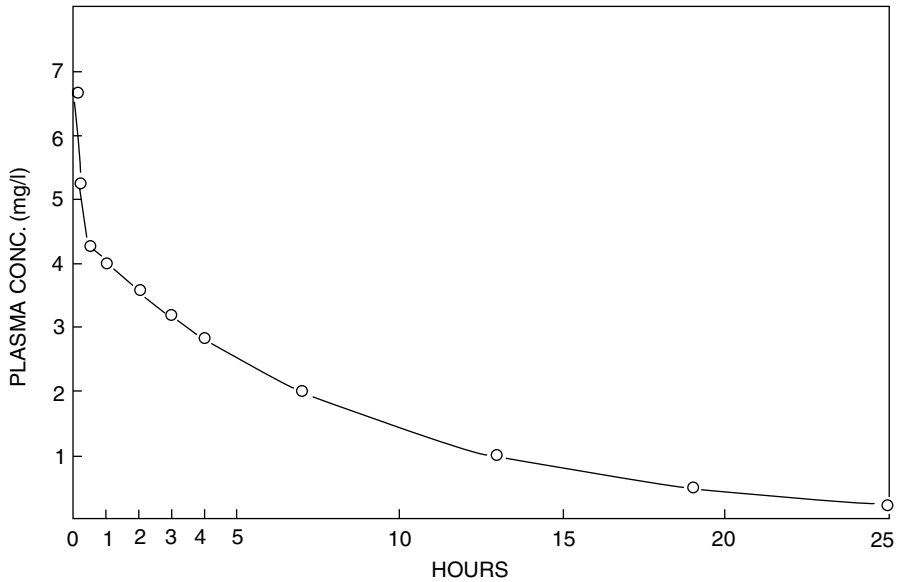


FIGURE 18.15 Rectilinear plot of the same data as Figure 18.14 with extension of the curve to 25 h. Example of the type of plot that is used for determination of the area under the plasma concentration–time curve (AUC).

18.7.2.1.4 Clearance (Cl)

When data have been obtained which allow estimates of V_d and k_{el} (as from Fig. 18.14), total body clearance for an agent may be calculated from the following relationship:

$$\mathrm{Cl} = V_d k_{el} \tag{18.10}$$

For the example illustrated in Figure 18.14:

$$\mathrm{Cl} = 40.0\,\mathrm{l} \times 0.1155/\mathrm{h} = 4.62\ \mathrm{l/h}$$

Total body clearance may also be calculated from Equation (18.11).

$$\mathrm{Cl} = \frac{\text{dose}}{\mathrm{AUC}\ (0-\infty)} \tag{18.11}$$

18.7.2.1.5 Analogy of Prolonged Inhalation Exposure to Intravenous Infusion

The pharmacokinetics of constant infusion provides a useful example for the discussion of continuous inhalation exposure. Constant intravenous infusion is an example of "zero-order" rate of administration, i.e., a constant absolute amount of an agent is administered per unit of time. This may be compared with continuous inhalation exposure in the situation where a constant fraction of the inhaled agent is absorbed per unit of time. The absorption may be from a combination of both respiratory and enteral routes.

The data obtained from the plasma concentration–time course after a bolus intravenous injection of an agent may provide useful information for anticipating the pharmacokinetic behavior of the agent with constant intravenous infusion or inhalation exposure. If an agent shows a first-order elimination

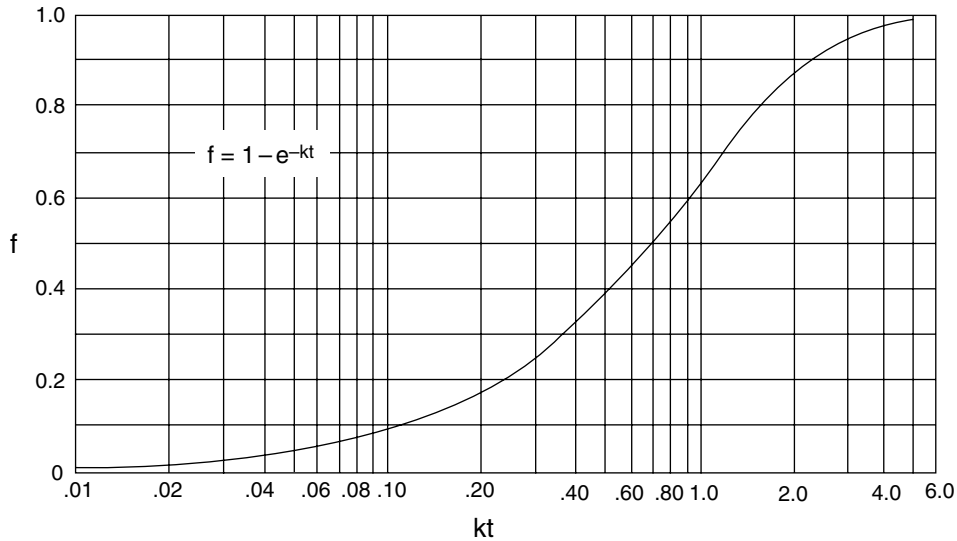


FIGURE 18.16 Generalized relationship for the changes in plasma concentration of an agent in relation to the period of administration. The relationship applies to any system in which there is zero-order rate of administration of the agent and first-order rate of elimination; it describes the accumulation of agent as the substance is continuously administered and also can be used to determine the time course of the shift from one steady state to another, if the rate of administration of the agent is changed. The curve is obtained from the equation $f = 1 - e^{-kt}$, where f = fractional attainment of steady state or change from one steady state to another; k is the elimination rate constant (designated k_{el} in the text); and t is time, with the same units as k. Real time may be obtained by dividing a particular value of kt from the graph by k. (From Goldstein et al., 1974, used with permission.)

rate, as illustrated in Figure 18.14, it can be predicted that the plasma concentration (or other measure of body burden) will eventually plateau. This information would be of particular value, for example, in considering the consequence of occupational inhalation exposure. The knowledge that a steady-state plasma plateau will result, regardless of the length of exposure, is of obvious importance.

The time of exposure required to attain the plasma steady-state concentration (C^{SS}), or any fraction of C^{SS}, can be determined from the relationship plotted in Figure 18.16. The only value needed for these determinations, k_{el}, is obtained from the plasma concentration–time course data (as illustrated in Figure 18.14). According to the relationship presented in Figure 18.16, 50% of the plateau is reached in one half-time, 90% in approximately 3.3 half-times, 97% in approximately 5 half-times, and 99% in approximately 7 half-times. Using these values, the plasma concentration–time course for zero-order rate of administration and first-order rate of elimination (generalized for fraction of plasma C^{SS} and for periods of exposure as multiples of $t_{1/2}$) is shown in Figure 18.17a and 18.17b.

These curves illustrate several important points. Figure 18.17a shows that the curve for the disappearance of the agent after the termination of infusion (or inhalation exposure, as the case may be) is the reverse of the curve for accumulation. The area under the curve from the termination of exposure until ∞ is the same as the observed area from the start of exposure until attainment of a virtual plateau. This proves to be a convenient situation for estimating the AUC for a given period of exposure. The AUC is simply a rectangle with the dimensions of the plasma concentration at the plateau and the total time of exposure. This fact will be applied to determine the "dose" from inhalation exposure. Figure 18.17b illustrates the same relationship as Figure 18.17a, but with a fraction of plasma C^{SS} on a log scale. A feature of this plot is that the disappearance curve following the termination of exposure is a straight line for a one-compartment open model and allows convenient estimation of k_{el}.

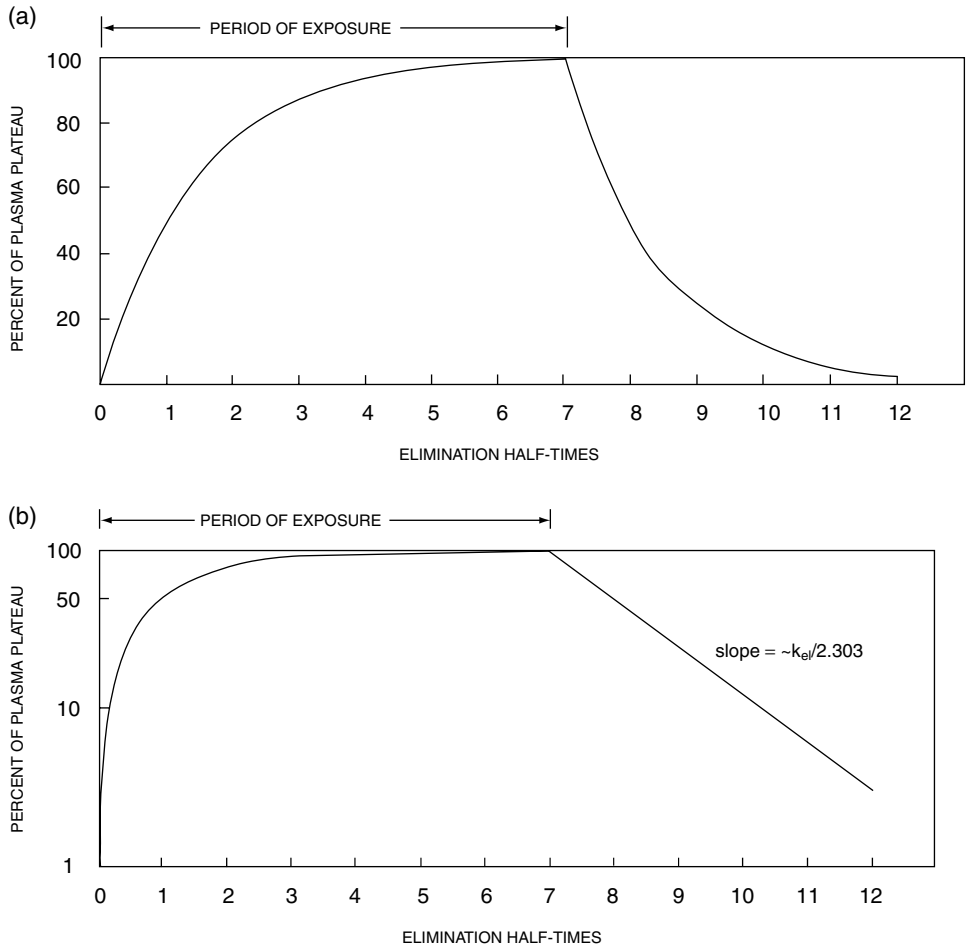


FIGURE 18.17 Theoretical curves for the change in plasma concentration (expressed as a percent of the steady-state plateau concentration) as a function of time of administration of agent (expressed as multiples of the elimination half-time, $t_{1/2}$) for a one-compartment open system. (a) Rectilinear plot. (b) Semilogarithmic plot of the relationship. The relationship applies to any system represented by zero-order administration (continuous i.v., inhalation, continuous transdermal, etc.) and first-order elimination.

18.7.2.1.6 Determination of "Dose" from an Inhalation Exposure

The pharmacokinetic principles presented may be applied toward estimations of a value that is usually difficult to obtain, i.e., the absolute "dose" received from an inhalation exposure. The *Ct* product, as discussed elsewhere, is an attempt to quantify inhalation exposure. However, because this measure only includes the concentration of agent in the inhaled atmosphere, *C*, and the time of exposure, *t*, it provides no quantitative information on the amount of agent absorbed systemically.

Several approaches may be used to determine the dose (either total dose or dose per unit of time) from inhalation exposure when certain plasma concentration data are also obtained.

18.7.2.1.6.1 Option 1

If, for example, plasma concentration–time data are available from an intravenous bolus injection that allows determination of the V_d and k_{el} for the agent, and a plasma concentration is determined at a time when a steady-state plateau would be expected (it is adequate to use five to seven elimination half-times for this measurement), then:

$$\text{Dose per unit of time} = V_d k_{el} C^{SS} \tag{18.12}$$

An example may be given with the data from Figure 18.14 in which the $V_d = 40.0$ l and the $k_{el} = 0.1155$/h. If plasma concentration after prolonged inhalation exposure is found to plateau at 10 μg/l, then:

$$\text{Dose} = 40.0\ \text{l} \times 0.1155/\text{h} \times 10\ \mu\text{g/l} = 46.2\ \mu\text{g/h}$$

Note that this discussion is based on the premise that the k_{el} is not influenced by dose over the range that would be encountered from inhalation exposure. This can be verified by giving a succession of intravenous bolus injections that span the expected or observed range of plasma concentrations. Of course, the same species should be used for both the intravenous bolus experiments and the inhalation exposures. Factors such as age, sex, etc., should be standardized. Animals may be prepared with indwelling venous catheters for the administration of agent and sampling of plasma (Upton, 1975). In some cases it may be possible to include a small group of animals that receive both intravenous injection and inhalation exposure.

18.7.2.1.6.2 Option 2

Because plasma C^{SS} is directly proportional to the dose of the agent (by rearranging Equation 18.12) the dose in an inhalation exposure may be determined by comparison with the C^{SS} that results from continuous intravenous infusion. If it is certain that the periods of intravenous infusion and inhalation exposure are adequate for plasma concentrations to plateau, the two periods do not have to be identical. Dose is then calculated from the relationship in Equation (18.13).

$$\frac{\text{Dose (by inhalation)}}{\text{Dose (i.v. infusion)}} = \frac{\text{C}^{\text{ss}}\ \text{(from inhalation)}}{\text{C}^{\text{ss}}\ \text{(i.v. infusion)}} \tag{18.13}$$

The dose by inhalation is expressed in the same units as the intravenous infusion dose.

18.7.2.1.6.3 Option 3

With agents that have relatively long half-times of elimination, it may not be practical to carry out inhalation exposure or intravenous infusion for the time required to reach plasma C^{SS}. In such cases it may be possible to compare the AUC resulting from a period of inhalation exposure with the AUC for an identical period of intravenous infusion. Then:

$$\frac{\text{Dose (by inhalation)}}{\text{Dose (i.v. infusion)}} = \frac{\text{AUC } (0-\infty)\ \text{(from inhalation)}}{\text{AUC } (0-\infty)\ \text{(i.v. infusion)}} \tag{18.14}$$

Alternately, for some applications, it might be adequate to simply compare the plasma concentrations obtained from inhalation exposure and intravenous infusion for identical periods; Equation (18.13) is then used, substituting plasma concentrations for plasma C^{SS} values.

18.7.2.2 Two-Compartment Open Model

The basic pharmacokinetic principles discussed for a one-compartment system must be modified when the diffusion of an agent into or out of certain peripheral compartments is not instantaneous. A two-compartment system is illustrated diagrammatically in Figure 18.18. The discussion of this model will be limited to the points necessary for applications to inhalation exposure; the reader is directed to some of the references noted previously for more comprehensive treatments of two-compartment and multicompartment models.

The equation for determining the plasma concentration at a time after administration of a bolus of drug in a two-compartment model is presented in Equation (18.15):

$$\text{Plasma concentration at time "}t\text{"} = A\,e^{-\alpha t} + B\,e^{-\beta t} \tag{18.15}$$

where A and B represent the extrapolated intercepts on the ordinate of the two exponential terms in the equation; α is a first-order distribution rate constant, and β is the first-order elimination rate constant (these constants are presented diagrammatically in Figure 18.19).

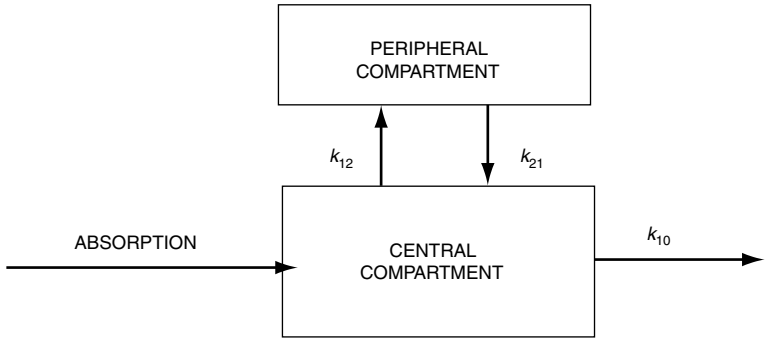


FIGURE 18.18 Diagram of two-compartment open model.

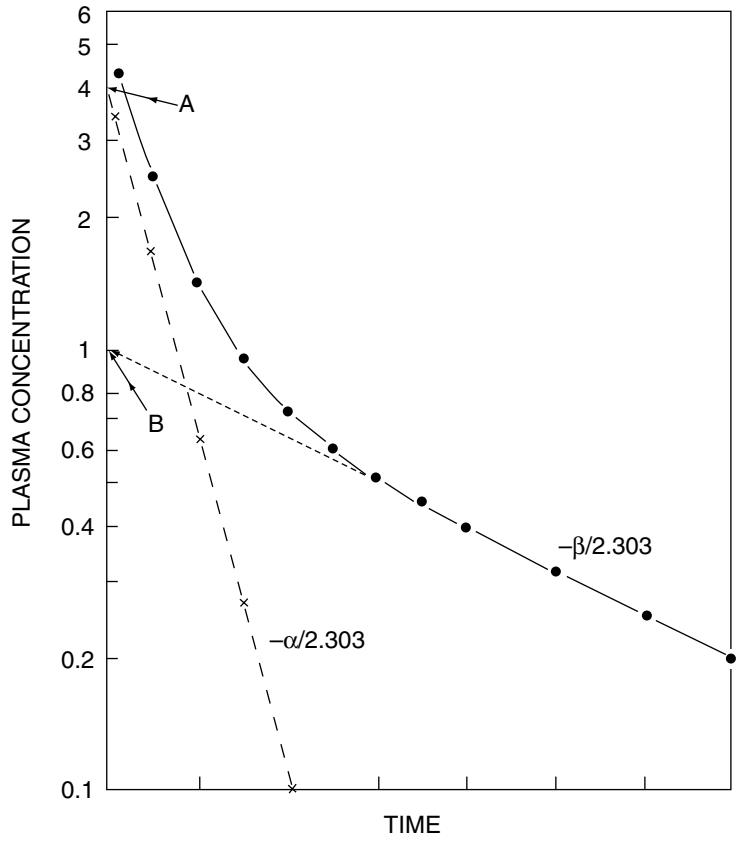


FIGURE 18.19 Semilogarithmic plot of the plasma concentration–time course of an agent after i.v. injection: an example of a two-compartment open system. Plasma concentration and time are in relative units. Plasma concentrations are indicated by filled circles. The values obtained directly or indirectly from these data (A, B, α, β) may be used to calculate the rate constants k_{12}, k_{21}, and k_{10} (see text for details).

18.7.2.2.1 Apparent Volume of Distribution (Bolus Intravenous Injection)

Figure 18.19 presents a plasma concentration–time course following a bolus intravenous injection that would be typical for a two-compartment system. This figure demonstrates the biexponential disappearance of the agent from plasma, which is characteristic of a two-compartment model.

The analysis of the biexponential plot in Figure 18.19 allows the illustration of several pharmacokinetic principles. (This analysis is not essential for the discussion of certain practical applications to inhalation exposure which will be continued below.) The rate constants for a two-compartment system (Figure 18.18), k_{12}, k_{21}, and k_{10} can be determined by graphical (or linear regression) analysis of data as presented in Figure 18.19 (Renwick, 1982, has provided well-illustrated examples of such analyses). The terminal disappearance phase, designated β in Figure 18.19, represents the overall rate at which the agent is eliminated from the body. It is not the same as the potential rate of elimination from the central compartment; i.e., it is not equivalent to k_{10}. The β constant is a composite rate, the result of the interaction of k_{12}, k_{21}, and k_{10}.

Back-extrapolation of the terminal phase to time 0 yields the intercept B; if the value of B is subtracted from each of the data points, a curve is constructed that has the rate constant designated α in Figure 18.19 (method of residuals) (see, e.g., Harvey, 1975). Although α is largely an expression of the distributive phase following an intravenous bolus injection, it is also a composite rate constant, as noted above for β. Back-extrapolation of this early phase yields the intercept A. The rate constants k_{12}, k_{21}, and k_{10} can now be calculated using the values determined from the actual data, as follows (see Wagner, 1975, for derivation of these formulas):

$$k_{21} = \frac{\alpha B + \beta A}{A + B} \tag{18.16}$$

$$k_{10} = \frac{\alpha\beta}{k_{21}} \tag{18.17}$$

$$k_{12} = \alpha + \beta - k_{21} - k_{10} \tag{18.18}$$

The apparent volume of distribution of an agent in a two-compartment open model is obtained using the formula:

$$V_d = \frac{\text{Dose}}{\text{AUC}\ (0-\infty)\cdot\beta} \tag{18.19}$$

18.7.2.2.2 Elimination Rate Constant

The overall elimination rate constant (not k_{10}) is β and is calculated from the $t_{1/2}$ of the terminal phase of the plasma concentration time course:

$$k_{el}\ (\text{or}\ \beta) = \frac{0.693}{t_{1/2}\ \text{for terminal phase}} \tag{18.20}$$

18.7.2.2.3 Clearance

Total body clearance in the two-compartment model is determined from:

$$\text{Cl} = \frac{\text{Dose}}{\text{AUC}\ (0-\infty)} \tag{18.21}$$

18.7.2.2.4 Determination of "Dose" from Inhalation Exposure in the Two-Compartment Open Model

The analogy between prolonged inhalation exposure and constant intravenous infusion which was developed above for the one-compartment model applies also for the two-compartment system (Section 18.7.2.1.5). The plasma concentration time course for zero-order administration and first-order elimination in the two-compartment open model is shown in Figure 18.20. The options listed above for estimating the inhalation dose in the one-compartment model may be utilized for the same purpose in the two-compartment model. Equations (18.12), (18.13), and (18.14) and the accompanying discussions apply; the value for clearance (Cl) is more properly substituted for $V_d K_{el}$ in Equation (18.12).

18.7.2.3 Three-Compartment Open Model

It is well recognized that the distribution and elimination kinetics of many chemicals can only be adequately described by representation as three pharmacokinetic compartments (Figure 18.21).

18.7.2.3.1 Determination of Pharmacokinetic Constants for the Three-Compartment Model

Nonlinear regression analysis of plasma concentrations after elimination of many drugs has demonstrated that three exponential terms are necessary to satisfactorily predict plasma concentrations after drug administration. The general formula for this relationship is as follows:

$$\text{Plasma concentration at time "}t\text{"} = A\,e^{-\alpha t} + B\,e^{-\beta t} + C\,e^{-\gamma t} \tag{18.22}$$

where, A, B, and C represent the extrapolated intercepts on the ordinate of the three exponential terms in the equation; α and β are first-order distribution rate constants, and γ is the first-order elimination rate constant. This relationship is illustrated in Figure 18.22. Derivation of the pharmacokinetic constants (volumes of the compartments and intercompartmental diffusion constants, as illustrated in Figure 18.21) from plasma–time concentration data requires specialized software that is supported by a Fortran compiler. One version of this software is NONMEM[1], but other programs are available. Discussion of the derivation of the constants is beyond the scope of this presentation. An extremely useful program is available (STANPUMP[2]) that allows one to simulate predicted plasma concentrations following administration of a number of anesthetic drugs. The program can simulate drugs that demonstrate either two or three pharmacokinetic compartments. The software includes pharmacokinetic parameters for approximately 20 important anesthetic drugs, but it also allows the option to load an external set of pharmacokinetic constants for other chemicals or drugs.

The volumes of the pharmacokinetic compartments and the intercompartmental and elimination rate constants can differ greatly among drugs. As examples, compartment volumes for four opioid analgesic drugs are presented in Figure 18.23. Although presented schematically, the relative areas in the drawing are proportional to the compartmental volumes of the actual drugs.

An important advance in appreciation of the implication of multicompartmental pharmacokinetics on expected plasma concentrations resulted from the work of Hughes et al. (1992). This work introduced the concept of the "context-sensitive half-time." The context-sensitive half-time is not the half-time of elimination of a drug from the body (the $t_{1/2}$) but is simply the time required for the plasma concentration to decrease by 50% following discontinuation of an infusion; the premise is that the drug is infused into the central compartment (V_1). The "context" referred to in this principle is the duration of the infusion, i.e., the total body burden of the drug at the time when the infusion is discontinued. With all drugs that demonstrate multicompartmental pharmacokinetics, the rate of decline of the plasma concentration is prolonged progressively as the period of infusion is increased. However,

[1] GloboMax, a Division of ICON plc, 7250 Parkway Drive, Suite 430, Hanover, MD 21076.

[2] Address requests for information to Steven L. Shafer, M.D., Palo Alto Veterans Affairs Medical Center, Department of Anesthesia, Stanford University, Stanford California. E-mail: steven.shafer@stanford.edu.

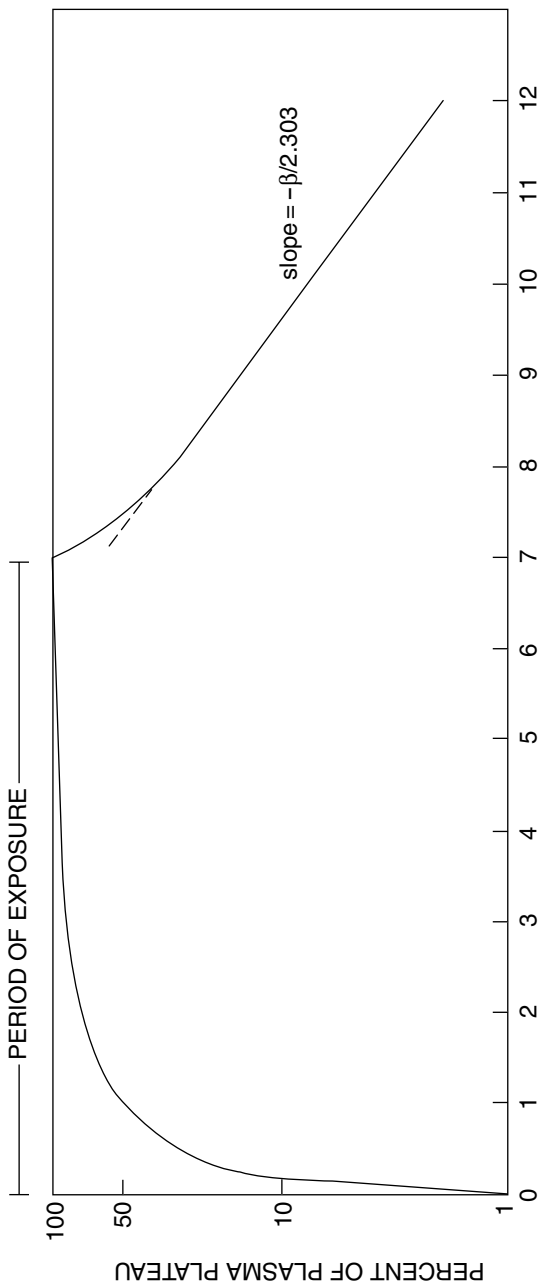


FIGURE 18.20 Semilogarithmic plot of the theoretical curve for the change in plasma concentration (expressed as a percent of the steady-state plateau concentration) as a function of time of administration of agent (expressed as multiples of the elimination half- time) for a two-compartment open system. (See the legend for Figure 18.17 for additional comments.)

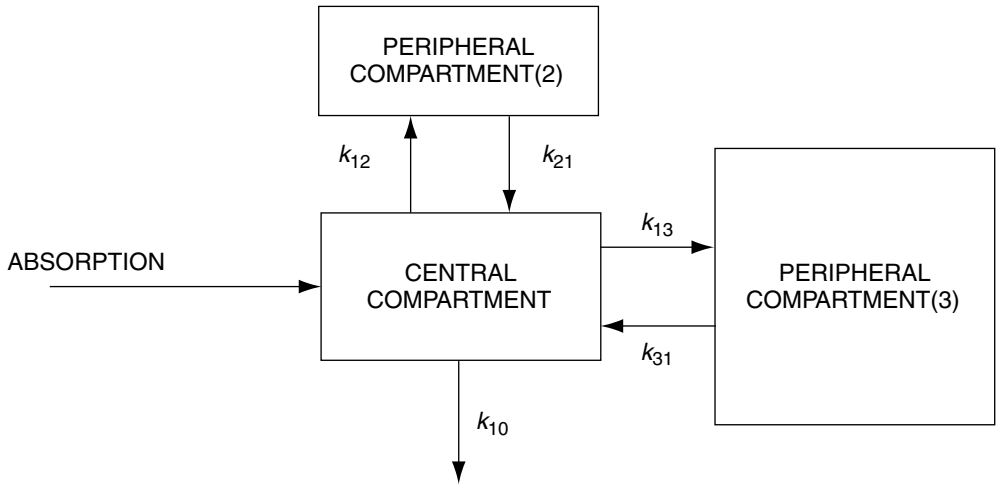


FIGURE 18.21 Diagram of three-compartment open model.

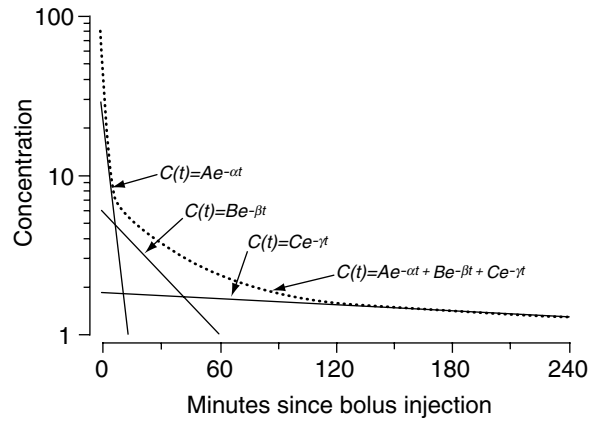


FIGURE 18.22 Representation of distribution and elimination constants for a three-compartment pharmacokinetic model. (From Schwinn and Shafer, 2000. Reproduced by permission of Churchill Livingstone.)

the extent of the prolongation (the context-sensitive half-time) varies considerably among drugs. One of the advantages of a drug that has a small influence of context (duration of infusion) is that recovery from side effects of a drug would be rapid (for example, the respiratory depressant effect of an opioid analgesic drug at the end of surgery) when its therapeutic effect is no longer needed.

The context-sensitive half-times for a number of drugs are illustrated schematically in Figure 18.24. It can be seen that fentanyl shows a large influence of the duration of infusion on the time for plasma concentration to decrease by 50% (from plasma concentration C_{p100} to C_{p50}). In comparison, sufentanil shows a much smaller influence of infusion duration on the rate of decrease in plasma concentration upon termination of the infusion. The reason for the differences in the behavior of these two drugs has to do with the net effects of their relative rates of clearance from the central compartment ($V_1 \times k_{10}$), and the rate of replenishment of the central compartment from the peripheral compartments ($V_2 \times k_{21}$ and $V_3 \times k_{31}$). Thus, the plasma concentration falls relatively rapidly with sufentanil because clearance of sulfentanil from the central compartment is somewhat faster, but, more importantly, the peripheral compartments give up sufentanil much more slowly back to the central compartment than with fentanyl.

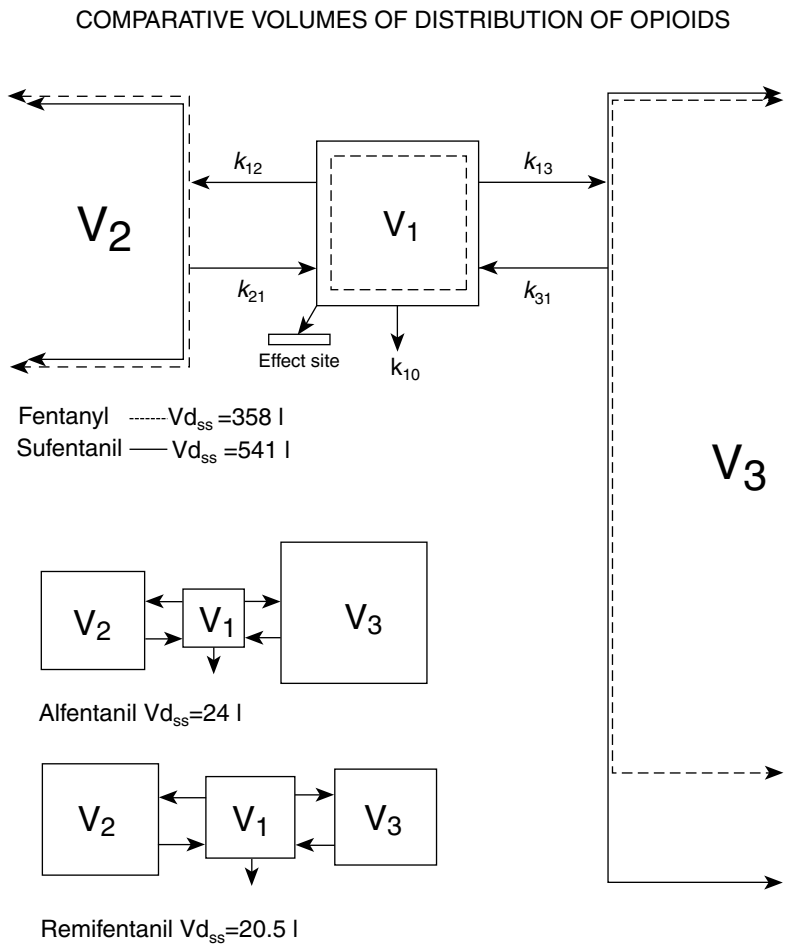


FIGURE 18.23 Schematic comparison of the compartmental volume sizes of four opioid analgesic drugs, drawn to approximate scale. Vd_{ss}, steady-state volume of distribution, i.e., total volume of distribution. Data for Vd_{ss} values are from Shafer and Varvel, 1991, and Minto et al., 1997.

It can be seen that the context-sensitive half-time is a manifestation of intercompartmental redistribution of drug when the input of drug into the central compartment is stopped. In fentanyl and sufentanil, this is important from a pharmacodynamic (or toxicodynamic) standpoint, because the central compartment is in communication with the cerebrospinal fluid, which is the target site for the action of these drugs.

The comparison between fentanyl and sufentanil demonstrates a paradox, which helps to emphasize the implications of the context-sensitive half-time. Although plasma concentrations of sufentanil fall more rapidly than of fentanyl when drug infusion into the central compartment is halted, the rate of drug *elimination* from the body is greater for fentanyl. This is because only the central compartment is sampled with the analysis of plasma; peripheral, and thereby total body, amounts of sufentanil decrease more slowly than for fentanyl.

The primary concept that, for multicompartmental models, the decrease in drug concentration in the central compartment usually occurs at a faster rate than the ultimate first-order rate of elimination of drug from the body (the true $t_{1/2}$ of elimination) was introduced in the discussion of two-compartment models. Note that Figure 18.20 shows that the initial rate of decrease in plasma concentration after the termination of inhalation exposure was more rapid than the final rate of drug elimination from the body.

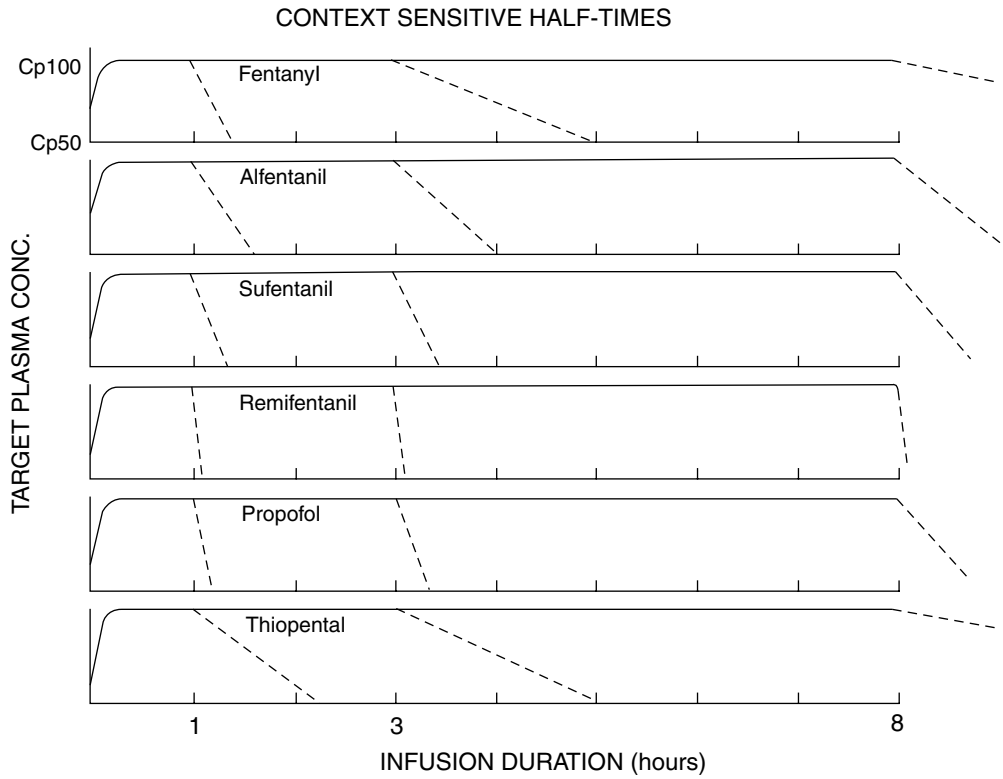


FIGURE 18.24 Schematic representation of the context-sensitive half-times of several drugs, following the termination of infusion after 1, 3, and 8 h. See the text for explanation. Data from Hughes et al., 1992, and Westmoreland et al., 1993.

18.7.2.3.2 Clearance

Total body clearance in the three-compartment model may be determined as for the one- and two-compartment models (Equation 18.23):

$$\text{Cl} = \frac{\text{Dose}}{\text{AUC}\ (0-\infty)} \tag{18.23}$$

When pharmacokinetic data are available, total body clearance can also be obtained from the product of the volume of the central compartment and the rate constant k_{10} ($V_1 \times k_{10}$).

18.7.2.3.3 Determination of "Dose" from Inhalation Exposure in the Three-Compartment Open Model

The analogies between prolonged inhalation exposure and constant intravenous infusion that were developed above for the one- and two-compartment models apply as well to the three-compartment model.

The options listed above for estimating the inhalation dose in the simpler models can be applied for the same purpose in the three-compartment model. Equations (18.13) and (18.14) apply without modification. In Equation (18.12) it is more appropriate to substitute total body clearance (Cl) for $V_d k_{el}$; the equation would then be as follows:

$$\text{Dose per unit of time} = \text{Cl} \times C^{SS}$$

18.8 SUMMARY

Factors involved in the absorption of agents from inhalation exposure, and in their distribution and elimination from the body, have been discussed in this chapter. Basic principles of pharmacokinetics have been reviewed and related to inhalation exposure. A special emphasis has been placed on the application of these principles to the determination of the systemic dose received by inhalation.

ACKNOWLEDGMENTS

The author received support from the Department of Anesthesiology, New York Medical College, for much of the original work contained in this chapter. I am also grateful to Mario E. Inchiosa, Ph.D., for helpful discussions on certain theoretical considerations and to Andrew S. Inchiosa for help in the preparation of the manuscript.

REFERENCES

Beattie, C., History and principles of anesthesiology, in *Goodman and Gilman's The Pharmacological Basis of Therapeutics,* 10th ed., Hardman, J.G., Limbird, L.E., and Goodman, A.G., Eds., McGraw-Hill Medical Publishing, New York, 2001, pp. 321–335.

Bowman, W.C. and Rand, M.J., *Textbook of Pharmacology,* 2nd ed., Blackwell Scientific Publishers, Oxford, 1980.

Breckenridge, R., Bending, M.R., and Brunner, G., Impact of drug monoxygenases in clinical pharmacology, In *Microsomes and Drug Oxidations,* Ullrich, V., Roots, I., Hildebrandt, A., Estabrook, R.W., and Conney, A.H., Eds., Pergamon Press, New York, 1977, pp. 385–394.

Burton, J.A. and Schanker, L.S., Absorption of antibiotics from the rat lung, *Proc. Soc. Exp. Biol. Med.,* 145, 752–756, 1974a.

Burton, J.A. and Schanker, L.S., Absorption of cortico-steroids from the rat lung, *Steroids,* 23, 617–624, 1974b.

Casarett, L.J., The vital sacs: alveolar clearance mechanisms in inhalation toxicology, in *Essays in Toxicology,* vol. 3, Hayes, W.J., Jr., Ed., Academic Press, New York, 1972, pp. 1–36.

Creasey, W.A., *Drug Disposition in Humans,* Oxford University Press, New York, 1979.

Foulkes, E.C., Mechanisms of renal excretion of environmental agents, in *Handbook of Physiology,* Section 9. *Reactions to Environmental Agents,* Lee, D.H.K., Ed., American Physiological Society, Bethesda, MD, 1977, pp. 495–502.

Gibaldi, M. and Perrier, D., *Pharmacokinetics,* Marcel Dekker, New York, 1975.

Goldstein, A., Aronow, L., and Kaplan, S.M., *Principles of Drug Action,* 2nd ed., John Wiley and Sons, New York, 1974.

Guyton, A.C., *Textbook of Medical Physiology,* 6th ed., W.B. Saunders Co., Philadelphia, 1981.

Harvey, S.C., Drug absorption, action, and disposition, in *Remington's Pharmaceutical Sciences,* 15th ed., Mack Publishing Co., Easton, PA, 1975, pp. 669–711.

Hill, D.W., *Physics Applied to Anesthesia,* 4th ed., Butterworths, Boston, 1980.

Hirom, P.C., Millburn, P., Smith, R.L., and Williams, R.T., Species variations in the threshold molecular-weight factor for the biliary excretion of organic anions, *Biochem. J.,* 129, 1071–1077, 1972.

Hogben, C.A.M., Tocco, D.J., Brodie, B.B., and Schanker, L.S., On the mechanism of intestinal absorption of drugs, *J. Pharmacol. Exp. Ther.,* 125, 275–282, 1959.

Hughes, M.A., Glass, P.S.A., and Jacobs, J.R., Context-sensitive half-time in multicompartment pharmacokinetic models for intravenous anesthetic drugs. *Anesthesiology,* 76, 334–341, 1992.

Inchiosa, M.A., Jr., Phang, W., and Bizzarri, D.V., Investigation of a new approach for predicting the anesthetic induction dose of thiopental, *Bull. N.Y. Acad. Med.*, 57, 349–354, 1981.

Kilburn, K.H., Clearance mechanisms in the respiratory tract, in *Handbook of Physiology,* Section 9: *Reactions to Environmental Agents,* Lee, D.H.K., Ed., American Physiological Society, Bethesda, MD, 1977, pp. 243–262.

Klaassen, C.D., Biliary excretion of metals, *Drug. Metab. Rev.,* 5, 165–196, 1976.

Klaassen, C.D., Absorption, distribution, and excretion of toxicants, in *Casarett and Doull's*, *Toxicology,* 2nd ed., Doull, J., Klaassen, C.D. and Amdur, MO, Eds. Macmillan Publishing Co., New York, 1980, pp. 28–55.

Larson, C.P., Eger, E.I., and Severinghaus, J.W., Ostwald solubility coefficients for anesthetic gases in various fluids and tissues, *Anesthesiology,* 23, 686–689, 1962.

Lu, A.Y.H. and West, S.B., Multiplicity of mammalian microsomal P-450, *Pharmacol. Rev.,* 31, 277–295, 1980.

Mason, R.J., Dobbs, L.G., Greenleaf, R.D., and Williams, M.C., Alveolar type II cells, *Fed. Proc.,* 36, 2697–2702, 1977.

Matin, S.B., Wan, S.H., and Karaim, J.J., Pharmacokinetics of tolbutamide: prediction by concentration in saliva, *Clin. Pharmacol. Ther.,* 16, 1052–1058, 1974.

Menzel, D.B. and McClellan, R.O., Toxic responses of the respiratory system, in *Casarett and Doull's Toxicology,* 2nd ed., Doull, J., Klaassen, C.D., and Amdur, M.O., Eds., Macmillan Publishing Co., New York, 1980, pp. 246–274.

Minchin, R.F. and Boyd, M.R., Localization of metabolic activation and deactivation systems in the lung: Significance to the pulmonary toxicity of xenobiotics, *Annu. Rev. Pharmacol. Toxicol.,* 23, 217–238, 1983.

Minto, C.F., Schnider, T.W., Egan, T.D., Youngs, E., Lemmens, H.J.M., Gambus, P.L., Billard, V., Hoke, J.F., Moore, K.H.P., Hermann, D.J., Muir, K.T., Mandema, J.W., and Shafer, S.L., Influence of age and gender on the pharmacokinetics and pharmacodynamics of remifentanil. I. Model development, *Anesthesiology,* 86, 10–23, 1997.

Nebert, D.W., Eisen, H.J., Negishi, M., Lang, M.A., Hjelmeland, L.M., and Okey, A.B., Genetic mechanisms controlling the induction of polysubstrate mono oxygenase (P-450) activities, *Annu. Rev. Pharmacol. Toxicol.,* 21, 431–462, 1981.

Newton, R., Broughton, L.J., Lind, M.J., Morrison, P.J., Rogers, H.J., and Bradbrook, I.D., Plasma and salivary pharmacokinetics of caffeine in man, *Eur. J. Clin. Pharmacol.,* 21, 45–52, 1981.

Pappenheimer, J.R., Renkin, E.M., and Borrero, L.M., Filtration, diffusion and molecular sieving through peripheral capillary membranes. A contribution to the pore theory of capillary permeability, *Am. J. Physiol.,* 167, 13–46, 1951.

Parsons, W.D. and Neims, A.H., Effect of smoking on caffeine clearance, *Clin. Pharmacol. Ther.,* 24, 40–45, 1978.

Piotrowski, J., *The Application of Metabolic and Excretion Kinetics to Problems of Industrial Toxicology*, National Library of Medicine, National Institutes of Health, Washington, DC, 1971.

Poland, R.E. and Rubin, R.T., Saliva cortisol levels following dexamethasone administration in endogenously depressed patients, *Life Sci.,* 30, 177–181, 1982.

Renkin, E., Transport of large molecules across capillary walls, *The Physiologist,* 7, 13–28, 1964.

Renwick, A.G., Pharmacokinetics in toxicology, in *Principles and Methods of Toxicology,* Hayes, A.W., Ed., Raven Press, New York, 1982, pp. 659–710.

Ritschel, W.A., *Handbook* of *Basic Pharmacokinetics,* Drug Intelligence Publications, Hamilton, IL 1976.

Schwinn, D.A. and Shafer, S.L., Basic principles of pharmacology related to anesthesia, in *Anesthesia,* 5th ed., Miller, R.D., Ed., Churchill Livingstone, Philadelphia, 2000, pp. 15–47.

Shafer, S.L. and Varvel, J.R., Pharmacokinetics, pharmacodynamics, and rational opioid selection, *Anesthesiology,* 74, 53–63, 1991.

Singer, S.J. and Nicolson, G.L., The fluid mosaic model of the structure of cell membranes, *Science,* 175, 720–731, 1972.

Stowe, C.M. and Plaa, G.L., Extrarenal excretion of drugs and chemicals, *Annu. Rev. Pharmacol.,* 8, 337–356, 1968.

Tanford, C., Swanson, S.A., and Shore, W.S., Hydrogen ion equilibria of bovine serum albumin, *J. Am. Chem. Soc.,* 77, 6414–6421, 1955.

Taylor, A.E. and Gaar , K.A., Jr., Estimation of equivalent pore radii of pulmonary capillary and alveolar membranes, *Am. J. Physiol.,* 218, 1133–1140, 1970.

Upton, R.A., Simple and reliable method for serial sampling of blood from rats, *J. Pharm. Sci.,* 64, 112–114, 1975.

von Hayek, H., *The Human Lung,* Hafner, New York, 1960.

Wagner, J.G., *Fundamentals of Clinical Pharmacokinetics,* Drug Intelligence Publications, Hamilton, IL, 1975.

Weibel, E.R., *Morphometry of the Human Lung,* Academic Press, New York, 1963.

Westmoreland, C.L., Hoke, J.F., Sebel, P.S., Hug, C.C., Jr., and Muir, K.T., Pharmacokinetics of remifentanil (GI87084B) and its major metabolite (GI90291) in patients undergoing elective inpatient surgery, *Anesthesiology,* 79, 893–903, 1993.

Wilkinson, G.R., Pharmacokinetics, in *Goodman and Gilman's The Pharmacological Basis of Therapeutics,* 10th ed., Hardman, J.G., Limbird, L.E., and Goodman, A.G., Eds., McGraw-Hill Medical Publishing, New York, 2001, pp. 3–29.

19 Mechanistic Determinants and Modeling of the Inhalation Pharmacokinetics of Volatile Organic Chemicals

Andy Nong and Kannan Krishnan

CONTENTS

19.1 INTRODUCTION

Pharmacokinetics involves the study of the rate and extent of absorption, distribution, metabolism and excretion of chemicals in biota (Wagner 1975). Inhalation pharmacokinetics relates to the characterization of the time-course of concentration in tissues, blood or excreta of inhaled chemicals and their metabolites. The pharmacokinetics or the processes of pulmonary uptake, tissue distribution, metabolism, urinary and fecal excretion as well as exhalation together determine the amount of inhaled chemicals available for interaction in the tissues. The adverse response in biota is more closely related to internal dose (e.g., concentration of the toxic chemical in the target tissue) rather than inhalation exposure concentration. In fact, dose-response relationships that often appear complex at the exposure dose level become simpler when expressed on the basis of internal dose of the chemical (Gehring et al. 1978, Andersen et al. 1987, Clewell et al. 2002). Figure 19.1 shows an example of refinement of the dose-response relationship for a volatile organic chemical (VOC) resulting from the use of internal dose. Panel A depicts the relationship between the exposure concentration and the observed cancer response for a VOC, which is neither clearly linear nor nonlinear. However, once the exposure concentration is related to the internal dose (e.g., area under the parent chemical concentration vs time in target tissue), the non-linearity due to pharmacokinetics is clearly evident (Panel B). Consequently, the relationship between the internal dose measures and adverse responses can be established more confidently (Panel C). One of the major advantages of constructing dose-response relationships on the basis of internal

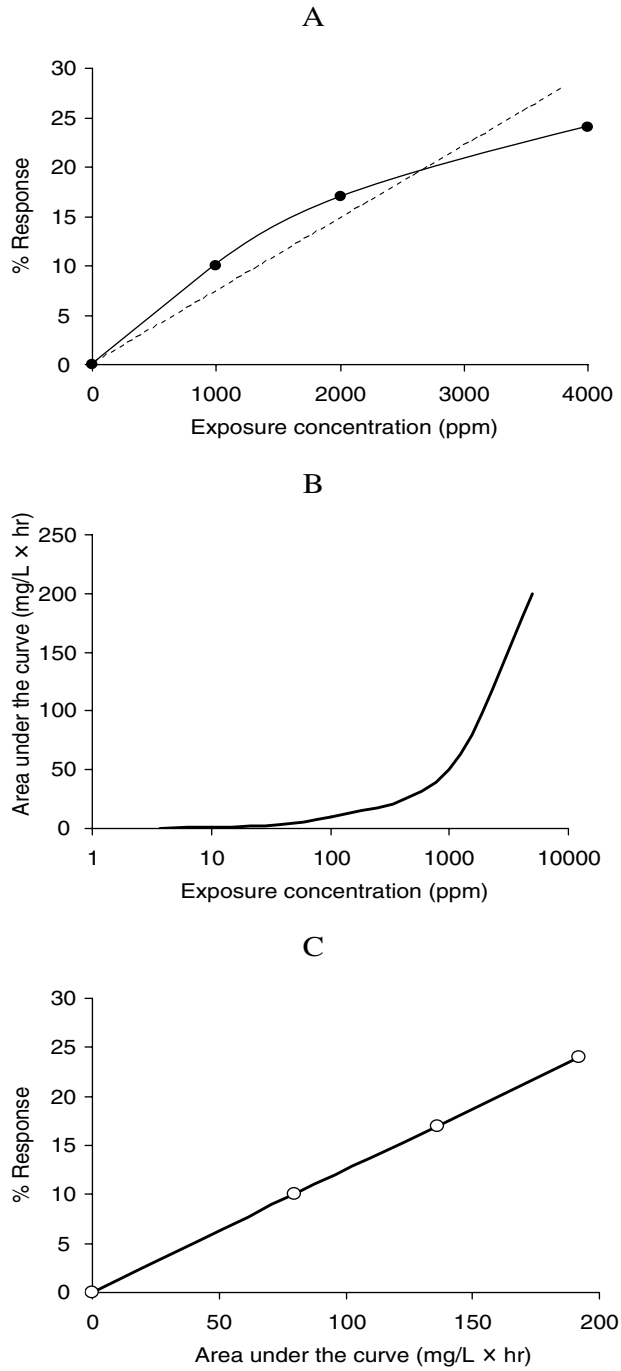


FIGURE 19.1 Relationship between the exposure concentration and adverse response for a hypothetical volatile organic chemical (Panel A). Panels B and C represent the relationship between exposure concentration and internal dose (area under the parent chemical concentration vs time curve in target tissue) as well as between internal dose and response.

or delivered doses is that it provides a scientifically-sound basis for conducting extrapolations and for comparing responses observed in various species, exposure concentrations and scenarios (Clewell and Andersen 1994, Medinsky 1995). Physiologically-based pharmacokinetic models are increasingly being used to simulate the internal dose of inhaled VOCs on the basis of mechanistic determinants of uptake and disposition (Ramsey and Andersen 1984, Andersen 1995, 2003, Filser et al. 1995, Leung and Paustenbach 1995, Andersen and Dennison 2001, Krishnan and Andersen 2001).

The internal dose, a key determinant of adverse tissue responses induced by systemically-acting inhaled chemicals, is the net result of pharmacokinetics or the processes of absorption, distribution, metabolism and excretion. This chapter provides an overview of the mechanistic determinants influencing the internal dose and pharmacokinetics of inhaled chemicals, as well as scientifically-sound models for their integration to provide predictions of the pharmacokinetics of inhaled VOCs.

19.2 MECHANISTIC DETERMINANTS OF INHALATION PHARMACOKINETICS OF VOCs

The kinetics of uptake, distribution, metabolism and excretion are determined by a number of factors. These factors are either related to characteristics of the exposed organism (e.g., breathing rate, cardiac output, tissue volumes, tissue blood flow rates, tissue content of metabolizing enzymes) or of the inhaled VOC (e.g., blood: air partition coefficient, tissue: blood partition coefficients, metabolic rate constants). The relative importance of these determinants as they contribute to an increase or a decrease in the internal dose of VOCs, may vary according to the chemical and exposure situations. The general features of these determinants of inhalation pharmacokinetics as well as common methods of assessing them for VOCs are provided in the following paragraphs.

19.2.1 Physiological Determinants

Physiological determinants of the inhalation pharmacokinetics of VOCs include: alveolar ventilation rate, cardiac output, tissue blood flow rates, and tissue volumes (Fiserova-Bergerova 1983). The ventilation rate not only determines the rate of uptake of VOCs but also the exhalation rate. The rate of exhalation or pulmonary clearance is determined by the breathing rate as well as the blood:air partition coefficients of VOCs. The ventilation rate in mammals, in general, scales to body surface or a fractional power of body weight ($BW^{0.7}$) (Fiserova-Bergerova 1995). The cardiac output or the systemic circulation rate determines the speed at which absorbed chemicals are transported to tissues. The blood circulation and therefore the delivery of chemicals to tissues is faster in smaller animals (e.g., mice) than in larger animals (e.g., humans).

Tissue volumes along with their composition determine the extent to which chemicals are diluted within the organism (i.e., volume of distribution). The larger the tissue volumes, the larger the volumes for distribution of chemicals. In addition, the greater the content of lipids in tissues, the larger the volume of distribution, resulting in lower blood concentrations. The tissue blood flow rates, on the other hand, influence the rate at which a chemical in systemic circulation is delivered to a tissue. In any given species, organs such as liver and kidney have the greatest blood perfusion rate compared to the muscle tissue (Table 19.1). Tissues with greater perfusion rates may attain the maximal level of accumulation for a given exposure situation (i.e., steady-state condition) quickly, depending upon their volumes and solubility characteristics.

Some of the physiological parameters that determine the inhalation pharmacokinetics of VOCs can be measured directly in the animal species of interest, whereas others may have to be inferred on the basis of body weight. For example, breathing rates can be measured with the use of a spirometer, plethysmograph, pneumotachograph, hotwire anemometer, or nonbreathing valves (ICRP 1975, Mauderly 1990). Cardiac output has been determined from dye dilution curves using oximeters (Delp et al. 1991). Compilations of physiological determinants and their relationship to body weight are available for a number of species including humans (e.g., Caster et al. 1956, Arms and Travis 1988, Davies and Morris 1993, Brown et al. 1997).

Table 19.1 Reference Physiological Parameters for Mice, Rats, and Humans

Physiological parameters	Mouse	Rat	Human
Cardiac output (L/hr)	1.02	4.98	372
Alveolar ventilation (L/hr)	1.5	7.02	300
Tissue perfusions (L/hr)			
Liver	0.258	1.248	97
Fat	0.09	0.45	18.6
Organs	0.522	2.538	164
Muscle	0.156	0.75	93
Tissue volumes (L)			
Liver	0.0014	0.01	2
Fat	0.0025	0.0175	13
Organs	0.0013	0.0125	4
Muscle	0.0175	0.1875	43

Based on Arms and Travis (1988) using typical body weights of 0.025, 0.25, and 70 kg for mouse, rat, and human, respectively.

19.2.2 Physicochemical Determinants

The physicochemical determinants such as the blood:air partition coefficients and tissue:blood partition coefficients influence the rate of respiratory uptake and distribution of VOCs. Partition coefficients, in general terms, refer to the relative distributions of chemicals between two phases at equilibrium.

The blood:air partition coefficient is a critical determinant of the pulmonary uptake of VOCs. The absorption of VOCs with a relatively high blood:air partition coefficient is limited by alveolar ventilation rate, whereas that of VOCs with low blood:air partition coefficients is limited by cardiac output (reviewed in Fiserova-Bergerova 1983). The blood:air partition coefficient, as described by Henry's law, is the ratio of solubility of a VOC in blood and air. The blood:air partition coefficients of VOCs have most commonly been determined *in vitro* by vial equilibration technique (Sato and Nakajima 1979b, Fiserova-Bergova and Diaz 1986, Johanson and Dynesius 1988, Gargas et al. 1989). Table 19.2 presents the blood:air partition coefficients as well as n-octanol:air partition coefficients for a number of VOCs. The variability of blood:air partition coefficients among VOCs is not explained solely by the differences in octanol or lipid solubility. Rather, the solubility in water should be accounted for, and in some cases binding to blood proteins needs to be accounted for additionally (Poulin and Krishnan 1996b).

Poulin and Krishnan (1996b) developed the following equation for predicting blood:air partition coefficients (P_b) of VOCs that do not bind significantly to blood proteins:

$$P_b = \left[P_{o:w} P_{w:a} F_{nl}\right] + \left[P_{w:a} F_w\right] \quad (1)$$

where $P_{o:w}$ = n-octanol:water or oil:water partition coefficient, $P_{w:a}$ = water:air partition coefficient, F_{nl} = neutral lipid-equivalent components in blood, calculated as the sum of neutral lipid content plus 30% of the phospholipid content (expressed as volume fraction), and F_w = water-equivalent components in blood, calculated as the sum of water content plus 70% of phospholipid content (expressed as volume fraction).

In the above equation, the first term represents the partitioning of VOCs between the blood lipids and air whereas the second term represents the partitioning between blood aqueous component and air. P_b of VOCs, according to the above approach, can be calculated with the knowledge of blood composition data, $P_{o:w}$ and $P_{w:a}$. The data on lipid and water content of rat and human blood are available in the literature and so are the numerical values of $P_{o:w}$ and $P_{w:a}$ at 37°C for several VOCs (Poulin and

Table 19.2 Blood: Air and n-Octanol:Air Partition Coefficients of Some VOCs

	Partition Coefficient	
Chemical	**Blood: Air**	**Octanol: Air**
Vinyl chloride	1.16 ±0.08	24.4 ±3.7
Vinyl bromide	2.27 ±0.16	56 ±1.5
Methyl chloride	2.48 ±0.23	8.57 ±0.22
Carbon tetrachloride	2.73 ±0.23	374 ±11
Chloroform	6.85 ±0.51	402 ±12
Trichloroethylene	8.11 ±0.17	553 ±46
Benzene	8.19 ±0.10	465 ±5
Tetrachloroethylene	10.3 ±1.10	2134 ±159
Toluene	15.6 ±1.70	1471 ±69
m-Xylene	32.5 ±1.60	3245 ±116
Styrene	51.9 ±2.00	5465 ±219

Source: Gargas et al. (1989) and Sato and Nakjima (1979a, 1979b).

Krishnan 1995, 1996a,b). For lipophilic low molecular weight VOCs, binding to blood proteins should be accounted for, particularly for predicting P_b in rodents (Poulin and Krishnan 1996b).

Tissue:blood partition coefficients represent another set of physicochemical characteristics that influence the pharmacokinetics of inhaled VOCs. These determinants represent the equilibrium ratio of concentration of a VOC between the tissues and blood. The tissue:blood partition coefficients of adipose tissue and muscle are more important than those for smaller tissues, as critical determinants of the blood concentration of lipophilic VOCs. The larger the tissue:blood partition coefficients, the greater their affinity for tissues. This, of course, is further influenced by tissue volumes. For example, the volume of adipose tissue represents about 7% and 19%, respectively, in adult rats and humans (Table 19–1).

The tissue:blood partition coefficients for VOCs have been determined by dividing the tissue:air partition coefficients by the blood:air partition coefficient, determined by vial equilibration method (Sato and Nakajima 1979b, Fiserova-Bergova and Diaz 1986, Gargas et al. 1989, Krishnan and Andersen 2001). Tissue:blood, tissue:air and blood:air partition coefficients can also be determined using empirical or semi-empirical methods based on molecular structure information or physicochemical characteristics (Poulin and Krishnan 1998, 1999, Basak et al. 2002, Payne and Kenny 2002, Beliveau et al. 2003, 2005).

19.2.3 Biochemical Determinants

Biochemical processes such as biotransformation, macromolecular binding, and renal clearance are key determinants of the inhalation pharmacokinetics of VOCs. Metabolism and renal clearance contribute to decrease in the concentration of a VOC in systemic circulation whereas tissue binding may contribute to increased retention in the body. These biochemical processes will likely be the rate-limiting step of the kinetics of poorly cleared VOCs. Using time-course data obtained under *in vivo* or *in vitro* conditions, the rates of biochemical processes can be estimated (Dedrick et al. 1972, 1973, Sato and Nakajima 1979a, Reitz et al. 1996, DeJongh and Blaauber 1997, Lipscomb et al. 1998). A strategy that has often been used, involves analysis of data obtained under *in vivo* conditions where pharmacokinetic behavior of a VOC is related to one or two dominant factors and thereby derive estimates of these parameters.

Closed chamber or gas uptake method has commonly been used for estimation of rates of metabolism of VOCs. This method uses a desiccator-type chamber with recirculating atmosphere for exposing groups of animals to VOCs (Andersen et al. 1980; Filser and Bolt 1981, Dennison et al. 2005).

Periodic monitoring of the chamber concentration of VOCs during experimental conditions is performed both in the absence and in the presence of animals, for various starting concentrations. The net difference between these two sets of data is attributed to uptake and metabolism by the animals. Since pulmonary uptake is accounted for by the PBPK models, an optimization of metabolic parameters (the only unknown) is done until adequate fit of model simulations to experimental data on chamber concentrations is obtained (Andersen et al. 1980; Filser and Bolt 1981, Gargas et al. 1986). This method has been used successfully to obtain metabolic rate constants of VOCs that are biotransformed by a single first-order process, a saturable process, or a combination of both (Andersen et al. 1987, Gargas et al. 1990, Krishnan et al. 1992). Any chemical or scenario exhibiting a spontaneous loss in excess of ~2% per hour may not be conducive to the use of gas uptake data for the determination of metabolic rate constants. It is also important to monitor the oxygen concentration, humidity level, chamber pressure as well as breathing rates during gas uptake studies (Johanson and Filser 1992, Dallas et al. 1994, Dennison et al. 2005). Since the gas uptake studies involve whole-body exposures, adsorption to fur and dermal uptake may occur. To determine the contribution of adsorption to fur to VOC uptake during whole-body exposures, the exposed animals should be placed in a clean chamber (following the termination of gas uptake exposures), and the time course of the appearance of the chemical characterized (Gargas and Andersen 1989). If the role of dermal absorption is important, then it should be additionally taken into account in the PBPK model (Krishnan and Andersen 2001).

The gas uptake method is not suitable for estimation of metabolic rates of organic chemicals that (i) have low vapor pressure, (ii) exhibit high chamber loss rates, or (iii) possess high blood: air partition coefficient (>60). In such cases, the metabolic rate constants have been assessed using an alternative method, namely, the exhaled breath chamber method (Gargas and Andersen 1989). In this approach, the animals are administered the chemical, and then the time course of chemical in exhaled air is monitored. The data are then analyzed with a PBPK model that contains all parameters except the metabolic constants. By statistical optimization of model fit to experimental data, the metabolism rate constants are determined (Gargas and Andersen 1989, Gargas 1990). The pharmacokinetic data collected following conventional, constant whole body exposures may also be analyzed with a PBPK model to estimate the metabolic constants (e.g., Tardif et al. 1993).

19.3 MODELING OF INHALATION PHARMACOKINETICS OF VOCs

The physiological, physicochemical and biochemical determinants, discussed in the previous section, together influence the absorption, distribution, metabolism and elimination of VOCs. The internal dose of VOCs (parent chemical or metabolite concentration in target tissues) is determined essentially by the interplay among these factors. Mathematical models that integrate information on these mechanistic determinants are particularly useful not only in facilitating a better understanding of the inhalation pharmacokinetics of VOCs but also in conducting extrapolations essential for risk assessment purposes (interspecies, high to low exposure concentration and exposure scenario extrapolations). In this regard, physiologically-based pharmacokinetic (PBPK) models are important.

For simulating the inhalation pharmacokinetics of VOCs, PBPK models describe the organism as a set of compartments, each of which is characterized physically, physiologically, and biochemically (Figure 19.2, Ramsey and Andersen 1984). Each of the compartments represents an individual tissue or a group of tissues with similar characteristics. For example, fat depots such as perirenal, epididymal, and omental fat are frequently grouped and represented as a single "fat" compartment (Figure 19.2). If necessary, a "fat" compartment may be subdivided into two or more groups according to the perfusion rates (e.g., inner and subcutaneous adipose tissues). Another example of lumped compartment is the "richly perfused tissues" which consists of adrenal glands, kidney, thyroid, brain, lung, heart, testis, and hepatoportal system. Tissues with poor blood perfusion characteristics (muscle, skin)—that have fairly similar concentrations vs time profiles—are grouped as "poorly perfused tissues". Since the skeletal and

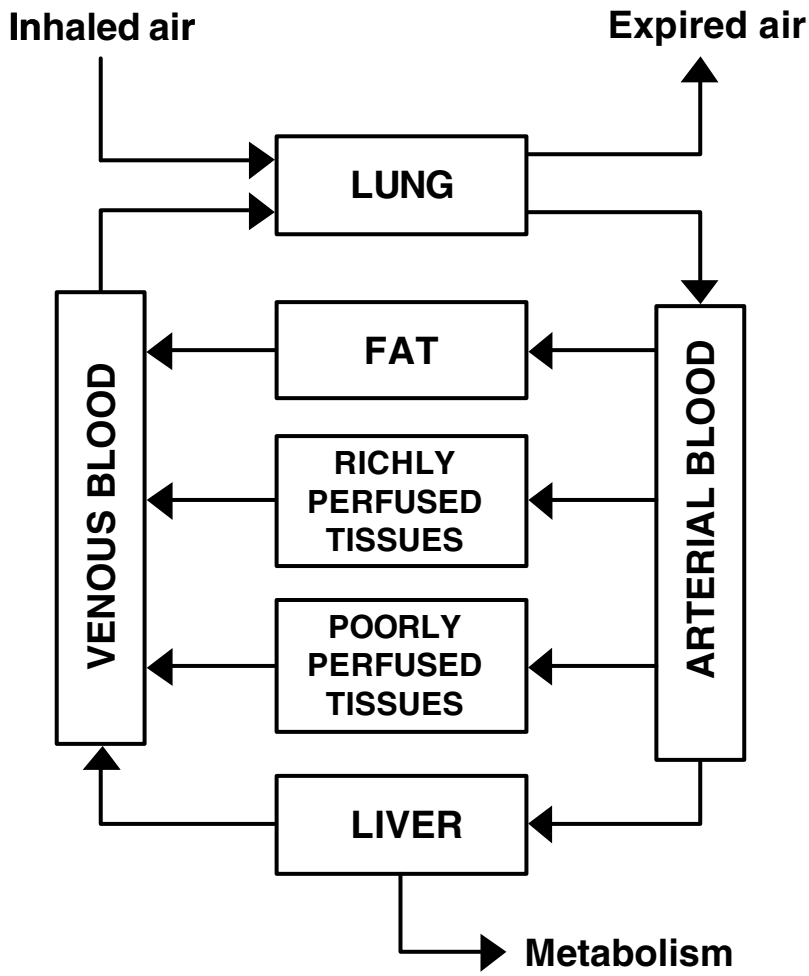


FIGURE 19.2 Conceptual representation of a physiologically-based pharmacokinetic model for inhaled VOC. Based on Ramsey and Andersen (1984).

structural components of the body have only negligible perfusion and do not contribute significantly to the pharmacokinetic behavior of VOCs, they have not been routinely included in the PBPK models (Krishnan and Andersen 2001).

In the PBPK model illustrated in Figure 19.2, the input results from the inspiration of VOC in the inhaled air at a flow rate equal to the alveolar ventilation rate. The VOC in alveolar air is assumed to be in equilibrium with arterial blood which flows at a rate equal to the cardiac output, and distributes the chemical to liver, fat, richly perfused tissue, and poorly perfused tissue compartments as a function of tissue-specific perfusion rates. The tissue uptake of VOCs appears to be frequently limited by tissue perfusion and therefore has been described according to Fick's law (Table 19.3). Chemicals in venous effluents of the various tissue compartments contribute to the mixed venous concentration that returns to the lung compartment at a flow rate equal to cardiac output (Ramsey and Andersen 1984). Examples of equations constituting PBPK models of VOCs are listed in Table 19.3.

With knowledge of the various input parameters (exposure concentration and duration, physiological parameters, partition coefficients, and metabolic constants), the equations of PBPK models can be solved to provide predictions of the kinetic behavior of chemicals in the test species. Simulation requires solving the set of mass balance differential equations simultaneously by

Table 19.3 Examples of Equations Used in Inhalation PBPK Models for VOCs[a]

Compartment	Equations
Arterial blood	$Ca = \frac{Qc \times Cv + Qp \times Cinh}{Qc + \frac{Qp}{Pb}}$
Liver	$\frac{dC_l}{dt} = \frac{\left(Ql \times (Ca - Cv_l) - \frac{V_{max} \times Cv_l}{Km + Cv_l}\right)}{V_l}$ $Cv_l = \frac{C_l}{P_l}$
Fat	$\frac{dC_f}{dt} = \frac{Qf}{V_f} \times (Ca - Cv_f)$ $Cv_f = \frac{C_f}{P_f}$
Richly perfused tissues	$\frac{dC_r}{dt} = \frac{Qr}{V_r} \times (Ca - Cv_r)$ $Cv_r = \frac{C_r}{P_r}$
Poorly perfused tissues	$\frac{dC_s}{dt} = \frac{Qs}{V_s} \times (Ca - Cv_s)$ $Cv_s = \frac{C_s}{P_s}$
Venous blood	$Cv = \frac{Ql \times Cv_l + Qf \times Cv_f + Qr \times Cv_r + Qs \times Cv_s}{Qc}$
Alveolar air	$Calv = \frac{Ca}{Pb}$

[a] C: Concentration (mg/liter or mmol/liter) , Q: Flow rate (liters.hr^{-1}), V: Volume (liters), P: Tissue:blood partition coefficient, Pb: Blood:air partition coefficient, A Amount (mg), V_{max}: Maximal velocity of enzymatic reaction (mg.hr^{-1}), Km: Michealis Menten affinity constant (mg/L); Subscripts: a = arterial blood, alv = end-alveolar air, f = fat, inh = inhaled air, l = liver, r = richly perfused tissues, s = slowly perfused tissues, v = mixed venous blood, vf = venous blood leaving fat, vl = venous blood leaving liver, vr = venous blood leaving richly perfused tissue, vs = venous blood leaving poorly perfused tissue.

numerical methods. Several commercially-available simulation or programming software packages can be used for conducting PBPK model simulations (Menzel et al. 1987, Johanson and Naslund 1988, Easterling et al. 2000).

Table 19.4 presents a list of VOCs for which PBPK models have been developed. Current practices for PBPK modeling of various VOCs can be found in Reddy et al. (2005). While most of the PBPK models for VOCs have been validated in rodents, some models have also been extrapolated to humans and validated with limited human pharmacokinetic data. An example of such an effort is illustrated in Figure 19.3. This figure represents PBPK model simulations of the inhalation pharmacokinetics of toluene and *m*-xylene in human volunteers exposed to 17 ppm and 33 ppm for 7 hr (Tardif et al. 1995).

One of the advantages of PBPK models relates to their use in the conduct of extrapolations on the basis of equivalent internal dose metrics. For example, duration-specific inhalation concentrations of a VOC that would give same internal dose metrics can be simulated using PBPK models.

Table 19.4 Volatile Organic Chemicals for which PBPK Models Have Been Developed in One or More Species

Chemicals	Species[a]
Acetone	H
Acrylonitrile	R, H
Benzene	R, M, H
Bromochloromethane	R
Bromodichloromethane	R
Bromotrifluoromethane	R
Butadiene (1,3)	R, M, H
Butanol (2-)	R
Carbon tetrachloride	R
Chlorobenzene	H
Chloroethane	R
Chloroform	R, M, H
Chloromethane	R
Cyclohexane	H
Dibromomethane	R
Dichloroethane (1,1-, 1,2-)	R, M
Dichloroethylene (cis, trans)	R
Dioxane (1,4-)	R, M, H
Isopropene	R, M, H
Methanol	R, M, H, Mk
Methyl chloroform	R, M, H
Methyl t-butyl ether	R
Methylene chloride	R, M, H
Methyl ethyl ketone	H
m-Xylene	R, H
n-Hexane	R, H
Tetrachloroethane	R
Tetrachloroethylene	R, M, H, D
Tetrahydrofuran	H
Toluene	R, H
Trichloroethylene	R, M, H
Trifluoroethane	R
Trimethyl benzene	H
Vinyl acetate	R
Vinyl chloride	R
Vinyl fluoride	R

[a] R = rat, M = mouse, H = human, Mk = monkey, D = dog

Source: Krishnan and Andersen (2001).

Figure 19.4 depicts an example of 7-hr to 24-hr extrapolation for toluene in humans based on equivalent 24-hr area under the curve (AUC = 0.88 mg/L × hr). These simulations suggest humans exposed to 17 ppm for 7 hr would receive the same internal dose as the humans exposed to 5.38 ppm for 24 hr. These kinds of simulations would not only contribute to refine/reduce animal use in experiments but also help in the design of experiments.

In the PBPK model for toluene described above, pulmonary uptake is represented by assuming that the entire chemical disappearing from the inspired air appears in the arterial blood and that the chemical in alveolar air and arterial blood are in instantaneous equilibrium. Airways such as nasal passages, larynx, trachea, bronchi, and bronchioles are considered as inert tubes that carry the chemical to the pulmonary region, where diffusion occurs. There is evidence that the

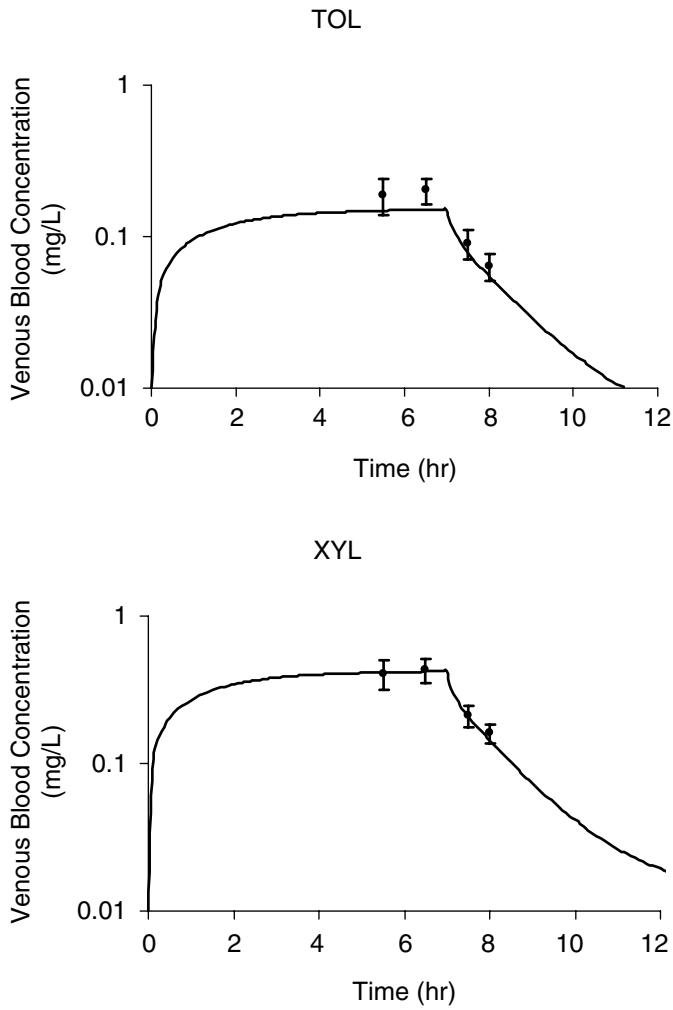


FIGURE 19.3 Comparison of PBPK model simulations (solid lines) with experimental data on the venous blood concentrations in humans exposed to 17 ppm of toluene (TOL) or 33 ppm of m-xylene (XYL). Based on Tardif et al. (1995).

simple continuous ventilation equilibration models do not adequately predict either total respiratory uptake or regional uptake of highly soluble polar solvents and these solvents appear to have complex relationships between uptake and the blood-air partition coefficient (Johnason 1991). The lower pulmonary uptake of polar solvents is, in part, due to their adsorption and/or dissolution in the surface of the respiratory epithelium during inhalation and their desorption during exhalation. This adsorption-desorption mechanism is a consequence of both the aqueous solubility of the chemicals and the cyclic nature of respiratory exchange.

PBPK models for polar solvents, in addition to accounting for the anatophysiological characteristics of the respiratory tract, blood flow rates, and partition coefficients of the chemical do take into account the adsorption of vapors during inhalation and desorption during exhalation. The PBPK model for polar solvents developed by Johanson (1991) consists of nine compartments each one representing an anatomical level of the respiratory tree, with the last compartment corresponding to the gas exchange region of the respiratory tract (respiratory bronchioles, alveolar ducts, and alveoli).

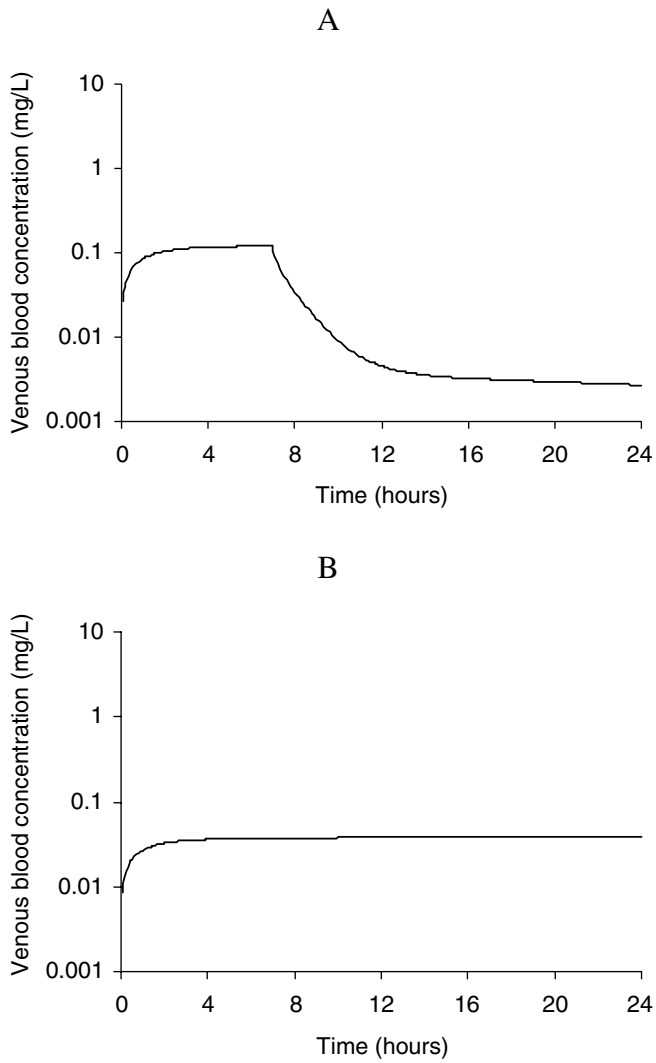


FIGURE 19.4 PBPK model-derived duration extrapolation (7-hr to 24-hr) of toluene exposures in human based on equivalent 24-hr area under the curve. Panel A displays the simulated blood concentration profile in humans exposed to 17 ppm for 7 hr whereas Panel B depicts the human blood concentration profile associated with 24-hr exposure to 5.38 ppm. Based on Tardif et al. (1997).

Each of the first eight compartments is linked with a peripheral compartment facilitating the description of radial diffusion of solvent from the outermost layer and deeper portions of the airway wall. The peripheral compartment of the ninth region corresponds to the rest of body, represented as a single or a multicompartmental PBPK model (Johanson 1991).

The major advantage of PBPK models is their usefulness in predicting tissue dosimetry of chemicals for untested exposure scenarios in laboratory animals, and possibly in humans as well. The validated PBPK models have been used to predict the tissue dose of the potential toxic moiety of VOCs for various exposure concentrations, scenarios and species. This is particularly important since health risk assessments are frequently based on toxicology studies conducted in laboratory

animals exposed to high concentrations of VOCs often by scenarios different from anticipated human exposures.

19.4 PREDICTION AND EXTRAPOLATION OF INHALATION PHARMACOKINETICS OF VOCs

PBPK models are uniquely useful in facilitating the conduct of extrapolations of inhalation pharmacokinetics and tissue dosimetry of VOCs from high to low exposure concentrations, species to species and one exposure scenario to another (Clewell and Andersen 1994). These extrapolations are conducted on the basis of internal dose simulations (e.g., area under the parent chemical vs time curve, amount metabolized per unit time). The following sections highlight the use of PBPK models in high dose to low dose and interspecies extrapolations of the inhalation pharmacokinetics of VOCs.

19.4.1 High Dose to Low Dose Extrapolation

At the high concentrations of VOCs to which animals are exposed during toxicology studies, the internal dose and adverse responses may not be directly proportional to the exposure concentration and could be a result of potentially complex, nonlinear pharmacokinetic processes occurring in the organism at such exposure concentrations (Clewell and Andersen 1984, Andersen et al. 1987). A scientifically-sound extrapolation of internal dose in such cases can be achieved by using PBPK models. These models facilitate the extrapolation of internal dose from high exposure concentration to low exposure concentrations by accounting for the dose-dependency of relevant processes (Clewell and Andersen 1987, D'Souza et al. 1988, Krishnan et al. 1992, Tardif et al. 1995). The description of hepatic metabolism in PBPK models has often included a capacity-limited process, characterized by a maximal velocity and a Michaelis constant, that facilitates simulation of saturation at high exposure concentrations. As such, for the conduct of high dose to low dose extrapolation, no change in the parameters of PBPK model is required, with the exception of the exposure concentration.

The use of PBPK models in the conduct of high dose to low dose extrapolation is exemplified in Figure 19.5. Here, the area under the blood concentration vs time curve (AUC) and the total amount metabolized as a function of exposure concentrations of toluene are presented. These simulation results are indicative of the nonlinear behavior of toluene in this range of exposure concentrations (1 to 10000 ppm). In such instances, the use of models based on the qualitative and quantitative information on the mechanism of non-linearity should be sought for conducting extrapolation of tissue dose from high exposure concentration to low exposure concentration.

19.4.2 Interspecies Extrapolation

The tissue dosimetry associated with a given exposure concentration of a VOC may differ between species. The interspecies differences in tissue dosimetry may be due to qualitative or quantitative differences in absorption, distribution, metabolism or excretion (ADME) processes. Accounting for the interspecies differences in the occurrence, the magnitude and rate of ADME processes or their parameters allows the prediction of the impact on tissue dosimetry across species. The usefulness of PBPK models for this purpose has been demonstrated with a number of VOCs (Andersen et al. 1991, Reitz et al. 1996, Tardif et al. 1997, Clewell et al. 2002). The procedure initially involves the development of an inhalation PBPK in the test animal species, and then scaling of the physiological parameters of the model as well as replacement of chemical-specific parameters for the species of interest (e.g., humans).

For conducting rat to human extrapolation of inhalation pharmacokinetics of VOCs, quantitative estimates of chemical-specific parameter values (i.e., partition coefficients and metabolic rate constants) in humans are required. The tissue-air partition coefficients of VOCs appear to

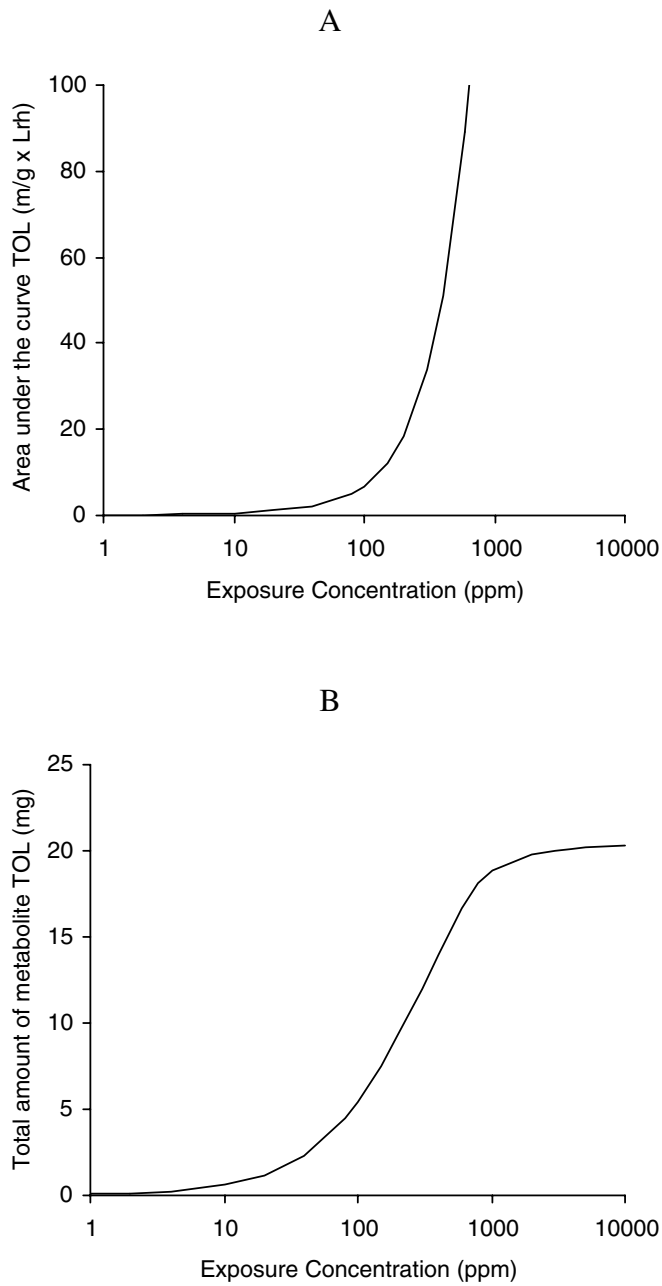


FIGURE 19.5 High dose to low dose extrapolation of internal dose of toluene using a rat PBPK model. (A) Area under the blood concentration vs time curves (AUCs) and (B) total amount metabolized associated with 6-hr inhalation exposure to toluene were calculated for a period of 24-hr. Simulations were based on PBPK model published by Tardif et al. (1997).

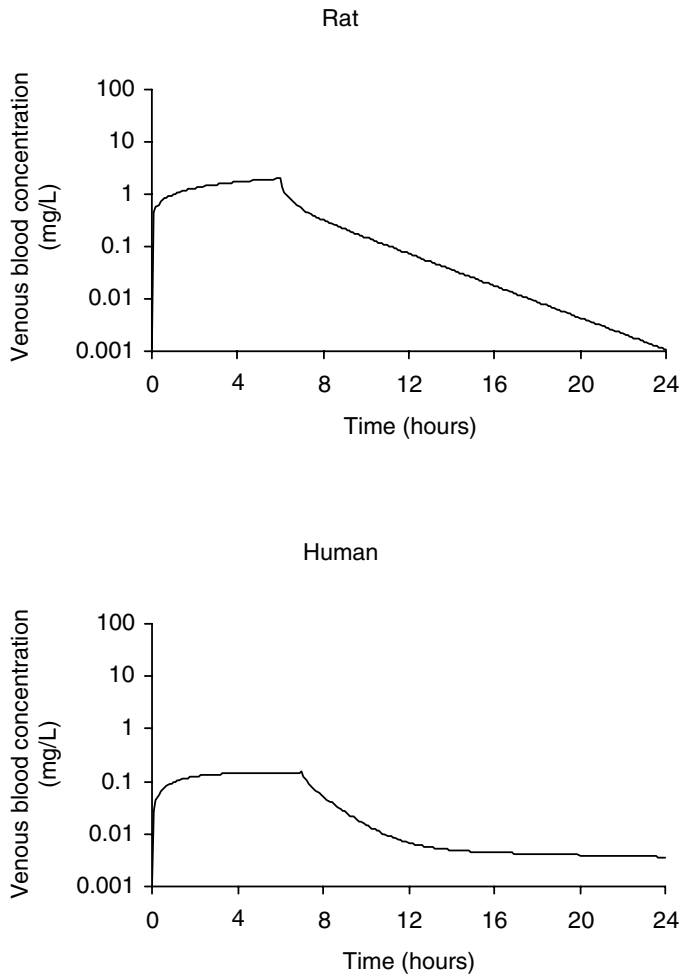


FIGURE 19.6 Illustration of the use of PBPK models for the conduct of rat to human extrapolation of the inhalation pharmacokinetics of toluene. The simulation in the rat was obtained for 6 hr exposure to 100 ppm of toluene and in human for 7 hr exposure to 17 ppm of toluene. Simulations were obtained using the PBPK model published by Tardif et al. (1997).

be fairly constant across species, whereas blood-air partition coefficients show some species-dependency (Gargas et al. 1989, Poulin and Krishnan 1996c). Therefore, the tissue:blood partition coefficients for humans have been calculated by dividing the rodent tissue:air partition coefficients by the human blood:air partition coefficients (Krishnan and Andersen 2001). The species differences in tissue:blood and blood:air partition coefficients for VOCs may also be predicted using species-specific data on the content of neutral lipids, phospholipids, protein and water (Poulin and Krishnan 1995,1996c).

Whereas the adult physiological parameters vary coherently across species, the kinetic constants for metabolizing enzymes do not necessarily follow any type of readily predictable pattern, making the interspecies extrapolation of xenobiotic metabolism rates somewhat difficult. Therefore the metabolic rate constants for VOCs should preferably be obtained in the species of interest. *In vivo* approaches for determining metabolic rate constants are not always feasible or

ethical for application in humans. In such cases, metabolism rates in humans may be estimated from *in vitro* data or using a parallelogram approach based on in vivo data in rodents and in vitro data obtained using rodent and human tissue microsomal fractions (Reitz et al. 1996, DeJongh and Blaauber 1997, Lipscomb et al. 1998, 2003). In the case of highly metabolized VOCs, most of which are substrates of CYP 2E1, the maximal velocity has been scaled to the 3/4th power of body weight, keeping the affinity constant species-invariant. This approach has proved to be an useful approximation of metabolism rates across species particularly in the absence of direct measurements of metabolic rates (e.g., Andersen et al. 1987, Tardif et al. 1997).

Figure 19.6 illustrates the use of PBPK models in the conduct of rat to human extrapolation of the inhalation pharmacokinetics of toluene. In this case, the PBPK model was first developed and validated with inhalation pharmacokinetic data in the test species, i.e., rat (Tardif et al. 1995, 1997). Subsequently, physiological parameters and blood:air partition coefficients for humans were introduced into the model while the metabolic constants were computed on the basis of body weight to the power of 0.7. The extrapolated rat PBPK model adequately predicted the inhalation pharmacokinetics of toluene in human volunteers exposed to 17 ppm for 7 hr (Figure 19.6).

19.5 CONCLUDING REMARKS

The tissue dosimetry and pharmacokinetics of inhaled VOCs are determined by the interplay of several physiological, physicochemical and biochemical factors. PBPK models allow the integration of these factors specific for each chemical and species, thus facilitating the prediction and extrapolation of tissue dosimetry and pharmacokinetics of inhaled VOCs. Using the tissue dose of the toxic moiety of an inhaled toxicant in health risk assessment calculations provides a better basis than the exposure or atmospheric concentrations of the parent chemical to relate to the observed toxic effects. The development of scientifically-sound approaches for estimation of the pharmacokinetic determinants of VOCs should help *a priori* prediction of their *in vivo* pharmacokinetics in view of helping the design of inhalation toxicology studies and better interpretation of toxicity data resulting from such studies.

ACKNOWLEDGMENT

Dr. Krishsnan's work in the subject area has been partly supported by Health Canada and U.S. EPA (4W-0322-NASX).

REFERENCES

Andersen, M.E., Development of physiologically based pharmacokinetic and physiologically based pharmacodynamic models for applications in toxicology and risk assessment, *Toxicol. Lett.,* 79, 35–44, 1995.

Andersen, M.E., Toxicokinetic modeling and its applications in chemical risk assessment, *Toxicol. Lett.,* 138, 9–27, 2003.

Andersen, M.E., Clewell, H.J., Gargas, M.L., MacNaughton, M.G., Reitz, R.H., Nolan, R.J., and McKenna, M.J., Physiologically based pharmacokinetic modeling with dichloromethane, its metabolite, carbon monoxide, and blood carboxyhemoglobin in rats and humans, *Toxicol. Appl. Pharmacol.*, 108, 14–27, 1991.

Andersen, M.E., Clewell, H.J. I., Gargas, M.L., Smith, F.A., and Reitz, R.H., Physiologically-based pharmacokinetics and risk assessment process for methylene chloride, *Toxicol. Appl. Pharmacol.,* 87, 185–205, 1987.

Andersen, M.E. and Dennison, J.E., Mode of action and tissue dosimetry in current and future risk assessments, *Sci. Tot. Environ.,* 274, 3–14, 2001.

Andersen, M.E. Gargas, M.L., Jones, R.A., and Jenkins, L.J., Determination of the kinetic constants for metabolism of inhaled toxicants in vivo by gas uptake measurements, *Toxicol. Appl. Pharmacol.,* 54,116, 1980.

Arms, A.D. and Travis, C.C., Reference Physiological Parameters in Pharmacokinetic Modeling. Office of Health and Environmental Assessment, EPA, EPA/600/6–88/004, 1–1-7.16, US Environmental Protection Agency, Washington, DC, 1988.

Basak, S.C., Mills, D., Hawkins, D.M., and El-Marsi, H.A., Prediction of tissue-air partition coefficients: A comparison of structure-based and property-based methods, *SAR QSAR Environ. Res.,* 13, 649–665, 2002.

Beliveau, M., Lipscomb, J., Tardif, R., and Krishnan, K., Quantitative structure-property relationships for interspecies extrapolation of the inhalation pharmacokinetics of organic chemicals, *Chem. Res. Toxicol.,* 18,475–85, 2005.

Beliveau, M., Tardif, R., and Krishnan, K., Quantitative structure-property relationships for physiologically based pharmacokinetic modeling of volatile organic chemicals in rats. J. Toxicol. Appl. Pharmacology., 189, 221–232, 2003.

Brown, R.P., Delp, M.D., Lindstedt, S.L., Rhomberg, L.R., and Beliles, R.P., Physiological parameter values for physiologically based pharmacokinetic models, *Toxicol. Ind. Health,* 13, 407–484, 1997.

Caster, W.O., Poncelet, J., Simon, A.B., and Armstrong, W.B., Tissue weights of the rat. I. Normal values determined by dissection and chemical methods, *Proc. Soc. Exp. Biol. Med.,* 91, 122–126, 1956.

Clewell, H.J. and Andersen, M.E., Dose species and route extrapolations using physiologically-based pharmacokinetic modeling, *Drinking Water and Health,* 8,159–184, 1987.

Clewell, H.J. and Andersen, M.E., Physiologically-based pharmacokinetic modeling and bioactivation of xenobiotics, *Toxicol. Ind. Health,* 10, 1–24, 1994.

Clewell, H.J., Andersen, M.E., and Barton, H.A., A consistent approach for the application of pharmacokinetic modeling in cancer and noncancer risk assessment, *Environ. Health Perspect.,* 110, 85–93, 2002.

D'Souza, R.W., Francis, W.R., and Andersen, M.W., Physiological model for tissue glutathione depletion and increased resynthesis after ethylene dichloride exposures, *J. Pharmacol. Exp. Ther.,* 245,563–568, 1988.

Dallas, C.E., Muralidhara, S., Chen, X.M., Ramanthan, R., Varkonyl, P., Gallo, J M., and Bruckner, J.V., Use of a physiologically based model to predict systemic uptake and respiratory elimination of perchloroethylene, *Toxicol. Appl. Pharmacol.,* 128, 60–68, 1994.

Davies, B. and Morris, T., Physiological parameters in laboratory animals and humans, *Pharm. Res.,* 10, 1093–1095, 1993.

DeJongh, J. and Blaauboer, B.J., Evaluation of in vitro-based simulations of toluene uptake and metabolism in rats, *Toxicol. in Vitro,* 11, 485–489, 1997.

Dennison, J.E., Andersen, M.E., and Yang, R.S., Pitfalls and related improvements of in vivo gas uptake pharmacokinetic experimental systems, *Inhal. Toxicol.,* 17,539–48, 2005.

Dedrick, R.L., Forrester, D.D., and Ho, D.H.W., In vitro-in vivo correlation of drug metabolism: Deamination of 1-β-D-arabinosyl cytosine, *Biochem. Pharmacol.,* 21, 1–16, 1972.

Dedrick, R.L., Zaharko, D.S., and Lutz, R.J. Transport and binding of methotrexate in vivo, *J. Pharm. Sci.,* 62, 882–890, 1973.

Delp, M.D., Manning, R.O., Bruckner, J.V., and Armstrong, R.B., Distribution of cardiac output during diurnal changes in activity in rats, *Am. J. Physiol.,* 261, H1487–1493, 1991.

Easterling, M.R., Evans, M.V., and Kenyon, E.M., Comparative analysis of software for physiologically based pharmacokinetic modeling: simulation, optimization, and sensitivity analysis, *Toxicol. Meth.*, 10, 203–229, 2000.

Filser, J.G. and Bolt, H.M., Inhalation pharmacokinetics based on gas uptake studies. I. Improvement of kinetic models, *Arch. Toxicol.,* 47, 279–292, 1981.

Filser, J.G., Csanady, G.A., Kreuzer, P.E., and Kessler, W., Toxicokinetic models for volatile industrial chemicals and reactive metabolites, *Toxicol. Lett.,* 82/83, 357–366, 1995.

Fiserova-Bergerova, V., *Modeling of inhalation exopsure to vapors: uptake, distribution and elimination,* Vol. 1, CRC Press Inc., Boca Raton, FL, pp.73–100, 1983.

Fiserova-Bergerova, V., Extrapolation of physiological parameters for physiologically based simulation models, *Toxicol. Lett.,* 79, 77–86, 1995.

Fiserova-Bergerova, V. and Diaz, M.L., Determination and prediction of tissue-gas partition coefficients. Int Arch Occup Environ Health 58, 75–87, 1986.

Gargas, M.L., An exhaled breath chamber system for assessing rates of metabolism and rates of gastrointestinal absorption with volatile chemicals, *J. Am. Coll. Toxicol.,* 9, 447–453, 1990.

Gargas, M.L. and Andersen, M.E., Determinating the kinetic constants of chlorinated ethane metabolism in the rat from rates of exhalation, *Toxicol. Appl. Pharmacol.*, 99, 344–353, 1989.

Gargas, M.L., Andersen, M.E., and Clewell, H.J., A physiologically-based simulation approach for determining metabolic rate constants from gas uptake data, *Toxicol. Appl. Pharmacol.*, 86, 341–352, 1986.

Gargas, M.L., Burgess, R.J, Voisard, D.E, Cason, G.H, and Andersen, M.E., Partition coefficients of low molecular weight volatile chemicals in various liquids and tissues, *Toxicol. Appl. Pharmacol.*, 98, 87–99, 1989.

Gargas, M.L., Clewell, H.J., and Andersen, M.E., Gas uptake inhalation techniques and the rates of metabolism of chloromethanes, chloroethanes and chloroethylenes in the rat, *Inhal. Toxicol.*, 2, 319, 1990.

Gehring, P.J., Watanabe, P.G., Park, C.N., Resolution of dose-response toxicity data for chemicals requiring metabolic activation: example—vinyl chloride, . *Toxicol Appl Pharmacol.,* 44, 581–91, 1978.

International Commission on Radiation Protection (ICRP), *International Commission on Radiation Protection: Report of the task group on reference man.* Pergamon Press, New York, 1975.

Johanson, G., Modelling of respiratory exchange of polar solvents, *Ann. Occup. Hyg.*, 35, 323–339, 1991.

Johanson, G. and Dynesius, B., Liquid: air partition coefficients for six commonly used glycol ethers, *Br. J. Ind. Med.,* 45, 561–564, 1988.

Johanson, G. and Filser, J.G., Experimental data from closed chamber gas uptake studies in rodents suggest lower uptake rate of chemical than calculated from literature values on alveolar ventilation, *Arch. Toxicol.*, 66, 291–295, 1992.

Johanson, G. and Naslund, P.H., Spreadsheet programming: A new approach in physiologically based modeling of solvent toxicokinetics, *Toxicol. Lett.*, 41, 115–127, 1988.

Krishnan, K. and Andersen, M.E. Physiologically based pharmacokinetic modeling in toxicology. In *Principles and methods of toxicology* (A.W. Hayes, Ed.), Taylor & Francis, Philadelphia, PA, pp. 193–241, 2001.

Krishnan, K., Gargas, M.L., Fennell, T.R., and Andersen, M E., A physiologically-based description of ethylene oxide dosimetry in the rat, *Toxicol. Ind. Health,* 8, 121–140, 1992.

Leung, H.W. and Paustenbach, D.J., Physiologically based pharmacokinetic and pharmacodynamic modeling in health risk assessment and characterization of hazardous substances, *Toxicol. Lett.*, 79, 55–65, 1995.

Lipscomb, J.C., Fisher, J.W., Confer, P.D., and Byczkowski, J.Z., In vitro to in vivo extrapolation for trichloroethylene metabolism in humans, *Toxicol. Appl. Pharmacol.*, 152, 376–387, 1998.

Lipscomb, J.C., Teuschler, L.K., Swartout, J., Popken, D., Cox, T., and Kedderis, G.L., The impact of cytochrome P450 2E1-dependent metabolic variance on a risk-relevant pharmacokinetic outcome in humans, *Risk Anal.,* 6, 1221–1238, 2003.

Mauderly, J.L., Measurement of respiration and respiratory responses during inhalation exposures, *J. Am. Coll. Toxicol.,* 9, 397–406, 1990.

Medinsky, M.A., The application of physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) modeling to understanding the mechanism of action of hazardous substances, *Toxicol. Lett.,* 79, 185–191, 1995.

Menzel, D.B., Wolpert, R.L., Boger, J.R., and Kootsey, J.M., Resources available for simulation in toxicology: specialized computers, generalized software and communication networks, *Drinking Water and Health,* 8, 229–254, 1987.

Payne, M.P. and Kenny, L.C., Comparison of models for the estimation of biological partition coefficients, *J. Toxicol. Environ. Health A.* 65,897–931, 2002.

Poulin, P. and Krishnan, K., A biologically-based algorithm for predicting human tissue: blood partition coefficients of organic chemicals, *Hum. Exp. Toxicol.,* 14, 273–280, 1995.

Poulin, P. and Krishnan, K., A tissue composition-based algorithm for predicting tissue: air partition coefficients of organic chemicals, *Toxicol. Appl. Pharmacol.*, 136, 126–130, 1996a.

Poulin, P. and Krishnan, K., A mechanistic algorithm for predicting blood: air partition coefficients of organic chemicals with the consideration of reversible binding in hemoglobin, *Toxicol. Appl. Pharmacol.*, 136, 131–137, 1996b.

Poulin, P. and Krishnan, K., Molecular structure based prediction of the partition coefficients of organic chemicals for physiological pharmacokinetic models, *Toxicol. Methods.,* 6, 117–137, 1996c.

Poulin, P. and Krishnan, K., A quantitative structure-toxicokinetic relationship model for highly metabolised chemicals, *Altern. Lab. Anim.,* 26, 45–59, 1998.

Poulin, P. and Krishnan, K., Molecular structure-based prediction of the toxicokinetics of inhaled vapors in humans, *Int. J. Toxicol.*, 18, 7–18, 1999.

Ramsey, J.C. and Andersen, M.E., A physiologically-based description of the inhalation pharmacokinetics of styrene in rats and humans, *Toxicol. Appl. Pharmacol.,* 73, 159–175, 1984.

Reddy, M., Yang, R.S., Andersen, M.E., and Clewell, H.J., *Physiological based pharmacokinetic modeling: science and applications,* John Wiley & Sons, Inc., Hoboken, NJ, 2005.

Reitz, R.H., Gargas, M.L., Mendrala, A.L., and Schumann, A.M., In vivo and in vitro studies of perchloroethylene metabolism for physiologically based pharmacokinetic modeling in rats, mice, and humans, *Toxicol. Appl. Pharmacol.,* 136, 289–306, 1996.

Tardif, R., Lapare, S., Krishnan, K. and Brodeur J., Physiologically based modeling of the toxicokinetic interaction between toluene and m-xylene in the rat, *Toxicol. Appl. Pharmacol.,* 120, 266–73, 1993.

Tardif, R., Lapare, S., Charest-Tardif, G., Brodeur, J., and Krishnan, K., Physiologically-based pharmacokinetic modeling of a mixture of toluene and xylene in humans, *Risk Anal.* 15, 335–342, 1995.

Tardif, R., Charest-Tardif, G., Brodeur, J., and Krishnan, K., Physiologically based pharmacokinetic modeling of a ternary mixture of alkyl benzenes in rats and humans, *Toxicol. Appl. Pharmacol.,* 144, 120–134, 1997.

Sato, A. and Nakajima, T., A vial equilibration method to evaluate the drug metabolizing enzyme activity for volatile hydrocarbons, *Toxicol. Appl. Pharmacol.,* 47, 41–46, 1979.

Sato, A. and Nakajima, T., Partition coefficients of some aromatic hydrocarbons and ketones in water, blood and oil, *Br. J. Ind. Med.,* 36, 231–234, 1979.

Wagner J.G., *Fundamentals of Clinical Pharmacokinetics,* Drug International. Hamilton, IL, 1975.

20 Inhalation Toxicology of an Irritant Gas—Historical Perspectives, Current Research, and Case Studies of Phosgene Exposure

Alfred M. Sciuto

CONTENTS

20.1 INTRODUCTION

In 1812 John Davy prepared phosgene ($COCl_2$, MW = 98.92) by mixing equal volumes of carbon monoxide and chlorine dried over calcium chloride. During World War I (WWI) phosgene (CAS 75-44-5) was produced as a result of passing carbon monoxide and chlorine over activated charcoal ($CO + Cl_2 \rightarrow COCl_2$). Once in solution phosgene produces carbon dioxide and hydrochloric acid via the following reaction:

$$COCl_2 + H_2O \rightarrow CO_2 + 2HCl$$

The hydrolysis reaction rate of phosgene is exceptionally rapid with a half-life calculated to be 0.026 sec at 37ºC. The National Institute for Occupational Safety and Health (NIOSH)–established permissible exposure limit (PEL) and threshold limit values (TLV) are 0.1 ppm and 0.4 mg/m^3, respectively (NIOSH, 1976). The physical characteristics of phosgene can be found in Table 20.1

For nearly 80 years, it has been known that phosgene has the capacity to cause severe lung injury. Phosgene has been known as an irritant as well as an asphyxiating gas based on the symptoms

TABLE 20.1 Physical and Chemical Properties of Phosgene

Synonyms	Carbonyl chloride, chloroform chloride
Chemical formula	$COCl_2$
Formula weight	98.9
Boiling point (1 atm)	7.5°C
Density, liquid, 0°C	1.4187 g/ml
Density, gas, 20°C	4.39 g/l
Specific gravity, gas, 20°C (air = 1)	3.4
Specific gravity, liquid,	
19°C/4°C	1.392
Solubility	Slightly soluble in water; hydrolyzes to hydrochloric acid and CO_2. Soluble in carbon tetrachloride, chloroform, acetic acid, benzene, toluene
Color	Colorless
Odor	Sweet in low concentrations, sharp, pungent in higher concentrations
Flammability	Nonflammable
(PEL) (NIOSH)	0.1 ppm (0.4 mg/m^3)
LD_{50} for humans	500 ppm × min

Source: Modified from NIOSH (1976).

observed in soldiers exposed during WWI. It was one of the weaponized respiratory threat agents in use during WWI and at times was also mixed with other gases such as chlorine. Battlefield exposure to gas mixtures was responsible for numerous casualties, approximately one-third of all casualties entering the hospital. In one instance, phosgene was directly responsible for more than 1100 casualties when 88 tons of gas was used against British soldiers at Wieltje, Belgium on December 19, 1915 (Spiers, 1986). Thereafter, it remained the war gas of choice during WWI and was used in shells, trench mortar bombs, and projector drums. It has been estimated that phosgene was responsible for nearly 85% of all respiratory-related deaths attributable to chemical weapons (Karalliedde et al., 2000). Based on military service medical records, phosgene gassing during WWI caused servicemen to lose 311,000 days due to hospitalization (Jackson, 1933).

With the expectation that phosgene would again be used as a chemical warfare gas, the U.S. government sponsored research during World War II on the physiological effects of phosgene poisoning. Most of the resulting data were "classified" and therefore did not appear in the literature until the late 1940s and early 1950s. These early studies provided the necessary background for future work concerning the toxicology of inhaled chemicals.

20.2 INDUSTRIAL USES

Although phosgene has been a military and potential terrorist threat for many years, it is also an occupational and an environmental hazard. Despite its well-publicized toxicity, phosgene gained widespread industrial use as a chemical intermediate in the production of pharmaceuticals, dyes, pesticides, polycarbonate plastics, and polyurethane for foam rubber products. As of 1998, it was estimated that 4.3 million pounds were produced each year in the United States alone (Chemical

Market Report [CMR], 1999). The Environmental Protection Agency (EPA) has estimated that approximately 40 tons of phosgene are released annually into the environment during manufacturing processes (EPA-450/4-84-0074) based on data through the 1970s (NIOSH, 1976). These data also show that less than 9 tons/year per facility are released during industrial processing. Approximately 85% of the U.S.-produced phosgene is used for the manufacture of polymeric isocyanates and toluene diisocyanates by phosgenation reactions with primary amines with 99.9% of the phosgene consumed where it is made (CMR, 1999). Dinitrotoluene isomers are reduced to toluene diamines that are then dissolved in monochlorobenzene, reacted with phosgene, and heated to form toluene diisocyanate. This product then contains approximately 80% of the 2,4-isomer and 20% of the 2,6-isomer. These compounds are further extended to hard plastics such as those used in car bumpers and high-impact-resistant plastic windows.

20.3 ENVIRONMENTAL ISSUES

Although phosgene is nearly completely consumed during industrial use, it can be released through failed processes. Phosgene is an environmental pollutant and has been detected in ambient air samples taken from the Los Angeles area (Singh, 1976). In Poland, for example, because of heavy industrialization and its proximity to densely populated areas, phosgene, along with chlorine, ammonia, and sulfur dioxide, has been identified as one of the most significant threats to the environment (Krajewski, 1997). In Bhophal, India, in 1984, approximately 150,000–200,000 people were accidentally exposed to nearly 50,000 pounds of methylisocyanate (MIC) released over a 2-h period. Based on the chemical reactions at the site, MIC, phosgene, and hydrogen cyanide all played a major role in the possible 3300 deaths that resulted from this catastrophe (Schelble, 1990; Rorison and MacPherson, 1997). In an earlier occurrence, on May 20, 1928, in Hamburg, Germany, 24,640 lb (11 metric tons) of phosgene was accidentally released from a storage tank. The toxic gas plume affected people as far as 6 miles away under light-wind conditions. Three hundred people were reported ill; 195 were seen at local hospitals that evening. There were seven deaths (Hegler, 1928). Although it has never been scientifically verified, the estimated 50% lethal concentration over time in humans is 800 ppm $\times$ min (3200 mg/m^3) for a 2-min exposure (Cucinell, 1974). For a complete review of the chemical reactions of phosgene with organic functional groups and inorganic compounds in industrial applications, see the work of Babad and Zeiler (1973).

20.4 EARLY WORK ON THE PHYSIOLOGICAL EFFECTS OF PHOSGENE

In 1921 a key publication by Laqueur and Magnus showed that phosgene produces variation in breathing patterns of exposed, isolated, and perfused cat lungs. Respiration was frequent and shallow and bronchoconstriction was apparent with exposure greater than 200 ppm (800 mg/m^3). These authors were also the first to determine that HCl liberation, which was considered to be the primary chemical mechanism of phosgene toxicity, may actually not be involved at all in this injury. They reasoned that when phosgene, in a miscible solvent, is reacted with water the hydrolysis rate is extremely rapid (Laqueur and Magnus, 1921). This indicated that HCl has a short-term effect and therefore does not explain the long-term adverse physiological effects commonly observed with phosgene poisoning.

Experiments published after World War II demonstrated that phosgene had wide-ranging effects, in particular, on the cellular architecture of the lung, whereas it had no effect on hemodynamic processes. Isolated dog lung experiments showed that doses of phosgene necessary to cause desquamation of bronchial mucosa increased fluid flux into the lung and that bronchoconstriction had no effect on the biochemical or physical state of the blood or pulmonary vascular bed (Daly de Burg et al., 1946). In 1948, Gibbon et al., using phosgene-exposed rat lungs, examined

the effects of 2.5 mg/l (617 ppm; 2468 mg/m^3) phosgene on pulmonary artery pressure and its effect on pulmonary edema formation. They concluded that phosgene did not increase pulmonary artery pressure and that pulmonary edema formation was not related to increases in hydrostatic pressure.

Important pharmacological experiments by Trethewie (1947a) demonstrated that isolated guinea pig lungs liberated histamine and a slow-reacting substance (SRS) when exposed to phosgene. Lung perfusate histamine release was measured from 5 min to 5 h after exposure to 2.5–6.5 ppm·min (10–26 mg·min/m^3) phosgene. There was an earlier and slightly larger output of histamine when guinea pigs were exposed to the higher phosgene concentrations. However, decreased histamine release was observed 5 h after exposure to 3.7–6.5 ppm·min (14.8–26 mg·min/m^3) phosgene. Five days after exposure, lung perfusate effluent from these experiments was administered to isolated jejunum from guinea pigs. It was observed that the magnitude of jejunum contraction increased as the concentration of phosgene administered to the guinea pig increased. These effects were not observed when phosgene was directly mixed with perfusate and administered to the jejunum. Thymoxyethyldiethylamine, a specific inhibitor of histamine, was administered to the isolated jejunum and blocked the contractile effect of perfusate containing histamine that was liberated by phosgene, but it did not block the effect of SRS. It was concluded that a contractile substance was released from the lung upon exposure to phosgene (Trethewie, 1947a).

In another paper the same year, Trethewie demonstrated that vitamin C did not protect the lungs of monkeys or guinea pigs from phosgene toxicity. He also observed a "capillary-damaging" substance in the perfusate, which caused the leakage of trypan blue into the perfusate. He likened this substance to "leukotaxin," which was described in an earlier paper by Menkin (1937). However, he concluded that this was not the substance responsible for damaging the capillaries because it was of short duration (Trethewie, 1947b). In the 1970s, leukotaxin was further analyzed and identified as the leukotrienes, reactive metabolites of the arachidonic acid cascade. These were eventually implicated as several of the causative biological compounds responsible for increased plasma leakage and pulmonary edema formation in experimental animals exposed to phosgene.

These early experiments were crucial for two main reasons: (1) they gave clues that a contractile substance, and not HCl, may be responsible for pulmonary capillary damage caused by phosgene, and (2) they also provided researchers with a mechanistic starting point, i.e., that arachidonic acid metabolites such as the leukotrienes could play a crucial role in phosgene toxicity. This was supported by evidence presented by Potts et al. in 1949, when they examined the chemical properties of phosgene in rats. They compared the results of enzymatic reactions in rat lung homogenates mixed with phosgene to lung tissue homogenates mixed with ketene, a known acylating agent. Ketene is 20 times more toxic than phosgene and presents a similar clinical picture. Both ketene and phosgene inhibited methylene blue reduction two to five times more than HCl-treated homogenates. They also tested 77 substances for their ability to be acylated by phosgene. The extent of acylation depended on the presence of amino, mercapto, or hydroxyl groups. Therefore, they demonstrated that the lethal action of phosgene is due directly to acylation of essential lung components such as the amino, hydroxyl, and sulfhydryl functional groups of proteins.

Exposure to phosgene may develop into fulminating and life-threatening pulmonary edema 1–24 h after initial exposure in both humans and animals (Tobias, 1945; Diller, 1978; Sciuto, 1998). Mechanisms related to this "latency" phase have eluded investigators. Research during the past eight decades has shown that direct exposure to phosgene does not cause systemic poisoning. Its primary effects were observed and measured in the deep-lung compartments (Daly de Burg et al., 1946; Potts et al., 1949). While the primary effect of phosgene intoxication is in the deep-lung compartments, early research unequivocally demonstrated that if the exposure is of sufficient duration and concentration the result can be catastrophic. In the dog, postexposure events consist of hypercellular aggregation, hemoconcentration, plugging of the capillaries, reduction in blood volume, and decreased heart size (Meek and Eyster, 1920).

20.5 EXPERIMENTAL MODELS AND TECHNIQUES

Numerous techniques have been used to determine the mechanisms of toxicity and the development of lung injury from exposure to phosgene. These have historically involved large-animal studies. As early as 1920 (Meek and Eyster), and in response to the many of the residual phosgene-related casualties remaining from WWI, the progression and intensity of phosgene exposure was experimentally determined in dogs, as mentioned earlier. As time passed, animal models included mice, rats, guinea pigs, swine, and monkeys. The rationale behind these studies involved the use of models that would better predict the temporal effects of the progression and scope of lung injury in humans. Many of these models involved the use of inhalation techniques and in past decades focused essentially on the establishment of a lethal concentration (c, in parts per million) over time (t, in minutes) relationship across species. This is also known as Haber's Law (Haber, 1924). The product of $c \times t$ corresponded to a standard to describe a biological end point that could be edema formation or death. Conceptually, this product simply meant that the same biological end point would occur whether the animals were exposed to 100 ppm (400 mg/m^3) phosgene for 10 min, 50 ppm (200 mg/m^3) for 20 min, or 20 ppm (80 mg/m^3) for 50 min all resulting in a $c \times t$ of 1000 ppm·min (4000 mg·min/m^3). However, later experiments have shown that for phosgene and other irritant gases Haber's Law is only applicable over short-exposure times (Rhinehart and Hatch, 1964; Gelzleichter et al., 1998). In a subchronic phosgene-exposure study using rats, Kodavanti et al. (1997) examined the effects of low concentrations on structural and extracellular alterations. Rats were exposed for 6 h/d to 0.1 ppm (0.4 mg/m^3) for 5 d/week, 0.2 ppm (0.8 mg/m^3) for 5 d/week, 0.5 ppm (2.0 mg/m^3) for 2 d/week or 1.0 ppm (4.0 mg/m^3) for 1 d/week for either 4 or 12 weeks. The authors found that phosgene caused extensive thickening and inflammation of the terminal bronchioles as well as the walls of the alveolar tissue at 1.0 ppm (4 mg/m^3). Much of the damage occurred over the 4-week period with little or no additional damage seen after continuous exposure of up to 12 weeks. The conclusion was that the actual phosgene concentration rather than the product of the concentration and the time ($C \times T$) was largely responsible for the injury.

During WWII, with the thought that chemical agents would be weaponized, much work was done to establish battlefield doses of phosgene that would cause incapacitation and even death. The main problem with these studies was that the doses of phosgene were not very well quantified and in many instances only nominal concentrations were described. This presented a serious problem when attempting to arrive at doses to extrapolate to humans because a clear species difference exists in response to the toxicity of phosgene. A review of the phosgene LCt_{50} literature by Diller and Zante has shown that cats appear to be the most sensitive to phosgene and goats the least sensitive (Diller and Zante, 1982). Guinea pigs, mice, and humans appear to be equally responsive at about 500 ppm·min (2000 mg·min/m^3). More recently, Schroeder and Gurtner (1992) showed that dogs are probably not a good animal model to study phosgene toxicity because they are, as Diller and Zante (1982) showed, not as sensitive as humans, mice, and guinea pigs. In 1990, Keeler et al. used a nose-only exposure method to determine the LCt_{50} for sheep, an animal not listed in the Diller and Zante review (Keeler et al., 1990). Keeler found that sheep were even more resistant to phosgene than dogs based on the data tables from Diller and Zante. The justification behind various animal models was to obtain the best and most accurate assessment of exposure dose-and-response effects with an attempt to extrapolate these to humans.

During the past 20 years many variations in animal models and methods of exposure have been used to address the elusive mechanisms of phosgene toxicity especially with respect to its latency phase. From the industrial point of view, many early studies focused on low levels of concentrations administered over several hours, days, or weeks and measured biochemical end points out to many weeks to more closely mimic a workplace exposure. Models and methods centered on issues such as the determination of exposure/responses using low-level concentrations over short-exposure durations such as minutes or several hours out to several weeks. Many of these studies also focused on histopathological and ultrastructural responses, as well as on the biochemical effects such as in

energy metabolism, lung mechanical function, inflammation, and the signal transduction pathways of mediator release. These studies were designed in large part to determine the mechanism responsible for the latent toxicity observed after exposure, because knowledge of the temporal changes could lead to more efficacious treatments. Although a specific need exists to have knowledge of the biochemical effects of phosgene poisoning, the basic physiological response is also very crucial. Brown et al. (2002, 2003) have established a state-of-the-art intensive care unit to investigate the effects of phosgene exposure on the large anesthetized and mechanically ventilated swine. Pulmonary architecture in swine is very similar to that of adult humans, which makes these types of studies and comparisons vital. In this model it has been demonstrated that swine show a marked decrease in pH, Po_2, O_2 saturation, dynamic compliance, and increased lung wet weight beginning at 6 h after a 10-min exposure to 2443 mg·min/m^3 (611 ppm·min) phosgene. This model has been extended to show that phosgene causes a decrease in shunt fraction and oxygenation index, with alterations in diffusion and respiratory mechanics along with ventilation perfusion mismatches. These animals eventually deteriorate into an adult respiratory distress syndrome (ARDS)-like condition by 21 h after exposure. It is hoped that the knowledge gained from these studies will ultimately lead to therapies that will be effective in counteracting the effects not only of phosgene toxicity, but also of ARDS, because their physiological conditions and symptoms largely overlap.

20.5.1 Mechanistic Toxicology

In 1973, Babad and Zeiler published an extensive report on the various chemical reactions of phosgene with industrial compounds as well as important cellular components of biomolecules, such as sulfhydryl, amine, and hydroxyl groups. Research related to the mechanistic toxicology of phosgene exposure began to emerge in the 1980s. Experimental results regarding the effects of phosgene exposure on lung tissue have ranged over various animal models and exposure conditions. Experimental investigators such as Frosolono, Currie, Pawlowski, Burleson, Selgrade, Madden, Ghio, Kennedy, and Guo set the stage for investigative mechanistic toxicology of phosgene exposure. Chemically, phosgene is extremely reactive. It is believed that phosgene can undergo hetero- and/or homolytic cleavage into a reactive carbamoyl monochloride radical, which may be partly responsible for its reactivity with lung tissue components (Arroyo et al., 1993). However, these data have not been corroborated. Considering this chemical reactivity, there is little surprise that exposure has been found to directly affect type I pneumocytes (Pawlowski and Frosolono, 1977; Diller et al., 1985), increase lavage polymorphonuclear phagocytes (Currie et al., 1987a), decrease both cytochrome *c* oxidase and ATPase activity (Frosolono and Pawlowski, 1977), and significantly reduce lung ATP concentrations (Currie et al., 1987b). Pulmonary host defense systems are also affected by exposure to phosgene. The long-term effects of phosgene inhalation are also known to eventually result in immunosuppression that may increase mortality via infection and compromised lung function in rats exposed to 1 ppm (4 mg/m^3) for 4 h (Burleson and Keyes, 1989) and female mice exposed to phosgene at 0.1, 0.25, or 0.5 ppm (0.4, 1.0, or 2.0 mg/m^3) for 4 h/d for 5 consecutive days (Selgrade et al., 1989).

Recent experimental work in animals has shown that bronchoconstriction, enhanced pulmonary edema formation, elevated leukotriene production, increased lipid peroxidation by-products, and decreases in both dynamic compliance and lung tissue cyclic adenosine 3,5-monophosphate (cAMP) are several of the major responses of the lung to phosgene inhalation (Kennedy et al., 1989; Guo et al., 1990; Sciuto et al., 1996a). Phosgene has been found to be toxic through normal metabolic detoxification mechanisms unrelated to a direct phosgene inhalation exposure event. In hepatocytes, DeCurtis et al. (1994) and Guastadisegni et al. (1996, 1998) have determined that, as the oxidative metabolite of chloroform, phosgene forms adducts with phospholipids such as phosphotidylcholine (PC) and phosphotidylethanolamine (PE) under hypoxic or normoxic conditions. Adduct formation could be mechanistically important during the injury process because alveolar surfactant is largely phospholipid in content and alveolar edema causes a locally hypoxic environment. In humans, 50% of the lung tissue content is PC, 19% is PE, and surfactant is about 68% PC (Rooney, 1992).

Experimentally, several groups have determined that phosgene exposure causes surfactant dysfunction. In rats exposed to phosgene at 240 ppm·min (960 mg·min/m^3 or 1 ppm for 4 h), phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine were all significantly above controls at 1–3 d after exposure (Frosolono and Currie, 1985). Ghio and Hatch also showed in rats exposed to 0.5 ppm (2 mg/m^3) phosgene for 60 min (3 ppm·min or 12 mg·min/m^3) that pretreatment with colchicine, which binds to tubulin to disrupt formation and the transport function of microtubules, inhibited the phosgene-induced elevation of surfactant in the lavage fluid one day after exposure (Ghio and Hatch, 1992). In a more recent investigation, rats exposed to an LCt_{30} of phosgene over 10 min had alterations of surfactant phospholipid composition in the lung as measured in the lavage fluid after exposure. Phosphatidylglycerol and phosphatidylcholine along with other phospholipids increased from 6 h. The authors concluded that damage to the surfactant system could be due to reduced generation or release of free radicals by diseased pneumocytes, the presence of plasma proteins known to inhibit surfactant function, increased surfactant secretion by damaged type II cells, or the presence of inflammatory mediators (Jugg et al., 1999).

Phosgene exposure has also been shown to alter the respiratory breathing mechanics in rodents. Vital capacity, diffusing capacity, and total lung capacity were significantly decreased in rats at 24 h after exposure to 30 ppm·min (120 mg·min/m^3 or 0.5 ppm for 60 min) phosgene (Ghio et al., 1991). Lung compliance was also reduced in rabbits exposed to 1500 ppm·min (6000 mg·min/m^3) phosgene within 1 h of exposure (Guo et al., 1990). More recently Sciuto et al. (2002) examined the effects of phosgene in mice using the whole-body plethysmograph. Male mice were exposed to 32 mg/m^3 (8 ppm) phosgene for 20 min (640 mg·min/m^3). After exposure, conscious mice were placed unrestrained in a whole-body plethysmograph where significant differences between phosgene and air-exposed controls were measured for inspired and expired times, tidal volume, minute ventilation, end inspiratory pause, end expiratory pause, peak inspiratory flows, peak expiratory flows, and a measure of bronchoconstriction (termed Penh) across 12 h. Mice exposed to phosgene showed marked increases (approximately double) in Penh across all time points beginning at 5 h, when compared with air-exposed mice. These results indicate that exposure to phosgene causes early bronchoconstriction, a temporal obstructive-like injury pattern, and disruption of mechanical rhythm largely regulated by the progressive production of pulmonary edema that affects airway flow. In addition, exposed mice showed an early-exposure phase of sensory irritation followed by a pulmonary irritation effect over time. This is a combination of sensory effects characterized by increased respiratory rate and decreased tidal volume followed by pulmonary irritation effects over time.

Identifying the mechanistic pathways of toxicity following the inhalation of phosgene is important because it allows for rational therapeutic medical countermeasures to be investigated based on defects or changes in those pathways. Correction or protection of these pathways by certain classes of drugs has been investigated in many animal models. Under these set experimental conditions Food and Drug Administration (FDA)-approved compounds have been found to be protective against phosgene-induced acute lung injury as measured by the reduction of pulmonary edema. Kennedy et al. (1989) and Sciuto et al. (1996a, 1997b, 1998) have shown that the postexposure treatment with compounds that up-regulate cAMP, such as isoproterenol, terbutaline, or aminophyline, reduce lung water content in rabbits by lowering vascular pressure, decreasing leukotriene production and reducing lipid peroxidation. Guo et al. (1990) have also shown reduced lung injury in rabbits using drugs that block specific pathways of the arachidonic acid cascade. Treatments with drugs that up-regulate glutathione (GSH) have also been shown to be protective when administered after exposure to phosgene. *N*-Acetylcysteine (NAC), in general, considered to be an up-regulator of glutathione, was demonstrated to be highly effective in reducing lung edema when given as a bolus in the rabbit airway and as an intraperitoneal (i.p.) treatment in rats. It is not that NAC increases GSH to any great extent, but that NAC itself is a very good antioxidant (Sciuto et al., 1995; van Helden et al., 2004). Current unpublished work from our Institute has shown that lung tissue from mice exposed to 32 mg/m^3 phosgene had significantly enhanced mRNA expression of the enzymes required to drive the GSH redox

cycle (Sciuto et al., 2005). Genetic expressions of GSH reductase, GSH peroxidase, and GSH synthetase are significantly up-regulated in a temporal manner and agree very well with those same enzymes measured in lavage fluid (Sciuto et al., 2003b). These data suggest that free radical injury is an early and significant mechanism of phosgene toxicity and may indicate that compounds like NAC would be beneficial as treatments for lung injury. As mentioned earlier, it is believed that phosgene and similarly acting edemagenic gases react with the phospholipid content to the lung surfactant. Experiments by Currie et al. (1997) and van Helden et al. (2004) have demonstrated that this is indeed the case. In both models, increased rat survival rates were observed after treatment with surfactant replacement therapy against toxic gas-induced lung injury. A crucial question of the beneficial effect of any drug therapy treatment, especially against a gas as toxic as phosgene, would be how does treatment affect survival rates? This has been examined for NAC, as mentioned above, and for ibuprofen. Ibuprofen has been shown to be highly effective in enhancing the 12-h survival rates in mice (Table 20.2) when given intraperitoneally beginning at 20 min after exposure (Sciuto, 1997a). Ibuprofen is a known anti-inflammatory drug, ·OH radical scavenger, and free iron chelator, all of which are important components of phosgene-induced acute lung injury. In addition to the mouse model, ibuprofen has also been shown to be an effective postphosgene exposure therapy in the rabbit model (Kennedy et al., 1990) and the rat model (Sciuto et al., 1996c). Effective treatments have also been examined in relation to free radical scavenging and antioxidant properties. Sciuto and Moran (1999) fed mice two different levels of butylated hydroxyanisole (BHA) in rodent chow for 3 weeks and measured survival rates (Table 20.3). At both dietary levels of BHA, 0.75% and 1.5%, survival rates were significantly higher in phosgene-exposed diet-treated than in phosgene-alone controls at both 12 and 24 h after exposure. Although BHA pretreatment was extremely impressive, it is unlikely that preexposure treatment of phosgene is a realistic approach to prophylaxis. What needs to be considered is the delivery of a NAC/BHA/ibuprofen/isoproterenol/surfactant cocktail directly to the site of injury, the airway side of the lung, as soon as possible after phosgene exposure to counteract the identified mechanisms that could potentially lead to life-threatening lung injury. Brown et al. (2003) have shown that improvement in the pathophysiological response after phosgene inhalation can be achieved not by intervening with drugs, but by using "protective ventilation strategy." After exposing instrumented and anesthetized swine nose-only to phosgene at 2500 mg·min/m^3 (625 ppm·min) for 10 min, reduction in tidal volume, positive end expiratory pressure, and respiratory rate significantly aided in improving oxygenation and subsequently reducing mortality, lung inflammation, and pulmonary edema.

20.5.2 Lavage Studies

In earlier experiments using rats exposed whole-body to phosgene, lung tissue levels of ATP were significantly decreased immediately after exposure to low levels of phosgene, 0.05–1 ppm (0.2–4 mg/m^3) given over 4 h. These changes in ATP levels preceded edema as measured by lavage protein concentrations and lung wet weight, suggesting that biochemical changes occur much earlier and at lower ppm·min levels than the official published threshold limit value (Currie et al., 1987a). Currie and workers also observed that when rats were exposed to phosgene for 4 h to 0.125–1 ppm (from 20–240 ppm or 120–960 mg/m^3), polymorphonuclear lymphocytes (PMNs), lavage fluid protein, and wet/dry weight (a measure of pulmonary edema) ratios were all enhanced. The authors concluded that elevated lavage protein levels were the best early indication of phosgene-induced lung injury. In longer exposure experiments, 17 d at 0.125 ppm (0.5 mg/m^3) and 1 ppm (4 mg/m^3) for 7 h, lung tissue glucose-6-phosphate dehydrogenase levels and nonprotein sulfhydryls were significantly increased for as long as 14 d after exposure. Madden et al. (1991) measured decreases in lung lavage of prostaglandins and leukotrienes concomitantly with increases in PMNs and decreases in macrophages after rats were exposed whole-body to phosgene for 4 h in concentrations ranging from 0.01 (0.04 mg/m^3) to 1 ppm (4 mg/m^3).

TABLE 20.2 Mice 12- and 24-h Survival Rates Following Posttreatment of Phosgene Exposure with Ibuprofen (IBU)

	12 h				24 h			
IBU conc.[a]	Alive/total	Survival (%)	(χ^2)	Survival odds ratio[b]	Alive/total	Survival (%)	(χ^2)	Survival odds ratio[b]
0/0 saline-treated + phosgene	4/16	25	—	—	1/16	6.2	—	—
3/1.5 mg/mouse + phosgene	7/16	44	N.S.	2.3	2/16	12.5	N.S.	2.1
9/4.5 mg/mouse + phosgene	10/16	63	$p < 0.05$	5.0	3/16	19	N.S.	3.5
15/7.5 mg/mouse + phosgene	13/16	82	$p < 0.005$	13	3/16	19	N.S.	3.5

IBU administered at 0, 3, 9, or 15 mg/mouse at 20 min after exposure and again at 5 h after exposure at 0, 1.5, 4.5, or 7.5 mg/mouse.

[a] Phosgene exposure was for 20 min at 32–42 mg/m^3 (640–840 mg·min/m^3).

[b] Odds ratio for survival at each drug dose for 12 or 24 h was calculated based on the ratio of (total alive/total dead) for each IBU concentration versus exposed and saline treated. For example, at 15/7.5 mg IBU/mouse, this is (13/3)/(4/12) = 13. N.S. = not significant.

Source: From Sciuto (1997).

TABLE 20.3 The Effect of Phosgene Exposure on 12- and 24-h Mouse Survival Rates. Dietary Pretreatment with BHA Significantly Enhanced Survival Rates at Both 12 and 24 h When Compared with the Phosgene-Exposed Control Diet (5002) Group[a]

	12 h				24 h			
	Alive/ Total	Survival (%)	(χ^2)	Survival Odds Ratio[ba]	Alive/ Total	Survival (%)	(χ^2)	Survival Odds Ratio
Control diet (5002) + phosgene	11/30	36	—	—	7/30	23	—	—
0.75% BHA (5002) + phosgene	16/20	80	$p < 0.01$	6.9	11/20	55	$p < 0.05$	4.0
1.5% BHA (5002) + phosgene	14/14	100	$p < 0.0005$	46.6	13/14	92	$p < 0.0001$	42

[a] Odds ratios were analyzed by χ^2. All mice were exposed to a concentration × time product (LCt) of 640 mg·min/m^3 (32 mg/m^3 for 20 min).

[b] Odds ratio (OR) for survival at each diet level at 12 or 24 h was calculated based on the ratio of (total alive/total dead). For example, at 0.75% BHA + phosgene at 12 h, this is (16 ÷ 4)/(11 ÷ 19) = 6.9 compared with the control 5002 diet group. Note: It is not possible to calculate an OR in the 12-h 1.5% BHA because the total survival rate was 100%. We subtracted 0.5 from the alive total and added 0.5 to the dead total such that the actual OR calculation was (13.5 ÷ 0.5/11 ÷ 19) = 46.6 compared with the control 5002 diet group.

Source: From Sciuto and Moran (1999).

Duniho et al. (2002) examined lung tissue and bronchoalveolar lavage fluid at 1, 4, 8, 12, 24, 48, and 72 h after mice were exposed to 32 mg/m^3 (8 ppm) phosgene for 20 min. Over time, the histopathological lesions were characterized by acute changes consisting of alveolar and interstitial edema, fibrin, and hemorrhage followed by significant alveolar and interstitial flooding with inflammatory cell infiltrates, scattered bronchiolar and terminal airway epithelial degeneration, and necrosis (Table 20.4). From 48 to 72 h, partial resolution of the edema and degenerative changes occurred, followed by epithelial and fibroblastic regeneration centered on the terminal bronchiolar areas. Bronchoalveolar lavage was processed for cell differential counts, lactic dehydrogenase (LDH), and protein determination (see Figures 20.1–20.5). Comparative analysis revealed significant increases in both postexposure lung wet/dry weight ratios, and early elevations of lavage LDH and protein, and later elevations in leukocytes. This work attempted to describe the use of histopathology to chronicle the temporal pulmonary changes subsequent to whole-body exposure to phosgene, and correlate these changes with lavage constituents and postexposure lung edema in an effort to temporally characterize phosgene lung injury and identify early markers of exposure.

More recent studies by Sciuto et al. (2003a) analyzed lavage fluid from mice across a range of lavage exposure–response elements. In male mice exposed to 32 mg/m^3 (8 ppm) phosgene for 20 min, it was shown that for cytokines at 4 h postexposure, interleukin-6 (IL-6) was 15-fold higher

TABLE 20.4 Summary of Temporal Histopathology Scores for Mice Lungs

				Infiltrates	
Time	**Gas exposure**	**Edema**	**Fibrin**	**Bronchiolar**	**Interstitial**
1 h					
	Air	0 ± 0^a	0 ± 0^b	0 ± 0	0 ± 0
	Phosgene	0.0 ± 0.2	1.6 ± 0.2	0 ± 0	0 ± 0
4 h					
	Air	0 ± 0^c	0.6 ± 0.4^a	0 ± 0	0 ± 0
	Phosgene	3.3 ± 0.5	2.2 ± 0.2	0 ± 0	0 ± 0
8 h					
	Air	0 ± 0^c	1.0 ± 0.7^a	0 ± 0^d	0 ± 0^c
	Phosgene	3.9 ± 0.1	3.1 ± 0.1	0.5 ± 0.17	1.7 ± 0.2
12 h					
	Air	0.2 ± 0.2^c	0.8 ± 0.4^c	0 ± 0	0 ± 0^c
	Phosgene	3.7 ± 0.3	3.1 ± 0.3	0.3 ± 0.2	1.3 ± 0.2
24 h					
	Air	0.2 ± 0.2^b	1.0 ± 0.6	0 ± 0^a	0 ± 0^a
	Phosgene	3.8 ± 0.3	3.0 ± 0.4	1.0 ± 0	1.0 ± 0
48 h					
	Air	0 ± 0^a	0.2 ± 0.2^a	0 ± 0^a	0 ± 0^a
	Phosgene	3.0 ± 0.7	4.0 ± 0	1.3 ± 0.4	1.7 ± 0.4
72 h					
	Air	0.6 ± 0.6	1.0 ± 0.6	0 ± 0^d	0 ± 0^d
	Phosgene	1.5 ± 0.7	2.5 ± 0.7	2.0 ± 0	1.5 ± 0.7

[a] Probability air $<$ phosgene, $p \leqslant 0.05$.

[b] Probability air $<$ phosgene, $p \leqslant 0.001$.

[c] Probability air $<$ phosgene, $p \leqslant 0.005$.

[d] Probability air $<$ phosgene, $0.1 \leqslant p \leqslant 0.05$.

Source: From Duniho et al. (2002).

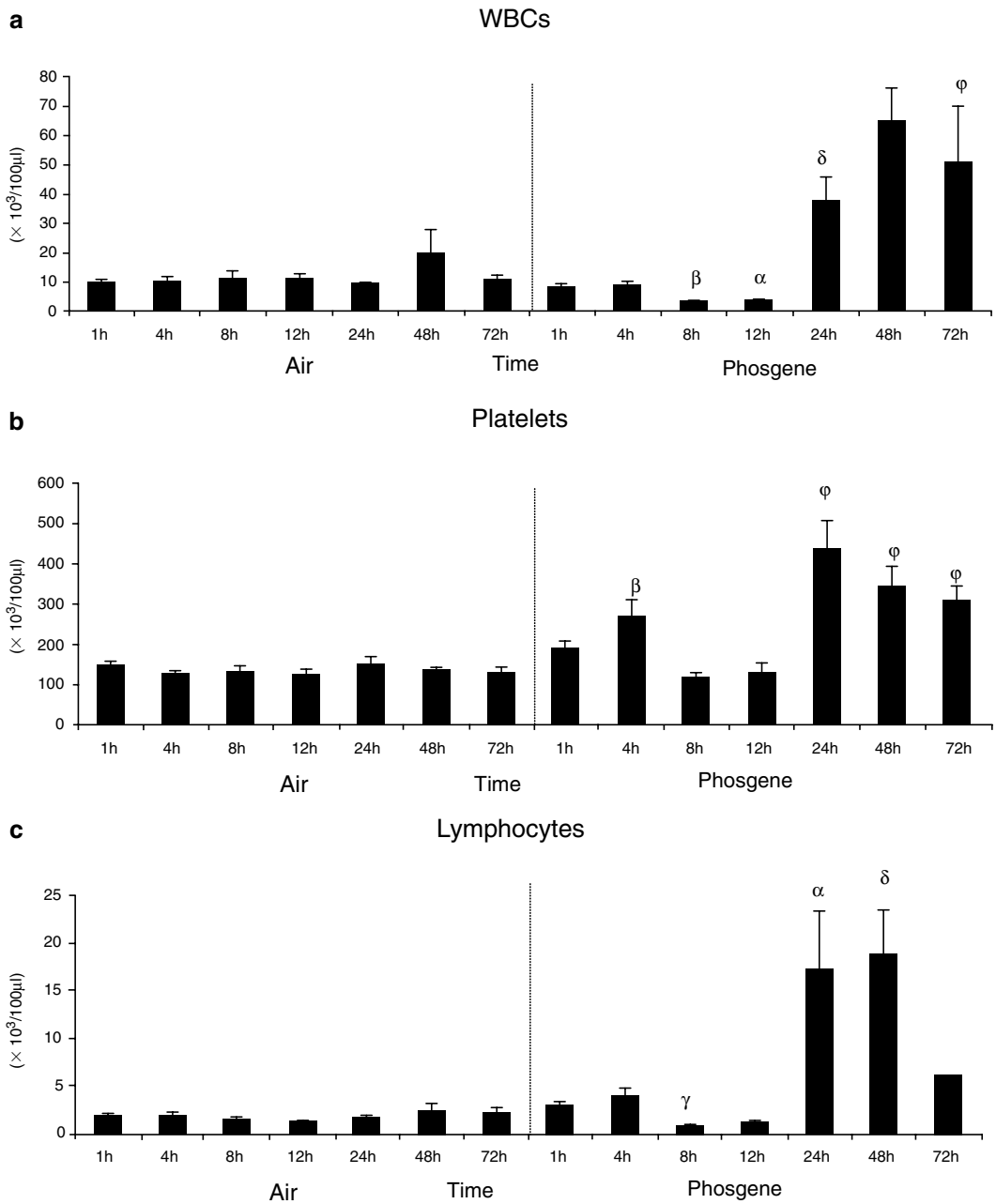


FIGURE 20.1 Phosgene- and air-exposure induced changes in lung lavage white blood cell population (WBCs) over time. (a) There was a significant downward trend in the WBC population in phosgene-exposed mice at both 8 and 12 h compared with air-exposed mice, $p \leqslant 0.01$ (β) and $p \leqslant 0.05$ (α), respectively. By 24 to 72 h this trend reversed with WBCs being significantly higher than in controls, $p \leqslant 0.005$ (δ) and $p \leqslant 0.001$ (ϕ) at 24 and 72 h, respectively. (b) Lavage platelets in phosgene-exposed mice were significantly increased compared with air-exposed controls at the early time point of 4 h, $p \leqslant 0.01$ (β), and then at the later time points from 24 to 72 h, $p \leqslant 0.001$ (ϕ). As is seen with WBCs and platelets, lymphocytes (c) were significantly elevated from 24 to 48 h compared with levels in air-exposed controls, $p \leqslant 0.05$ (α) and $p \leqslant 0.005$ (δ), respectively. However, at 8 h, the lavage lymphocyte population was actually marginally lower than that of air-exposed controls, $0.1 \leqslant p \leqslant 0.05$ (γ). Sample sizes range from 3 to 10; however, the 72-h time point for the lymphocyte population represents an $n = 1$. All data are plotted as mean ± SEM. (Figures adapted from Duniho et al., 2002.)

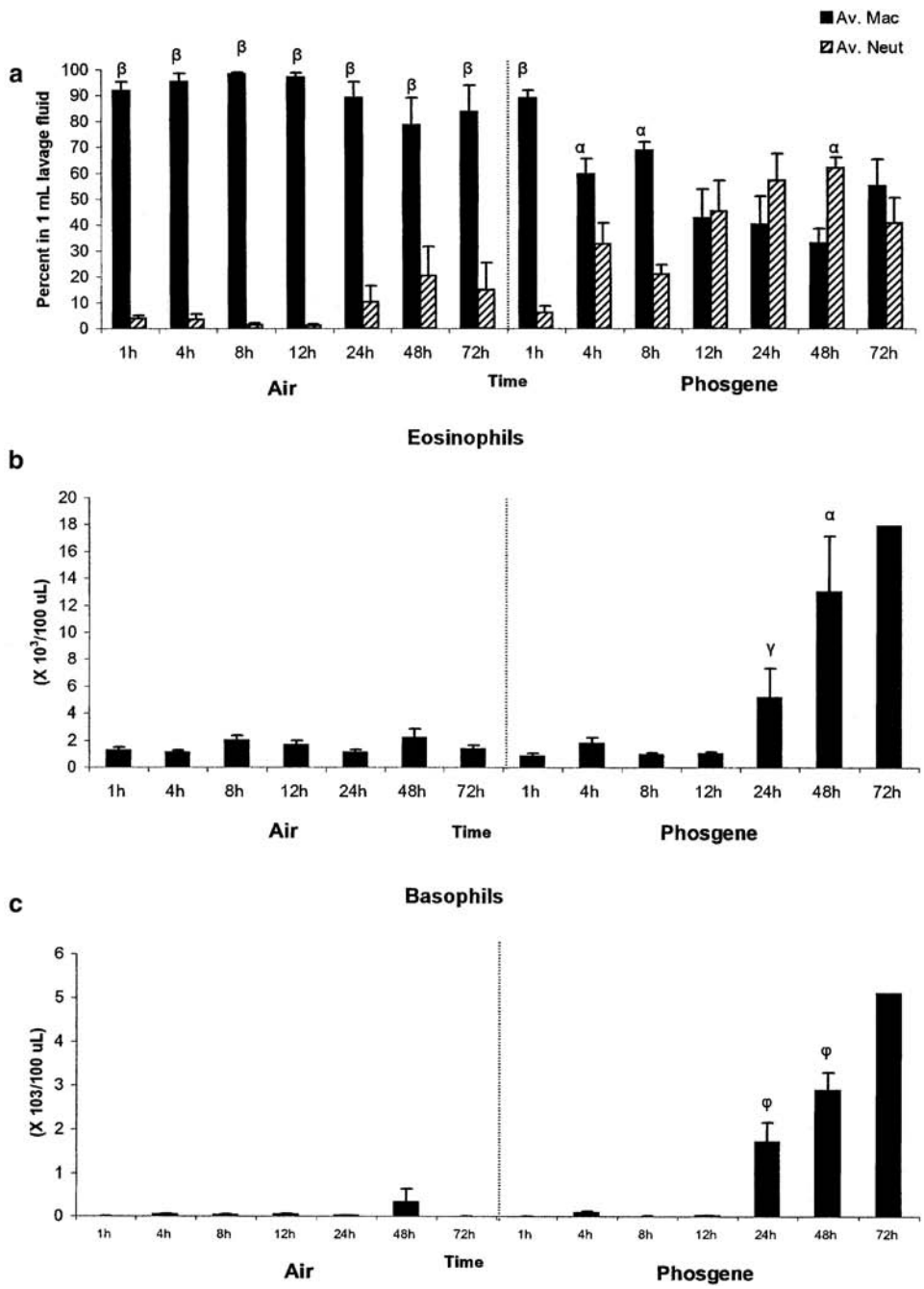


FIGURE 20.2 Phosgene- and air-exposure induced changes in lung lavage cell counts over time. (a) In general, lavage neutrophils exhibit an increasing trend to peak at 48 h, then start decreasing at 72 h. Conversely, lavage macrophages exhibit a decreasing trend to a low at 48 h and then begin to increase at 72 h; $p \leqslant 0.01$ (β), and $p \leqslant 0.05$ (α), respectively. (b) However, eosinophils showed a later increase in response at both 24 and 48 h. At 48 h increases in phosgene-exposed mice were significantly higher than in air-exposed mice, $p \leqslant 0.05$ (α). Although eosinophils increase at 24 h, change is only marginally significant, $0.1 \leqslant p \leqslant 0.05$ (γ). (c) Basophils are practically nonexistent in the air-exposed and early phosgene-exposed mice. However, basophils begin to increase significantly in number by 24 h $p \leqslant 0.001$ (ϕ). The sample size 72-h time point for b and c are $n = 1$. All data are plotted as mean $\pm$ SEM. (Figure adapted from Duniho et al., 2002.)

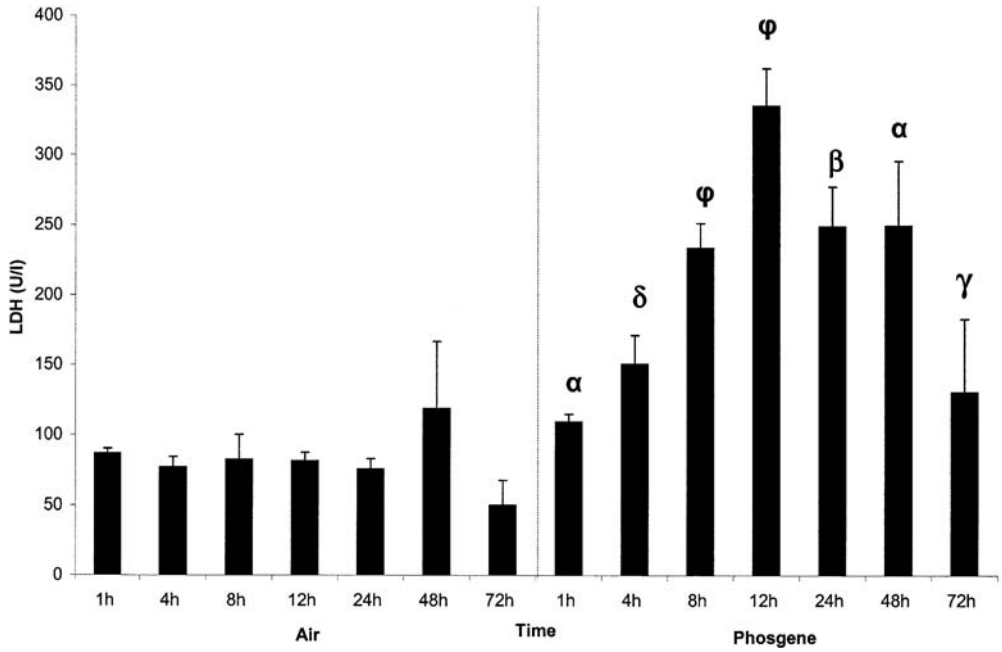


FIGURE 20.3 Phosgene exposure caused a significant increase in lung lavage LDH over time. Beginning as early as 1 h after exposure, LDH is significantly higher than in air-exposed controls, $p \leqslant 0.05$ (α). This trend increased steeply and began to decline and approach control levels by 72 h. LDH in phosgene-exposed mice was significantly higher than air-exposed controls at 4 h, $p \leqslant 0.005$ (δ); 8 to 12 h, $p \leqslant 0.001$ (φ); 24 h, $p \leqslant 0.01$ (β); and marginally higher at 72 h, $0.1 \leqslant p \leqslant 0.05$ (γ). All data are plotted as mean ± SEM. (Figure adapted from Duniho et al., 2002.)

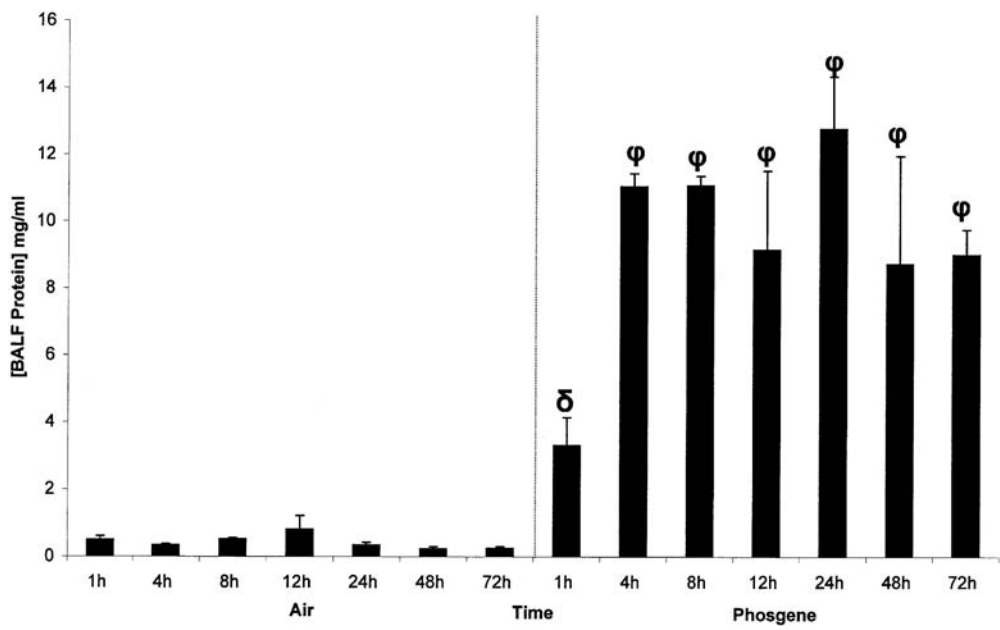


FIGURE 20.4 Phosgene exposure caused a highly significant, 4- to 10-fold increase in lung lavage protein concentration over the entire 72-h study compared with air-exposed controls, $p \leqslant 0.001$ (φ). All data are plotted as mean ± SEM. BALF, bronchoalveolar lavage fluid. (Figure adapted from Duniho et al., 2002.)

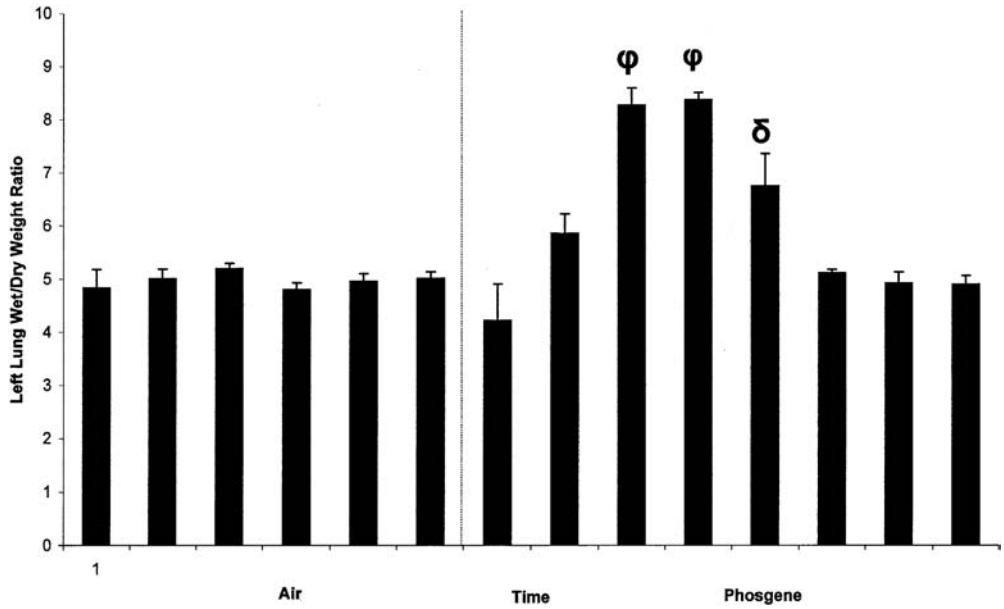


FIGURE 20.5 Phosgene exposure increases lung edema as measured by lung wet/dry weight (WW/DW) ratio compared with air-exposed controls. At both 4 and 8 h after exposure, WW/DW in the phosgene-exposed mice is significantly higher than in controls at matching time points, $p \leqslant 0.001$ (φ). Lung edema begins to resolve by 12 h where the significance level remains higher than in controls, $p \leqslant 0.005$ (δ). All data are plotted as mean ± SEM. (Figure adapted from Duniho et al., 2002.)

for phosgene-exposed mice than for the time-matched, air-exposed control group. At 8 and 12 h, IL-6, IL-1β, macrophage inflammatory protein 2 (MIP-2), and IL-10 were significantly higher in phosgene-exposed mice than in time-matched, air-exposed controls, whereas tumor necrosis factor α (TNFα) reached peak significance from 24 to 72 h. IL-4 was significantly lower in the phosgene-exposed mice than in the air-exposed mice from 4 to 8 h after exposure. These data show that lavage is an important tool in assessing pro- and anti-inflammatory markers of phosgene-induced acute lung injury and that knowledge of these temporal changes may allow for timely treatment strategies to be applied.

In a separate but similar mouse exposure model, lavage antioxidant enzymes glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), total GSH, and protein were determined from 1 to 72 h and at 7 d after phosgene exposure (Sciuto et al., 2003b). Phosgene exposure significantly enhanced both GPx and GR in phosgene-exposed mice compared with air-exposed mice from 4 to 48 h. Lavage GSH was also significantly increased between 4 and 24 h after exposure when compared with air-exposed mice. Lavage protein, an indicator of air–blood barrier integrity, was significantly higher than in air-exposed mice 4 h to 7 d after exposure. In contrast, lavage SOD was reduced by phosgene exposure from 4 to 24 h after exposure compared with air-exposed mice. Except for protein, all parameters returned to control levels by 7 d postexposure. These data indicate that the lung has the capacity to repair itself within 24–48 h after exposure by reestablishing a functional GSH redox system despite increased protein leakage. SOD reduction during increased leakage may indicate that barrier integrity is affected by superoxide anion production (Sciuto et al., 2003b).

One interesting aspect of lavage fluid analysis that has not been examined is the role of electrolytes in pulmonary edema formation and tissue injury following exposure to toxic gas. Na^+, Cl^-, K^+, and ionized Ca^{2+} concentrations were analyzed in lavage fluid and arterial blood in phosgene-exposed mice from 1 to 72 h (Sciuto et al., 2003c). Lavage Na^+ concentrations were significantly

higher in air than in phosgene-exposed mice at 4, 8, and 12 h postexposure, whereas both Ca^{2+} and K^+ were significantly higher in lavage fluid of phosgene-exposed mice than in that of air-exposed mice over 72 h. Significant changes in lavage K^+ and Ca^{2+} occurred as early as 4 h postexposure in phosgene-exposed mice when compared with air-exposed mice. Over time, there no significant changes were measured in arterial blood levels for Na^+, Cl^-, or Ca^{2+} for animals exposed to either air or phosgene. However, arterial K^+ concentrations in phosgene-exposed animals were significantly higher in phosgene-exposed mice than in the air-exposed animals across all time points with the highest K^+ levels of 7 mmol/l occurring at 8 and 24 h after exposure. Phosgene caused a time-dependent significant increase in lung edema from 4 to 12 h compared with air-exposed mice. It is evident from these experiments that blood K^+ levels measured as early as 1 h after exposure as well as lavage Na^+, K^+, and Ca^{2+} levels could serve as potential indicators of lung injury because both K^+ in lavage and blood and Ca^{2+} in lavage closely follow temporal increases in air–blood barrier permeability (edema) as measured by lung wet weight (Sciuto et al., 2003c).

20.6 MEDICAL EFFECTS

In terms of general population exposure estimates, the most quoted number comes from NIOSH where it has been projected that from 10,000 to 15,000 workers may have the potential for accidental exposure to phosgene (NIOSH, 1976). This includes firefighters, who are at risk because phosgene can be produced upon the thermal decomposition of chlorinated hydrocarbons (Noweir et al., 1973; Brown and Birky, 1980). Painters (Gerritsen and Buschman, 1960), car mechanics (Spolyor et al., 1950), and more recently welders (Nieuwenhuizen and Groeneveld, 2000) have also become exposed under some conditions. Commonly used industrial degreasers contain chlorinated hydrocarbons, such as perchloroethylene, which can form phosgene when heated or subjected to a heated surface.

Since World War I, the toxic effects of phosgene poisoning have been well documented (Gilchrist, 1933; Tobias, 1945; Bunting, 1945; Nash and Pattle, 1971). These biological effects have been observed in the industries previously described and are observed not only in humans but in various animals models as well (Boyland and McDonald, 1948; Rossing, 1964; Diller and Zante, 1982; Frosolono and Currie, 1985). However, little is known about the precise biochemical alterations induced by phosgene that lead to its latency and ultimate toxicity.

Upon initial exposure to phosgene, individuals may experience a range of symptoms. These include a decrease in systemic blood pressure, sinus and cardiac arrhythmias (Tobias, 1945; Brunner et al., 1948), shallow and frequent respiration, and decreases in vital capacity, respiratory

TABLE 20.5 Concentration–Effect Relationships of Phosgene Exposure

Perception of odor	>0.4 ppm
Recognition of odor	>1.5 ppm
Signs of irritation in eyes, nose, throat, and bronchi	>3 ppm
Beginning lung damage	>30 ppm·min
Clinical pulmonary edema	>150 ppm·min
L(CT)1	≈300 ppm·min
L(CT)50	≈500 ppm·min
L(CT)100	≈1300 ppm·min

Source: From Borak and Diller (2000).

volume, and arterial oxygen tension (Bunting, 1945; Everett and Overholt, 1968). With inhaled phosgene concentration >3 ppm (12 mg/m^3) there is a partial hydrolysis of phosgene to HCl that presumably is responsible for the initial irritation of mucous membrane surfaces of the eyes, nose, throat, and bronchi. A phosgene concentration >200 ppm (800 mg/m^3) may produce apnea of several seconds duration, bronchoconstriction (Banister et al., 1949), bronchial epithelium desquamation, and inflammation of the bronchi (Daly de Burg et al., 1946). Wells (1985) presents data on human exposures that indicate that pulmonary edema, arterial hypoxemia, and decreased fractional inspired oxygen (F_{IO_2}) were found. All patients exhibited initial symptoms of cough, chest tightness, and some nausea. Lung mechanics and volumes were negatively impacted by exposure within 1 d after exposure. Associated metabolic acidosis was treated with $NaHCO_3$ and pulmonary edema was corrected for by treatment with lasix. Borak and Diller (2000) identified several stages of graded and progressive physiological dysfunction as the phosgene concentration increases (Table 20.5).

During the past 20 years an immense effort has been made to identify treatments both preexposure and postexposure to ameliorate or reduce the effects of phosgene inhalation poisoning. These treatment therapies for phosgene exposure are used depending, of course, on the severity of exposure, because clinically there are no early diagnostic markers of exposure that would allow efficacious therapy to begin. The current dogma is to treat with the intravenous administration of sodium bicarbonate ($NaHCO_3$) and steroids. If severe exposure occurs and artificial ventilation is required, application of positive end expiratory pressure as well as O_2 therapy have been administered because patients have been found to be hypoxemic (Wells, 1985). Indeed, experiments on rodents have shown that a clear respiratory acidosis is present with increased P_{CO_2}, and decreased arterial pH and P_{O_2} that in mice lasts from about 4 to 12 h after exposure (Figures 20.6a–c). This effect of phosgene has also been seen in large-animal models such as the swine (Brown et al., 2002). The use of O_2 therapy, however, could be detrimental if continued for lengthy periods because its effects may exacerbate injury in a manner similar to that observed in paraquat poisoning. The current suggested treatment with steroids, such as methylprednisolone, involves intravenous (i.v.) therapy. However, in recent high-dose experiments in rabbits it was demonstrated that postexposure treatment with i.v. methylprednisolone was not effective (Kennedy et al., 1989). These data supported those of Mautone et al. (1985), who treated dogs with oral cortisone following exposure to phosgene and failed to observe any benefit.

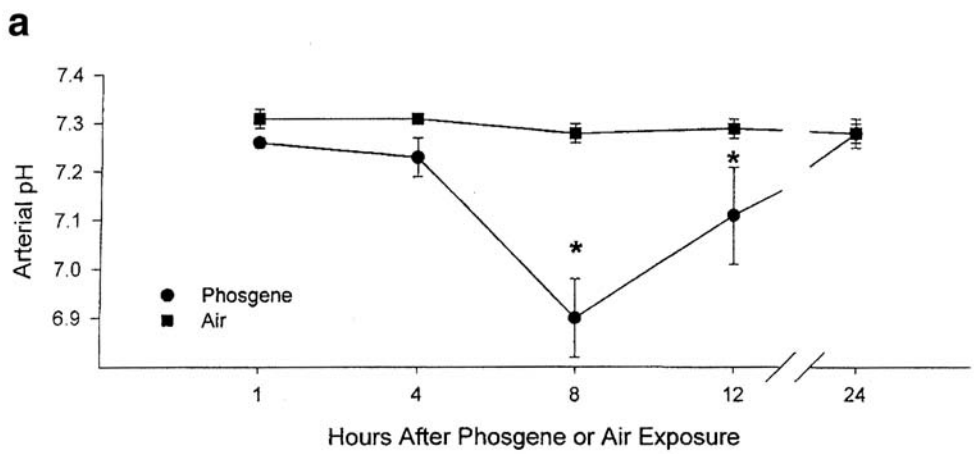


FIGURE 20.6a Temporal effects of phosgene inhalation on mouse arterial blood pH after a 20-min exposure to 32 mg/m^3 (8 ppm). Exposure to phosgene caused respiratory acidosis as early as 4 h after the start of exposure. At time points 8 and 12 h postexposure, phosgene-exposed mice had significantly lower arterial blood pH, $p < 0.05$ (*), respectively. Data are plotted as mean ± SEM. (Figure adapted from Sciuto et al., 2001.)

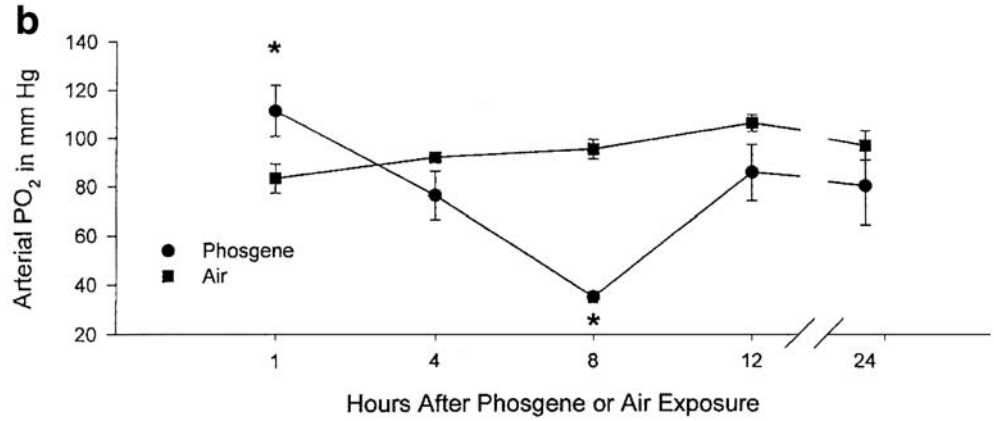


FIGURE 20.6b Temporal effects of phosgene inhalation on mouse arterial blood oxygen tension, Po_2, after a 20-min exposure to 32 mg/m^3 (8 ppm). Exposure to phosgene caused a marginal decrease in Po_2 beginning 4 h after the start of exposure compared with control. Similar to blood pH (Figure 6a), the most pronounced effect occurred at 8 h when Po_2 was significantly lower than the control value, $p < 0.05$ (*). Data are plotted as mean ± SEM. (Figure adapted from Sciuto et al., 2001.)

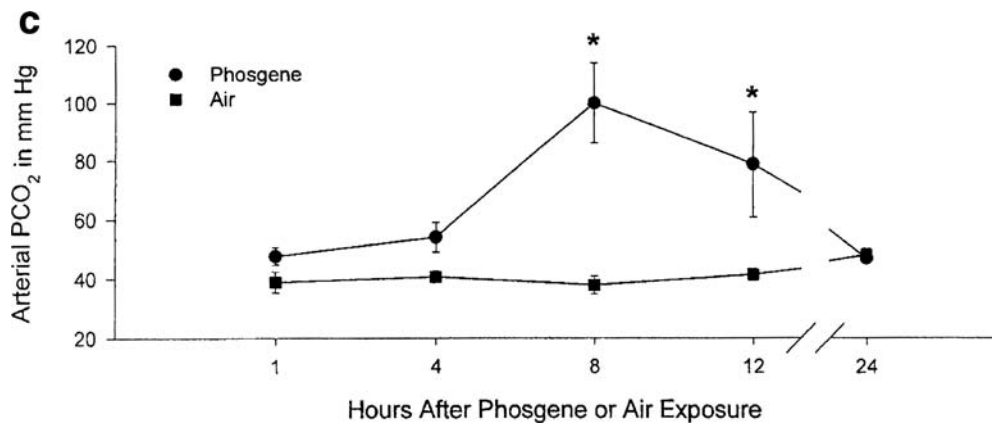


FIGURE 20.6c Temporal effects of phosgene inhalation on mouse arterial blood carbon dioxide tension, Pco_2, after a 20-min exposure to 32 mg/m^3 (8 ppm). Arterial blood Pco_2 begins to rise at 1 h, 47.7 ± 3 mmHg, and reaches a maximum of 99.7 ± 13.6 mmHg at 8 h, $p < 0.05$ (*), compared with the control levels of 38.4 ± 1.4 mmHg. Pco_2 begins to decrease to control levels at 12 h and reaches 47 ± 2 mmHg at 24 h. At 12 h Pco_2 levels remained significantly higher than controls, $p < 0.05$ (*). Data are plotted as mean ± SEM. (Figure adapted from Sciuto et al., 2001.)

20.6.1 Neurological Studies

Adverse pulmonary effects caused by phosgene inhalation may not be related to direct injury in the pulmonary tissue. Ivanhoe and Meyers (1964) showed that rabbits exposed to phosgene from 50 ppm (200 mg/m^3) for 14 min (700 ppm·min or 2800 mg·mg/min^3) to 200 ppm (400 mg/m^3) for 25 min (5000 ppm·min or 20,000 mg·mg/min^3) showed marked decreases in neurological sympathetic activity. They suggested that pulmonary edema associated with exposure resulted from neuroparalysis or a hypoactive sympathetic condition. This observation has yet to be confirmed by other investigators. Although, in an earlier study, Laqueur and Magnus (1921) exposed bilateral vagotomized cats to phosgene, and the pathophysiological response was not altered when compared with nonvagotomized animals. Anand et al. (1993) have shown in cats exposed through a range of phosgene

concentrations (320–1080 ppm or 1280–4320 mg/m^3) that C-fiber activation is enhanced during the course of enhanced capillary permeability. Recent work by Bauer et al. (1997) with guinea pigs exposed to phosgene for 10 min at concentrations ranging from 50 to 500 mg/m^3 (12.5–125 ppm) demonstrated that the tachykinin substance-P contributes not only to the induction of pulmonary edema, but also to the location of the lesion. These data suggest that nonadrenergic/ noncholinergic sensory mechanisms are activated by exposure to phosgene. Both the Anand and the Bauer studies strongly suggest that exposure to phosgene may be regulated through a sensory irritant neural pathway, which supports the respiratory dynamics data from Sciuto et al. (2002) in mice. Regarding the human exposure situation, few if any reports have appeared on the effects of phosgene exposure in the brain. The lone exception was an autopsy on a man who died 11 days after the May 20, 1928 phosgene exposure in Hamburg, Germany. The usual abnormalities were seen in the lung tissue, as would be expected; however, there were additional processes in brain gray matter and spinal cord as well as hyperemia and signs of bleeding in the white matter (EPA, 1993). At the time, it was believed that these were secondary to phosgene exposure. In recent studies in mice exposed to 32 mg/m^3 (8 ppm) phosgene for 20 min, brain tissue removed at 1, 4, 8, 12, 24, 48, and 72 h after exposure showed no light microscopic histopathological signs of toxicity when compared with brain tissue from the air-exposed controls (Sciuto, unpublished results, 2001).

20.7 CASE STUDIES

Phosgene gas has been and continues to be a potentially serious and occasionally fatal substance encountered in everyday life (Polednak and Hollis, 1985; Urbanetti, 1989). Polednak and Hollis did an extensive 30-year outcome review of over 800 Tennessee uranium-processing plant workers with known levels and duration of phosgene exposure from 1943 to 1945. Standardized mortality ratios for white males were assessed. They examined 694 males exposed to phosgene, which on occasion exceeded 1 ppm (4 mg/m^3) and compared them with 9280 controls. There were 107 respiratory diseases found in the exposed group versus 114 for controls. In an additional group of 106 males with a known acute exposure to high levels of phosgene, approximately 50 ppm (200 mg/m^3), there were 41 deaths versus an expected number of 34. Urbanetti (1989) discussed a case of accidental phosgene exposure resulting in a fatal outcome caused by a welding accident. This individual had been welding on a dipping basket for only one half-hour when symptoms of exposure began to appear. Following his release from the clinic, which initially determined that his chest x-rays were within normal limits, the patient was readmitted 3 h later when repeat x-rays showed substantial pulmonary edema with blood gases revealing a P_{O_2} of 30 mmHg and P_{CO_2} of 55 mmHg. Three days later the patient developed pneumonitis and respiratory failure that resulted in death.

Several single phosgene-exposure case reports have been published in the literature. Snyder et al. (1992) presented a case of possible phosgene exposure in a man who used a heat gun to scrape paint remover that contained methylene chloride (dichloromethane) from woodwork. The exposure was continuous over an 8-h period in an unventilated room. The patient exhibited all the typical signs of a phosgene exposure, i.e., chest discomfort, headache, and cough. Chest radiography revealed a slight increase in interstitial markings, and the patient was told to avoid paint remover exposure and was released. He returned the next day and examination showed that he suffered from diffuse wheezing with rales in all lung fields. His blood gases were pH 7.42, P_{CO_2} = 37 mmHg, and P_{O_2} was 67 mmHg, and a chest x-ray revealed diffuse interstitial edema and alveolar infiltrates. He improved over 48 h with oxygen supplementation and albuterol, with a chest x-ray showing a reduction of infiltrates. After one year the patient had episodic cough with wheezing and breathlessness that improved with albuterol. A methacholine challenge showed a hyperactive airway dysfunction.

Wyatt and Allister (1995) discuss the case of a welder who was exposed to a phosgene concentration that resulted from using a hot welding torch to cut through a refrigeration pipe that contained the freon chlorodifluoromethane. His symptoms were believed to be consistent with exposure to a concentration that exceeded 3 ppm (12 mg/m^3) based on the chart for exposure-response effects

(Table 20.5). He was dyspneic and tachycardic and suffered from pharyngeal erythema. Otherwise, electrocardiogram, chest x-ray, and arterial gases were within normal limits. The patient was discharged after 24 h observation complaining of lethargy and exertional dyspnea until his return to work 2 weeks later.

Chronic industrial exposure to phosgene is believed to cause reactive airways dysfunction syndrome (RADS) (Alberts and do Pico, 1996) and irritant-induced occupational asthma (Tarlo and Broder, 1989) and has been implicated in people suffering from multiple system atrophy (Hanna et al., 1999). Hanna and workers reviewed a case involving a chemical plant supervisor who developed parkinsonian clinical features following a 20-year history of daily exposure to phosgene, methyl isobutyl ketone, benzene, formaldehyde, and other solvents. It must be kept in mind that the exposure to phosgene cannot be the single causative agent under these multiple-chemical exposure conditions. However, the additive or synergistic effect of phosgene along with these other reagents is not known and has never been experimentally tested. The Hanna study corroborates an earlier clinical assessment of industrial plant workers by Callender et al. (1993). They provided some evidence of toxic encephalopathy and Parkinson disease in workers years after exposure to multiple solvents but to phosgene as well. However, caution should be exercised because as in the previous study long-term exposure to multiple solvents in an industrial environmental could confound the role of phosgene as a single causative agent of neurotoxicity.

Accidental phosgene exposure has been reported from international sources; however, these reports are few, especially from countries where release of such information is suppressed and where proper industrial controls are poorly implemented or nonexistent. Lihua et al. (1998) reported from China an analysis of 25 acute gas exposures during occupational accidents. These data cover poisonings from the city of Hangzhou between 1984 and 1996. They investigated poisoning from chemical industry, light industry, and the mining industry. There were 10 kinds of inhalation poisons with the top 5 being hydrogen sulfide, benzene, carbon monoxide, phosgene, and organophosphorus pesticides. Phosgene accounted for 12% of the accidents and 10% of the industrial poisonings. Although no associated mortalities resulted from phosgene exposure, it was the only industrially released gas that affected the general population when more than 20 kindergarten children were exposed during one accidental release. There was no mention of the outcome of their exposure. The authors stated that the top 5 gases released in Hangzhou City reflected the national picture for accidental industrial release. The percentage of industrial phosgene exposures observed by Lihua in China matches that described earlier in France by Faure et al. (1970). They showed that of the 99 industrial gas poisonings in the Lyon and Grenoble regions of France,12 were due solely to phosgene.

As can be seen from the previous discussions, all research was ultimately concerned with assessing mechanisms of toxicity in relation to two important themes: (1) determination of the reason(s) for the latency phase between exposure and the clinical appearance of toxicity and (2) development of appropriate medical countermeasures to treat victims of exposures. Although there have been some successes in the use of FDA-approved clinical drugs to delay or reduce toxicity in experimental models (discussed previously), there is limited evidence that these have been tested in cases of human exposure. Many of these studies were involved with postchallenge treatment to more closely mimic real case clinical situations observed in exposure-response-treatment approaches.

20.8 RISK ASSESSMENT

Phosgene is classified as a choking agent and irritant. It is regulated by the federally mandated Clean Air Act (1990) Amendment, Title I, Part A Section 112b. The single most difficult task establishing human risk assessment for phosgene exposures involves observational support backed by actual human exposure data. The latter is problematic in that estimation of exposure concentration and duration are rarely well established. As seen from the case studies, none of these variables is known with any degree of certainty, which makes definitive assessment of exposure for how long, to how much, or even to what very difficult to establish and quantify. Obviously, these are

extremely important parameters to know because they set the stage for and are directly responsible for the progression of acute lung injury from mild effects to even death. Currently, there are no early diagnostic markers of phosgene exposure for the emergency room physician to test. One recent experimental study in mice by Sciuto et al. (2003b) showed that arterial blood and lavage levels of K^+ are elevated within 1 h of exposure and may serve as an early marker of exposure, although it would not be specific for phosgene. Furthermore, Sciuto et al. (1996b) also showed that if the exposure is severe enough, phosgene could actually cause erythrocyte hemolysis as measured directly in the arterial blood. These results have been supported to a limited extent by showing that *in vitro* phosgene can form adducts with amino acid complexes of hemoglobin and albumin (Noort et al., 2000). However, all the results from this cited work are from carefully controlled laboratory experiments on rodents. If the dose of phosgene tested were based on body mass then an equivalent exposure in humans would necessarily have to be quite high. Therefore, caution must be used when extrapolating results from animals to humans. Another issue to consider would be minute ventilation of the test animal. The relationship between body mass and the product of the tidal volume and the respiratory rate is crucial, because this ultimately determines the amount of gas inhaled and therefore the dose. Based on body weight, rodents, especially mice, have a minute ventilation to body weight ratio at least five times that of a human, and rats about two to three times greater. Bide et al. (2000) raise these issues in their work on minute ventilation allometric estimates in mammals. Mautone and coworkers (1985) also raised this issue when they expressed inhaled phosgene concentration as an "exposure index" that took into account the relationship between body weight and inhaled gas volume. In earlier experiments, it was clearly demonstrated that when mice, rats, or guinea pigs were exposed to precisely equivalent phosgene concentrations, the hemolytic response due to exposure was inversely related to the mass of the rodent, i.e., as the mass increased the physiological response decreased (Sciuto et al., 1996b).

Case reports of actual phosgene exposures are not plentiful and, in many instances, could have been misdiagnosed as a genuine phosgene exposure. In the first large-scale human assessment of exposure, Gilchrist gave an excellent review of the medical follow-up of soldiers suspected of battlefield exposures to phosgene during WWI (Gilchrist, 1933). Veterans had presented with many of the classic signs of a phosgene exposure. However, the late sequelae described by Gilchrist could have been confounded by the possibility of exposure to a mixture of gases such as phosgene and chlorine or vesiccating agents as well as by a preexisting history of lung disease such as asthma and/or smoking habits.

In determining the risk of toxicity for humans following a phosgene exposure event, several issues need to be addressed. As has been pointed out, risk assessment in toxicology is a process whereby available and relevant biological, dose–response, and exposure data lead to a qualitative or quantitative estimate of an adverse outcome from exposure (Scala, 1991). Many of these estimates come largely from estimating human cancer risk following chemical exposures. As seen in the case studies cited earlier, neither dose–response nor exposure data were determined in nearly all instances nor was phosgene identified as the sole causative agent. Another aspect of risk is the characterization of the actual risk itself. Basically, this is the estimate of the incidence of an adverse effect of a phosgene exposure on a general or specific population. In the case of phosgene, although there have been few documented accidental releases of phosgene into a neighborhood as cited in the Hamburg or Bhopal incidents, it is the industrial worker, welder, fireman, etc., who are at the greatest risk of an accidental exposure. Moreover, exposure to phosgene itself can never be precisely known outside of a chemical plant where it is used in production processes and workers wear personal dosimeter badges. With regard to possible exposure during fire, it would be difficult to know whether the combusting material produces solely phosgene or a mixture of phosgene and other gases such as the Teflon® by-product perfluoroisobutylene, a gas that causes exposure sequelae nearly identical with those of phosgene (Lehnert et al., 1995; van Helden et al., 2004).

The issue of risk for phosgene exposure is discussed to some extent in the EPA health assessment document (EPA, 1993). Recently the EPA released data regarding acute exposure guideline

TABLE 20.6 Acute Exposure Guideline Levels (AEGL) Values for Phosgene[a]

	10-min	30-min	1-h	4-h	8-h	End point (Reference)
AEGL-1	NR	NR	NR	NR	NR	Not recommended (NR) due to lack to appropriate data and clinical latency period
AEGL-2	0.60 (2.4)	0.60 (2.4)	0.30 (1.2)	0.08 (0.32)	0.04 (0.16)	Chemical pneumonia in rats (Gross et al., 1965)
AEGL-3	3.6 (14.4.)	1.5 (6.0)	0.75 (3.0)	0.20 (0.8)	0.09 (0.36)	Highest concentration causing mo mortality in the rat after 30- or 10-min exposure (Zwart et al., 1990)

[a] Values are in ppm (mg/m^3).

Source: Modified from Bast and Bress (2003).

levels (AEGLs). AEGLs attempt to characterize the toxicity of one-time exposure limitations for the general population ranging from 10 min to 8 h (Bast and Bress, 2002). Data were generated by using an estimate published by ten Berge et al. (1986), in which, in place of Haber's $C \times T = K$, the data more closely fit the dose–factor relationship $C^n \times T = K$. An AEGL level 1 is the airborne concentration (mg/m^3 or ppm) that is predicted to cause some level of notable discomfort, irritation, or nonsensory effects that are reversible at the cessation of exposure. AEGL level 2 is the airborne concentration above which the general population and susceptible individuals could experience irreversible long lasting effects. AGEL level 3 is the airborne concentration of a substance above which it is predicted that the general population and certain individuals could experience a life-threatening event or death. Table 20.6, amended from Bast and Bress (2003), summarizes AEGL levels 1–3. They do not recommend establishing AEGL 1 values for phosgene because of the lack of sufficient animal studies. As can be seen from Table 20.6, AEGL values from 10- to 30-min exposures are well above the NIOSH established values of an 8-h time-weighted average of 0.1 ppm (0.4 mg/m^3). AEGL values were based on animal studies, which can be problematic in extrapolation to human exposure limits when considering the discussion above about the use of minute volume to body weight ratio promoted by Bide et al. (2000). Risk assessments from low-dose animal studies can be further confounded not only by the issue raised by Bide et al., but there could be differences in metabolism, exposure rates, genetic differences, and ranges of sensitivities as well. Data generated from animals consistently come from controlled-exposure conditions and nearly always to a single agent, both conditions that are unlikely to be duplicated in accidental environmental exposures. It would be difficult to assign a valid risk assessment for exposure guidelines for the general population for phosgene. At the present time, industry is considering the development of a database of past and present industrial exposure accidents. These data may be used in the future to arrive at a realistic risk for exposure to phosgene and possibly arrive at statistically based estimates of exposure-effects-responses that will be ultimately transitioned into beneficial treatment strategies.

20.9 CONCLUSIONS

The treatise above has spanned a large knowledge base regarding the history, experimental mechanistic toxicology, limited case summaries, and attempts at a risk assessment for phosgene inhalation. Although there remains no actual treatment against phosgene inhalation poisoning, the historical record indicates that large-scale exposures of the general public, while possible, are becoming less likely as safety controls and industrial processes improve. However, this does not rule out an intentional or terrorist event that could affect many people and cause a triage nightmare for local health authorities. Mechanistically, a great deal of recent data suggests that phosgene causes a multitude of adverse genetic, cellular, and systemic responses, especially at the air–tissue interface in the peripheral regions of the lung. These effects are seen across species and through a range of concentrations.

The singular and most important issue regarding phosgene inhalation is that is it very difficult to clinically diagnose or assess whether exposure has occurred and the extent of this exposure. As pointed out, experimental screening techniques such as blood electrolyte levels may show some promise. However, they are unlikely to be phosgene specific, because gases with comparable chemical reactivity and solubility react in a mechanistically similar manner. Assigning a risk of potential serious health effects for the general public for a phosgene release at this time is probably not practical as evidenced by the massive accidental release in Germany 75 years ago and by the EPA definitions of AEGLs. From the clinical data above, exposure to phosgene primarily occurs on an individual basis with many exposures from home-related activities. In conclusion, limited work will continue on the investigation of the early diagnosis of and treatments against phosgene-induced lung injury. It is believed that successful medical countermeasures, while not specific for phosgene, would be useful against any other mechanistically, comparably acting compound.

ACKNOWLEDGMENTS

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. In conducting the research described in this report, the investigator adhered to the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

REFERENCES

Alberts, W.M. and do Pico, G.A., Review: reactive airways dysfunction syndrome, *Chest.* 109, 1618–1626, 1996.

Anand, A., Paintal, A.S., and Whitteridge, D., Mechanisms underlying enhanced responses of J receptors of cats to excitants in pulmonary edema, *J. Physiol. (Lond.),* 471, 535–547, 1993.

Arroyo, C.M., Feliciano, F., Kolb, D.L., Keeler, J.R., Millette, S.R., and Stotts, R.R., Autoionization reaction of phosgene (OCCl2) studied by electron paramagnetic resonance/spin trapping techniques, *J. Biochem. Toxicol.* 8, 107–110, 1993.

Babad, H. and Zeiler, A.G., The chemistry of phosgene, *Chem. Rev.*, 73, 75–91, 1973.

Banister, J., Fegler, G., and Hebb, C., Initial respiratory responses to the intratracheal inhalation of phosgene, *Q. J. Exp. Physiol.*, 35, 233–250, 1949.

Bast, C.B. and Bress, W., Phosgene: acute exposure guideline levels (AEGLs) for phosgene, in *Acute Exposure Guideline Levels for Selected Airborne Chemicals*, Vol. 2, National Research Council, The National Academies Press, Washington, DC, 2002, pp. 15–70.

Bast, C.E., Bress, W., Garrett, R., Krewski, D., and Bakshi, K., Acute exposure guideline levels (AEGLs) for phosgene (abstr. 778), *Toxicol. Sci.,* 72 (Suppl 1), 160, 2003.

Bauer, R.M., Young, G.D., and Keeler, J.R., Tachykinins and pulmonary edema after acute phosgene exposure in guinea pigs, *J. Am. Coll. Toxicol.,* 15 (Suppl. 2), S99–S112, 1997.

Bide, R.W., Armour, S.J., and Yee, E., Allometric respiration/body mass data for animals to be used for estimates of inhalation toxicity to young adult humans, *J. Appl. Toxicol.*, 20, 273–290, 2000.

Borak, J. and Diller, W.F., Phosgene exposure: Mechanisms of injury and treatment strategies, *J. Occup. Environ. Med.*, 43, 110–119, 2000.

Boyland, E. and McDonald, E.F., The metabolism of the lungs from normal mice and from mice exposed to phosgene, *Biochem. J.*, 42, 68–71, 1948.

Brown, J.E. and Birky, M.M., Phosgene in the thermal decomposition products of poly (vinylchloride): generation, detection, and measurement, *J. Anal. Toxicol.*, 4, 166–174, 1980.

Brown, R.F.R., Jugg, B.J.A., Harban, F.M.J., Ashley, Z., Kenward, C.E., Platt, J., Hill, A., Rice, P., and Watkins, P.E., Pathophysiological responses following phosgene exposure in the anesthetized pig, *J. Appl. Toxicol.*, 22, 263–269, 2002.

Brown. R.F.R., Jugg, B.J., Harban, F.M., Platt, J., and Rice, P., Pathological improvement following protective ventilation strategy in an inhalationally induced lung injury model, *Am. J. Resp. Crit. Care Med.*, 167, A775, 2003.

Brunner, H.D., Boche, R.D., Gibbon, M.H., and McCarthy, M.D., Electrographic study of the heart and effect of vagotomy in phosgene poisoning, *Proc. Soc. Exp. Biol. Med.*, 68, 279–281, 1948.

Bunting, H., Changes in the oxygen saturation of the blood in phosgene poisoning, in *Fasiculus on Chemical Warfare,* Vol. II, *Respiratory Tract,* p. 484, National Research Council on Treatment of Gas Casualties, Washington, DC, 1945.

Burleson, G.R. and Keyes, L.L., Natural killer activity in fischer-344 rat lungs as a method to assess pulmonary immunocompetence: Immunosuppression by phosgene inhalation, *Immunopharmacol. Immunotoxicol.*, 11, 421–443, 1989.

Callender, T.J., Morrow, L., Subramanian, K., Duhon, D., and Ristovv, M., Three-dimensional brain metabolic imaging in patients with toxic encephalopathy, *Environ. Res.*, 60, 295–319, 1993.

Chemical Market Report, Chemical Profile for Phosgene, June 21, 1999.

Cucinell, S.A., Review of the toxicity of long-term phosgene exposure, *Arch. Environ. Health*, 28, 272–275, 1974.

Currie, W.D., Hatch, G.E., and Frosolono, M.F., Changes in lung ATP concentration in the rat after low-level phosgene exposure, *J Biochem Toxicol.*, 2, 105–114, 1987a.

Currie, W.D., Hatch, G.E., and Frosolono, M.F., Pulmonary alterations in rats due to phosgene inhalation, *Fundam. Appl. Toxicol.*, 8, 107–114, 1987b.

Currie, W.D., Pearlstein, R.D., Devlin, R.B., Becker, S., and Hatch, G.E., Effect of surfactant replacement on airway cytokine release after phosgene inhalation in rats (abstr.), *Am. J. Repsir. Crit. Care Med.*, 155 (Part II), A960, 1997.

Daly de Burg, I., Eggleton, B., Elsden, S.R., and Hebb, C.O., A biochemical study of isolated perfused lungs with special reference to the effects of phosgene, *Q. J. Exp. Physiol.*, 33, 215–240, 1946.

DeCurtis, V., Gemma, S., Sbraccia, M., Testai, E,. and Vittozzi, L., The contribution of electrophilic and radalic intermediates to phospholipid adducts formed by halomethanes *in vivo, J. Biochem. Toxicol.*, 9, 305–310, 1994.

Diller, W.F., Medical phosgene problems and their possible solutions, *J. Occup. Med.*, 20, 189–193, 1978.

Diller, W.F. and Zante, R., Dosis-wirkungs-beziehungen bei phosgeneinwirkung auf mensch und tier, *Zentbl. Arbmed. Arbshutz. Prophyl. Ergon.*, 32, 360–368, 1982.

Diller, W.F., Bruch, J., and Dehnen, W., Pulmonary changes in the rat following low phosgene exposure, *Arch. Toxicol.*, 57, 184–190, 1985.

Duniho, S.M., Martin, J., Forster, J.S., Cascio, M.B., Moran, T.S., Carpin, L.B., and Sciuto, A.M., Acute changes in lung histopathology and bronchoalveolar lavage parameters in mice exposed to the choking agent phosgene, *Toxicol. Pathol.*, 30, 339–349, 2002.

Everett, E.D. and Overholt, E.L., Phosgene poisoning, *JAMA*, 205, 243–245, 1968.

Faure, J., Sibille, M., Faure, H., Stephan, C., Yacoub, M., and Motin, J., L'Intoxication aigue par le chlore et le phosgene (etude clinique et experimentale), *Le Poumon et le Coeur. V*, 26, 913–929, 1970.

Frosolono, M. and Currie, W.D., Response of the pulmonary surfactant system to phosgene, *Toxicol. Ind. Health*, 1, 29–35, 1985.

Frosolono, M.F. and Pawlowski, R., Effect of phosgene on rat lungs after single high-level exposure. I. Biochemical alterations, *Arch. Environ. Health*, 32, 271–277, 1977.

Gelzleichter, T.R., Witschi, H., and Last, J.A., Concentration-response relationships of rat lung to exposure to oxidant air pollutants: a critical test of Haber's Law for ozone and nitrogen dioxide, *Toxicol. Appl. Pharmacol.*, 112, 73–80, 1998.

Gerritsen, W.B. and Buschman, C.H., Phosgene poisoning caused by the use of chemical paint removers containing methylene chloride in ill-ventilated rooms heated by kerosene stoves, *Br. J. Ind. Med.*, 17, 187–189, 1960.

Ghio, A.J., Kennedy, T.P., Hatch, G.E., and Tepper, J.S., Reduction of neutrophil influx diminishes lung injury and mortality following phosgene inhalation,. *J. Appl. Physiol.*, 71, 657–665, 1991.

Ghio, A. and Hatch, G., Colchicine inhibits elevations in both alveolar-capillary membrane permeability and lavage surfactant after exposure to phosgene, *Inhalation Toxicol.*, 4, 383–392, 1992.

Gibbon, M.H., Brunner, H.D., Boche, R.D., and Lockwood, J.S., Studies on experimental phosgene poisoning, *J. Thorac. Surg.*, 17, 264–273, 1948.

Gilchrist, H.L., The residual effects of warfare gases: the use of phosgene gas, with report of cases, *Med. Bull. Vet. Admin.*, 10, 1–36, 1933.

Guastadisegni, C., Balduzzi, M., and Vittozzi, L., Preliminary characterization of phospholipid adducts by [^{14}C]-CHCl3 reactive intermediates in hepatocyte suspensions, *J. Biochem. Toxicol.*, 11, 21–25, 1996.

Guastadisegni, C., Guidoni, L., Balduzzi, M., Viti, V., DiConsiglio, E., and Vittozzi, L., Characterization of a phospholipid adduct formed in Sprague Dawley rats by chloroform metabolism: NMR studies, *J. Biochem. Mol. Toxicol.*, 12, 93–102, 1998.

Guo, Y.-L., Kennedy, T.P., Michael, J.R., Sciuto, A.M., Ghio, A., Adkinson, N.F., Gurtner, G.H., Mechanisms of phosgene-induced toxicity: role of arachidonate mediators, *J Appl Physiol.*, 69, 1615–1622, 1990.

Haber, F.R., Zur geschichte des gaskrieges [On the history of the gas war], in *Fuenf Vortraege aus den Jahren 1920–23* (Five lectures from the years 1920–1923). Verlag von Julius Springer, Berlin, 1924. pp. 76–92.

Hanna, P.A., Jankovic, J., and Kirkpatrick, J.B., Multiple system atrophy: the putative causative role of environmental toxins, *Arch. Neurol.*, 56, 90–94, 1999.

Hegler, C., Ueber eine Massenvergiftung durch Phosgengas in Hamburg. I. Klinische Beobachtungen [on the mass poisoning by phosgene in Hamburg]. I. Clinical Observations, *Dtsch. Med. Wochenschr.*, 54, 1551–1553, 1928.Ivanhoe, F. and Meyers, F.H., Phosgene poisoning as an example of neuroparalytic acute pulmonary edema: The sympathetic vasomotor reflex involved, *Dis. Chest*, 46, 211–218, 1964.

Jackson, K.E., Phosgene. *J. Chem. Educ.*, Oct., 622–626, 1933.

Jugg, B., Jenner, J. and Rice, P., The effect of perfluoroisobutene and phosgene on rat lavage fluid surfactant phospholipids, *Hum. Exp. Toxicol.*, 118, 659–668, 1999.

Karalliedde, L., Wheeler, H., Maclehose, R., and Murray, V., Possible immediate and long-term health effects following exposure to chemical warfare agents, *Public Health*, 114, 238–248, 2000.

Keeler, J.R., Hurt, H.H., Nold, J.B., and Lennox, W.J., Estimation of the LCt50 of phosgene in sheep, *Drug Chem. Toxicol.*, 13, 229–239, 1990.

Kennedy, T.P., Michael, J.R., Hoidal, J.R., Hasty, D., Sciuto, A.M., Hopkins, C., Lazar, R., Bysani, G.K., Tolley, E., and Gurtner, G.H., Dibutyryl cAMP, aminophylline, and β-adrenergic agonists protect against edema caused by phosgene, *J. Appl. Physiol.*, 67, 2542–2552, 1989.

Kennedy, T.P., Rao, N.V., Noah, W., Michael, J.R., Jafri, M.K., Gurtner, G.H., and Hoidal, J.R., Ibuprofen prevents oxidant lung injury and *in vitro* lipid peroxidation by chelating iron, *J. Clin. Investig.*, 86, 1565–1573, 1990.

Kodavanti, U.P., Costa, D.L., Giri, S.N., Starcher, B., and Hatch, G.E, Pulmonary structural and extracellular matrix alterations in Fisher 344 rats following subchronic phosgene exposure, *Fundam. Appl. Toxicol.*, 37, 54–63, 1997.

Krajewski, J.A., Chemical accidents and catastrophes as a source of the greatest hazard to the environment and human health, *Med. Pract.*, 48, 93–103, 1997.

Laqueur, E. and Magnus, R., Uber kampfgasvergiftung. III. Experimentelle pathologie der phosgenvergiftung, *Z. Gesamte Exp. Med.*, 13, 200–290, 1921.

Lehnert, B.E., Archuleta, D., Gurley, L.R., Session, W., Behr, M.J., Lehnert, N.M., and Stavert, D.M., Exercise potentiation of lung injury following inhalation of a pneumoedematogenic gas: perfluoroisobutylene, *Exp. Lung Res.*, 21, 331–350, 1995.

Lihua, W., Xiaozhu, D., and Haiyan, Z., Analysis of 25 episodes of acute occupational poisoning accidents and studies on its preventive strategy, *Chung-hua yu fang hsueh tsa chih*, 32, 235–237, 1998.

Madden, M.C., Friedman, M., Keyes, L.L., Koren, H.S., and Burleson, G.R., Effects of phosgene exposure on lung aracidonic acid metabolism, *Inhalation Toxicol.*, 3, 73–90, 1991.

Mautone, A.J., Katz, Z., and Scarpelli, E., Acute responses to phosgene inhalation and selected corrective measures (including surfactant), *Toxicol. Ind. Health*, 1, 37–57, 1985.

Meek, W.J. and Eyster, J.A.E., Experiments on the pathologic physiology of acute phosgene poisoning, *Am. J. Physiol.*, 51, 303, 1920.

Menkin, V., Isolation properties of a factor responsible for increased capillary permeability in inflammation, *Proc. Soc. Exp. Biol. Med.,* 36, 164–167, 1937.

Nash, T. and Pattle, R.E., The absorption of phosgene by aqueous solutions and its relation to toxicity, *An. Occup. Hyg.*, 14, 227–233, 1971.

National Institute for Occupational Safety and Health (NIOSH), Occupational Exposure to Phosgene, Publication 76–137, U.S. Department of Health, Education and Welfare, U.S. Government Printing Office, Washington. DC, 1976.

Nieuwenhuizen, M.S. and Greoenveld, F.R., Formation of phosgene during welding activities in an atmosphere containing chlorinated hydrocarbons, *Am. Ind. Hyg. Assoc. J.*, 61, 539–543, 2000.

Noort, D., Hulst, A.G., Fidder, A., van Gurp, R.A., de Jong, L.P.A., and Benschop, H.P., In vitro formation of phosgene with albumin and hemoglobin in human blood, *Chem. Res. Toxicol.*, 13, 719–726, 2000.

Noweir, M.H., Pfitzer, E.A., and Hatch, T.F., Thermal decomposition of CCl4 vapors at its industrial limit concentration, *Am. J. Hyg. Assoc.*, 34, 25–37, 1973.

Pawlowski, R., and Frosolono, M.F., Effect of phosgene on rat lung after a single high level exposure. II. Ultrastructural alterations, *Arch. Environ. Health*, 32, 278–283, 1977.

Polednak, A.P. and Hollis, D.R., Mortality and cause of death among workers exposed to phosgene in 1943–45, *Toxicol. Ind. Health*, 1, 137–151, 1985.

Potts, A.M., Simon, W.S., and Gerard, R.W., The mechanism of phosgene and diphosgene, *Arch. Biochem.,* 24, 329–337, 1949.

Rhinehart, W.E. and Hatch, T., Concentration-time product (CT) as an expression of dose in sublethal exposures to phosgene, *Am. Ind. Hyg. Assoc. J.*, 25, 545–553, 1964.

Rooney, S.A., Phospholipid composition, biosynthesis, and secretion, in *Treatise on Pulmonary Toxicology: Comparative Biology of The Normal Lung*, Vol. 1, Parent, R., Ed., CRC Press, Boca Raton, FL, 1992, pp. 511–544.

Rorison, D.G. and McPherson, S.J., Acute toxic inhalations, *Environ. Emerg.*, 10, 409–435, 1997.

Rossing, R.G., Physiological effects of chronic exposure to phosgene in dogs, *Am. J. Physiol.*, 207, 265–272, 1964.

Scala, R.A., Risk assessment, in *Toxicology—the Basic Science of Poisons*, 4th ed., Amdur, M.O., Doull, J., and Klaassen, C.D., Eds., Pergamon Press, New York, 1991, pp. 985–996.

Schelble, D.T., Phosphine and phosgene, in *Clinical Management of Poisoning and Drug Overdose*, 2nd ed., Haddad, L.M. and Winchester, J.F., Eds., W.B. Saunders Co., Philadelphia, 1990, pp. 1235–1240.

Schroeder, S. and Gurtner, G.H., Evidence for a species difference in susceptibility and mechanism of phosgene toxicity between rabbit and dogs (abstr.), *Am. Rev. Repsir. Dis.*, 145 (Part II), A606, 1992.

Sciuto, A.M., Ibuprofen treatment enhances the survival of mice following exposure to phosgene, *Inhalation Toxicol.*, 9, 389–403, 1997a.

Sciuto, A.M., Assessment of acute lung injury in rodents exposed to phosgene, *Arch. Toxicol.*, 72, 283–288, 1998.

Sciuto, A.M., Carpin, L., Moran, T.S., and Forster, J.S., Chronological changes in electrolyte levels in arterial blood and broncheoalveoler lavage fluid in mice after exposure to an edemagenic gas, *Inhalation Toxicol.*, 15, 663–674, 2003c.

Sciuto, A.M., Cascio, M.B., Moran, T.S., and Forster, J.S., The fate of antioxidant enzymes in bronchoalveolar lavage fluid over 7 days in mice with acute lung injury, *Inhalation Toxicol.*, 15, 675–685, 2003b.

Sciuto, A.M., Clapp, D.L., Hess, Z.A., and Moran, T.S., The temporal profile of cytokines in the bronchoalveolar lavage fluid in mice exposed to the industrial gas phosgene, *Inhalation Toxicol.*, 15, 687–700, 2003a.

Sciuto, A.M., Lee, R.B., Forster, J.S., Cascio, M.B., and Moran, T.S., Temporal changes in respiratory dynamics in mice exposed to phosgene, *Inhalation Toxicol.*. 14, 487–501, 2002.

Sciuto, A.M. and Moran, T.S., BHA diet enhances the survival of mice exposed to phosgene: the effect of BHA on glutathione levels in the lung, *Inhalation Toxicol.*, 11, 855–871, 1999.

Sciuto, A.M., Moran, T.S., Narula, A., and Forster, J.S., Disruption of gas exchange in mice following exposure to the chemical threat agent phosgene, *Military Med.*, 166, 809–814, 2001.

Sciuto, A.M., Phillips, C.S., Dorsch, L.M., Hege, A.I., Moran, T.S., and Dillman, J.D., III., Genomic analysis of murene pulmonary tissue following carbonyl chloride inhalation, *Chem. Res. Toxicol.,* in press.

Sciuto, A.M., Stotts, R.R., Chittenden, V., Choung, E., and Heflin, M., Spectrophotometric changes in absorbance at 412–415 nm in plasma from three rodent species exposed to phosgene, *Biochem Biophys. Res. Commun.*, 226, 906–911, 1996b.

Sciuto, A.M., Stotts, R.R., and Hurt, H.H., The efficacy of ibuprofen and pentoxifylline in the treatment of phosgene-induced acute lung injury, *J. Appl. Toxicol.*, 16, 381–834, 1996c.

Sciuto, A.M., Strickland, P.T., and Gurtner, G.H., Postexposure treatment with isoproterenol attenuates pulmonary edema in phosgene-exposed rabbits, *J. Appl. Toxicol.*, 18, 321–329, 1998.

Sciuto, A.M., Strickland, P.T., Kennedy, T.P., Guo, Y.-L., and Gurtner, G.H., Intratracheal administration of Dibutyryl cAMP attenuates edema formation in acute phosgene-induced lung injury, *J. Appl Physiol.*, 80, 149–157, 1996a.

Sciuto, A.M., Strickland, P.T., Kennedy, T.P., and Gurtner, G.H., Protective effects of N-acetylcysteine treatment after phosgene exposure in rabbits, *Am. J. Respir. Crit. Care Med.*, 151, 768–772, 1995.

Sciuto, A.M., Strickland, P.T., Kennedy, T.P., and Gurtner, G.H., Postexposure treatment with aminophylline protects against phosgene-induced acute lung injury, *Exp. Lung Res.*, 23, 317–332, 1997b.

Selgrade, M.K., Starnes, D.M., Illing, J.W., Daniels, M.J., and Graham, J.A., Effects of phosgene exposure on bacterial, viral, and neoplastic lung disease susceptibility in mice, *Inhalation Toxicol.*, 1, 243–259, 1989.

Singh, H.B., Phosgene in the ambient air, *Nature*, 264, 428–429, 1976.

Snyder, R.W., Mishel, H.S., and Christensen, G.C., Pulmonary toxicity following exposure to methylene chloride and its combustion product, phosgene, *Chest*, 101, 860–861, 1992.

Spiers, E.M., *Chemical Warfare*, University of Illinois Press, Urbana, 1986, 277 pp.

Spolyar, L.W., Harper, R.N., Keppler, J.F., and Bumsted, H.E., Generation of phosgene during operation trichloroethylene degreaser, *Arch. Ind. Hyg.*, 4, 156–160, 1950.

Tarlo, S.M. and Broder, I., Irritant-induced occupational asthma, *Chest*, 96, 297–300, 1989.

tenBerge, W.F., Zwart, A., and Appleman, L.M., Concentration-time mortality response relationship of irritant and systemically acting vapours and gases, *J. Hazardous Mater.*, 13, 301–309, 1986.

Tobias, J.M., The pathological physiology of the lung after phosgene, in *Fasciulus on Chemical Warfare Medicine*, Vol. II. *Respiratory Tract*, National Research Council Committee on Treatment of Gas Casualties, Washington, DC, 1945, p. 331.

Trethewie, E.R., Pharmacology of phosgene, *Med. J. Aust.*, 20, 612–618, 1947a.

Trethewie, E.R., Histamine and phosgene poisoning, *Med. J. Aust.*, 20, 746–749, 1947b.

Urbanetti, J.S., Phosgene: clinical importance of and management, in *Proceedings of Workshop on Acute Lung Injury and Pulmonary Edema*, AD A216293, U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD, 1989, pp. 105–119.

U.S. EPA (U.S. Environmental Protection Agency), Health Assessment Document for Phosgene, EPA/8–86/022F, Office of Health and Environmental Assessment, Washington, DC, March 1993.

Van Helden, H.P.M., van de Meent, D., Oostdijk, J.P., Joosen, M.J.A., van Esch, J.H.M., Hammer, A.H., and Diemel, R.V., Protection of rats against perfluoroisobutene (PFIB)-induced pulmonary edema by curosurf and N-acetylcysteine, *Inhalation Toxicol.,* 16, 549–564, 2004.

Wells, B.A., Phosgene: a practitioner's viewpoint, *Toxicol. Ind. Health*, 1, 81–92, 1985.

Wyatt, J.P. and Allister, C.A., Occupational phosgene poisoning: a case report and review, *J. Accid. Emerg. Med.*, 12, 212–213, 1995.

Zwart, A., Arts, J.H.E., Klokman-Houweling, J.M., and Shoen, E.D., Determination of concentration-time-mortality relationships to replace LC_{50} values, *Inhalation Toxicol.*, 2, 105–117, 1990.

21 Inhalation Toxicology of Riot Control Agents

Harry Salem, Bryan Ballantyne, and Sidney A. Katz

CONTENTS

21.1 INTRODUCTION AND BACKGROUND

The *Encyclopedia of Chemical Technology* lists riot control agents as one of the five categories of chemicals used in war (Harris, 1992). They are described as nonlethal tear agents most effective against unprotected personnel. The term chemical warfare has been used since 1917. Irritant chemicals such as lacrimators and sternutators that were used in World War I are traditional examples of harassing agents. The effects of these are briefly incapacitating and reversible (Harris, 1992). In 1975, U.S. President Gerald Ford signed Executive Order 11850 renouncing first use of riot control agents in war except

in defensive military modes to save lives (Boyd, 2003). More recently, the Secretary of Defense must ensure that they will not be used unless there is presidential approval in advance (SECDEF, 2003).

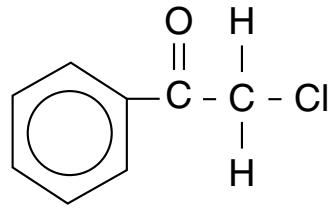
Riot control agents are those that cause disabling physiological effects when they come into contact with the eyes or skin, or when they are inhaled. They have the capacity to induce intense sensory irritation of the skin and mucous membranes of the eyes and respiratory tract. Riot control agents are peripheral sensory irritants that pharmacologically interact with sensory nerve receptors in skin and mucosal surfaces at the site of contamination, resulting in local pain and discomfort sensations with associated reflexes (Salem et al., 2001). The reflex most associated with the inhalation exposure of irritants is the Kratschmer reflex, first reported in 1870 (Kratschmer, 1870). He described this reflex response following the exposure of rabbits to chemical irritants such as chloroform and carbon dioxide. On exposure, the immediate response was apnea or cessation of respiration. This reflex is one of the protective reflexes or defense mechanisms programmed to prevent or reduce the amount of the noxious chemical reaching the lower respiratory tract and to maintain homeostasis. This effect is accompanied by bradycardia and a biphasic fall and rise in aortic blood pressure. The Kratschmer reflex is mediated by the olfactory (I), trigeminal (V), and glossopharyngeal (IX) cranial nerves and has also been demonstrated to occur in humans (Allen, 1928–29). The Kratschmer reflex also occurred in rodent and canine experiments after exposure to volatile solvents (Aviado, 1971). The cardiopulmonary receptors involved in the defense mechanisms prevent the absorption and distribution of the inhaled irritant to the vital organs and facilitate the expulsion of the irritant, whereas the extracardiopulmonary mechanisms promote metabolism and excretion of the absorbed chemicals. These have been described by Aviado and Salem, (1968, 1987) and Aviado and Aviado (2001). During apnea or cessation of respiration, blood levels of carbon dioxide increase and thus drive the respiratory center to restart the breathing. Individuals with compromised immune systems, nervous system depression as a result of alcohol or illicit drug consumption, or a combination of these, may prevent the restarting of respiration, resulting in death from asphyxia. This may be responsible, in part, for the over 100 in-custody deaths attributed by law enforcement agencies to positional asphyxia after exposure to "pepper" sprays.

21.2 RIOT CONTROL AGENTS

The Edgewood Chemical Biological Center (ECBC) and its predecessor organizations have more than 85 years experience in chemical and biological defense, including being the Nation's primary nonlethal agent development laboratory (Salem, 2003). Although they are considered military chemicals, riot control agents are distinct from chemical warfare agents (CWA). Chemical warfare agents include nerve agents such as Tabun (GA), Sarin (GB), Soman (GD), Cyclosarin (GF), and VX, nontraditional agents (NTA); blister agents such as mustard (HD) and lewisite (L); choking agents/lung irritants such as phosgene (CG); blood agents such as hydrogen cyanide (AC) and cyanogen chloride (CK); incapacitating agents such as adamsite (DM), 3-quinuclidinyl benzylate (BZ); and centrally acting anesthetics such as the fentanyls. Riot control agents are also designated as nonlethal and less-than-lethal agents, as well as incapacitating, immobilizing, and calmative agents. Their pharmacological classes include irritants, lacrimators, sternutators, emetics, sedatives, hynotics, serotonin antagonists, hypotensives, thermoregulatory disruptors, nauseants, vision disruptors, neuromuscular blockers, and malodorous substances. In addition to all the above, military chemicals also include training agents, smoke materials, and herbicides (Salem et al., 2001). The United States does not recognize riot control agents as chemical warfare agents as defined in the 1925 Geneva Convention (Sidell, 1997), and this is still the official U.S.-held policy (Office of the Press Secretary, White House, 1994). Riot control agents or water under pressure can be used in civil disturbances to distract, deter, incapacitate, disorient, or disable disorderly people, to clear facilities, to deny areas, or to rescue hostages. They can also be used in peacekeeping operations (Ballantyne, 1977a).

21.3 HISTORICAL

Although lacrimatory and irritant chemicals are considered to have a history dating from World War I, it appears that the first recorded effort to overcome an enemy by the generation of suffocating and



1 – chloroacetophenone (CN)

poisonous gases seems to have occurred in the wars of the Athenians and Spartans in 431–404 BCE. The Spartans, when besieging the cities of Plateau and Beluim, saturated wood with pitch and sulfur, and burned it under the walls of these cities in the hope of choking and incapacitating the defenders and rendering the assault less difficult. Similar uses of poison gases are recorded during the Middle Ages. In effect, they were like our modern stinkballs but were projected by squirts or in bottles in a manner similar to the hand grenade (West, 1919; Fries and West, 1921). The use of capsaicin as an irritant also predates World War I. When Christopher Columbus and his crew landed on Hispaniola in 1492, their behavior brought them into conflict with the local tribe of Arawak Indians. For their protection, Columbus and his men built a stockade. They were confronted by the Indians, who filled bottle gourds, or calabashes, with a paste of wood ash and ground jalapeno peppers, and lobbed them over the fence. The capsaicin-laden smoke, which was intended to encourage Columbus and his men to leave, was unsuccessful (Garrett, 2000). Capsaicin, which is considered the active ingredient of "pepper sprays," was synthesized at Edgewood Arsenal in the early 1920s and tested as an irritant. Following the synthesis of CS by British chemists Carson and Stoughton in 1928, the United States abandoned their work on capsaicin to develop CS.

Chloropicrin (trichloronitromethane) also known as Green Cross and PS, first synthesized around 1850, was used both as a harassing agent and lethal chemical in World War I, along with the other lethal agents, chlorine, phosgene, and trichlorethyl-chloroformate. Adamsite (diphenylamino-chlorarsine or DM), an arsenic-based compound, was developed for use in World War I. It is a vomiting and sneezing (sternutator) agent and was used as a riot control agent after the war. According to Swearenger (1966) ethyl bromoacetate (EBA) was considered the first riot control agent since the police in Paris, France used it in grenades in 1912 to temporarily disable lawless gangs. This tear gas was again used in the 1970s (Royer and Gainet, 1973). The other tear gases used in World War I included acrolein (Papite), bromoacetone (BA, B-stoff), bromobenzyl cyanide (BBC, CA), chloroacetone (A-stoff), and xylylbromide (T-stoff). Bromoacetone was the most widely used potent lacrimatory agent used in World War I (Salem et al, 2001). Chloroacetophenone (Mace) was invented by the German chemist, Graebe, in 1869 (Graebe, 1869, 1881). Other sources indicate it was originally synthesized in 1871 and 1881. The name MACE™ is derived from the chemical name methyl chloroacetophenone (methyl chloroacetophenone). This was the original chemical MACE product made by Smith and Wesson and was widely regarded as the original "tear gas."

Although MACE was trademarked for CN, it is commonly used as the generic term for riot control agents. After the United States entered the World War I, American chemists and the British investigated CN and found it to be one of the most effective lacrimators known. Its lacrimatory effects and persistence was equal to or slightly better than bromo benzyl cyanide (BBC) and it contained the less expensive chlorine instead of bromine. It is very stable under normal conditions and does not corrode steel. CN is a crystalline solid and can be dissolved in a solvent or delivered in thermal grenades.

CN was used as the tear gas of choice for the three decades after its introduction toward the later stage of World War I, but because of its relative toxic profile and the development of the more effective and safer tear agent CS, its use markedly declined (CHPPM, 1996).

21.4 CN (CHLOROACETOPHENONE, MACE)

Its CAS number is 532-27-4. It is also referred to as ω-chloroacetophenone, α-chloroacetophenone, phenacyl chloride, 2-chloro-1-phenylethanone, and phenyl chloromethyl ketone. It is a white solid with an apple blossom odor and a molar mass of 154.5 corresponding to a molecular formula of C_8H_7OCl. The molar solubility at 20°C is 4.4×10^{-3} mol/l (=68 mg/100 ml). Melting and boiling points are 54°C and 247°C, respectively. Density of the solid is 1.318g/cm^3 at 0°C, and density of the liquid 1.187g/m^3 at 58°C. The vapor is 5.3 times heavier than air. The vapor pressure of the solid is 2.6×10^{-3} torr at 0°C, 4.1×10^{-3} torr at 20°C, and 15.2×10^{-3} torr at 50°C.

21.4.1 CN Animal Toxicology

The toxicology of riot control agents, including CN, conducted by McNamara and his colleagues has been reviewed and summarized by the National Academy of Sciences (1984). In addition many reviews have been published on riot control agents (Swearinger, 1973; Ballantyne, 1977a; Verwey, 1977; Hu, 1992; Sidell, 1997, Olajos and Salem, 2001; Salem et al., 2001).

Toxicological studies were conducted on CN dispersed from commercially available thermal grenades or from acetone solutions. Acute and sublethal effects following aerosol exposure in experimental animals were lacrimation, conjunctivitis, copious nasal secretions, salivation, hyperactivity, lethargy, and dyspnea, which occurred in all animals. Effects on the skin of exposed animals were primarily erythema. The estimated LCt_{50} values calculated for CN in the various animal species were 8878 mg·min/m^3 in the rat, 7984 mg·min/m^3 in the guinea pig, and 7033 mg·min/m^3 in the dog. The pathological finding reported by Ballantyne and Swanston (1978) in the animals that died of inhalation of CN consisted of congestion of the alveolar capillaries, alveolar hemorrhage, and excessive secretions in the bronchi and bronchioles, as well as areas of acute inflammatory cell infiltration of the trachea, bronchi, and bronchioles. They also reported that CN was from 3- to 10-fold more toxic than CS in mice, rats, rabbits, and guinea pigs. The early deaths exhibited lesions of the upper respiratory tract, with marked pseudomembrane formation, excessive salivation, and nasal secretion. The animals that died later exhibited edema and hemorrhage of the lungs. In repeated exposures for 10 consecutive days in guinea pigs, dogs, and monkeys, the toxicity of CN was considerably less when administered in divided doses. Overall, these studies demonstrated a lack of cumulative toxicity (Salem et al., 2001).

Kumar et al. (1994) reported changes in biochemical end points measured after multiple exposures of CN and CR in mice for 15 min/d, for 5 and 10 d. They reported a decrease in hepatic glutathione and an increase in lipid peroxidation. Hepatic acid phosphatase increased after the 5-d exposure to CN, and the glutathione levels decreased after the 10-day CN exposure. CN-induced elevation in acid phosphatase levels reflected the release of lysosomal enzymes from the liver, indicative of tissue injury. CR exposure did not produce significant alterations in hepatic biochemical parameters. Additionally, hyperglycemia was observed after exposure to CN, an effect previously reported by Husain et al. (1991). Stress-mediated release of epinephrine is known to elevate glucose levels and thus may be responsible for the hyperglycemia. Significant decreases in body weight gain were also noted on exposure to these compounds, with CN having a more prominent effect on body weight. These findings were consistent with results reported by Ballantyne (1977a) on the repeated dose effects of orally administered CR in various animal species. Histopathological changes after CN exposures included hemorrhage, perivascular edema, and congestion of the alveolar capillaries, occluded bronchioles, and alveolitis. Renal histopathology demonstrated congestion and coagulative necrosis in the cortical renal tubules in CN-exposed mice. Hepatic histopathology consisted of cloudy swelling and lobular and centrolobular necrosis of hepatocytes after CN exposures.

CN is more likely to cause more serious eye effects than CS. At high concentrations, CN may result in chemical injury to the eye with corneal and conjunctival edema, erosion or ulceration, chemosis, and focal hemorrhages (Hoffman, 1967; Macrae et al., 1970; Leopold and Lieberman, 1971). CN-induced ocular effects on the rabbit eye following treatment with various formulations were investigated by Ballantyne et al. (1975) and Gaskins et al. (1972). Effects included lacrimation, chemosis, iritis, blepharitis, and keratitis, with severity dependent on the formulation.

CN is also a potent skin irritant and is more likely to cause more serious injury to the skin than CS. These effects include severe generalized itching, diffuse and intense erythema, severe edema, and vesication. CN is considered a more potent skin irritant and sensitizer than CS (Penneys et al., 1969; Chung and Giles, 1972; Holland and White, 1972).

In the two-year carcinogenicity inhalation bioassays in rats and mice, there was no indication of carcinogenicity in male rats, whereas equivocal evidence was found in female rats. These findings were evidenced by increased fibroadenomas of the mammary gland. In these two-year studies in mice, there was no evidence of carcinogenic activity in males and females (NTP, 1990). See also Chapter 9 by Weisburger and Hudson.

In the body, CN is converted to an electrophilic metabolite. It is a SN_2-alkylating agent that reacts with SH groups and other nucleophilic sites of biomolecules. Alkylation of SH-containing enzymes leads to enzyme inhibition with disruption of cellular processes. CN was found to inhibit human plasma cholinesterase via a non-SH interaction, and some of the toxic effects may be due to alkylation of SH-containing enzymes (Castro, 1968).

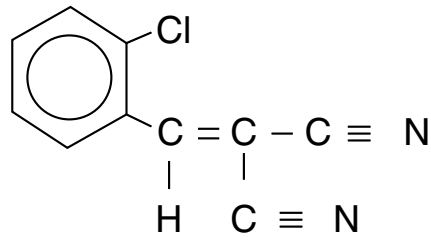
21.4.2 Human Volunteer Studies

The incapacitating effects of CN in human volunteers during exposure included lacrimation, some blurring of vision, and conjunctivitis. On the nose and throat, CN causes a tingling sensation, irritation, pain, and some increases in secretions, whereas in the respiratory tract it causes irritation, burning, and pain. CN on the skin causes burning in the periorbital area and other areas of tender skin, especially where sweating is present. Nausea and gagging occasionally occur during and soon after exposure. Most of these effects disappear within 20 min after exposure, but conjunctivitis and blepharospasm usually disappear after a few days leaving no after-effect. Incapacitating dosages (ICt_{50}) of CN have ranged from 20 to 50 mg·min/m^3. The ICt_{50} for CN is comparable with that for adamsite (DM), which was an early riot control agent that was replaced by CN. The ICt_{50} values for CN and DM are greater than for CS. The estimate of human LCt_{50} values extrapolated from animals exposed to CN dispersed from a solvent is 7000 mg·min/m^3, and when dispersed from commercially available grenades is 14,000 mg·min/m^3. Other estimates range from 8500 mg·min/m^3 to 25,000 mg·min/m^3. According to Punte et al. (1962a) the maximum safe inhalation dosage of CN for humans is estimated to be 500 mg·min/m^3.

Punte et al. (1962a) tested human volunteers in a wind tunnel at an air speed of 5 mph to establish the length of time a subject could remain in the CN-containing airstream. The tolerance time varied with the subject. CN aerosols were generated from acetone solutions at a Ct of 350 mg·min/m^3 with a mass median diameter of about 0.6 μm. The immediate effects of exposure were tingling of the nose and rhinorrhea, burning of the eyes and throat, lacrimation and blurred vision. Some subjects suffered dyspnea. These effects disappeared rapidly when the subjects left the wind tunnel. Acute injuries to the eyes, primarily from effects of blast and missiles may occur from tear-gas weapons, such as pen guns. The immediate effects of these injuries include swelling and edema of the lids with penetration of the skin, conjunctivitis, cornea, sclera, or globe by gunpowder and CN. Conjunctival ischemia and chemosis, corneal edema, erosion, inflammation or ulceration, and focal hemorrhage were reported by Oaks et al. (1960), Hoffman (1967), Levine and Stahl (1968), Laibson and O'Connor (1970), Leopold and Lieberman (1971), Oksala and Salminen (1975), Adams et al. (1966) reported three hand injuries resulting from accidental discharges of tear gas guns at close range. Surgery was required in all to relieve pain and to remove foreign material. They all suffered continuing pain and some loss of sensation, apparently from the toxic action of CN on nerves.

21.5 CS (CHLOROBENZYLIDENE MALONONITRILE)

The CAS number CS is 2698-41-1, and it is also known as β,β-dicyano-ortho-chlorostyrene, and 2-chlorobenzalmalononitrile. It is a white solid with molar mass of 188.5 corresponding to



2–chlorobenzylidene malononitrile (CS)

molecular formula of $C_{10}H_5N_2Cl$. The molar solubility in water at 20°C is 2.0×10^{-4} mol/l (= ~4 mg/100 ml). Dissolved in water CS is rapidly hydrolyzed; however, it may persist in the environment because its solubility in water is limited. The melting and boiling points are 72°C and 335°C, respectively. The vapor is several times heavier than air, and the vapor pressure of the solid is 0.00034 mmHg at 20°C.

In addition to the nonpersistent form of CS, two hydrophobic variations were created, CS1 and CS2. CS1 is a micronized powder formulation containing 5% hydrophobic silica aerogel, which can persist for up to 2 weeks in normal weather conditions (Ballantyne, 1977a), and CS2 is a siliconized microencapsulated form of CS1 with a long shelf life, persistence, resistant to degradation, and ability to float on water which could restrict or deny the use of water for military operations. CS is commonly used as a riot control agent and a simulant for training. Members of military organizations and law enforcement agencies are routinely exposed to heated CS during training. The heat vaporizes the CS for dispersion, which thus condenses to form an aerosol. Estimates of human toxicity for CN is 7000 mg·min/m^3 and for CS is from 25,000 to 100,000 mg·min/m^3 WHO (1970).

Both CS and CN are SN2-alkylating agents with activated halogen groups that react with nucleophilic sites and combine with intracellular sulfhydryl groups on enzymes such as lactic dehydrogenase to inactivate them. The effect is transient because the enzymes are rapidly reactivated. CS reacts rapidly with the disulfhydryl form of lipoic acid, a coenzyme in the pyruvate decarboxylase system. It has been suggested that tissue injury may be related to inactivation of certain of these enzyme systems. Pain can occur without tissue injury and may be bradykinin mediated. CS has been shown to cause the release of bradykinin, both *in vivo* and *in vitro*, and elimination of bradykininogen *in vivo* abolishes the systemic response to CS (U.S. Army Medical Research Institute of Clinical Defense [USAMRICD], 2000). The release of chlorine atoms on contact with skin and mucous membranes which are reduced to hydrochloric acid can cause local irritation and burns (Anderson et al., 1996).

21.5.1 Animal Toxicology

Repeat exposures of thermally dispersed CS were conducted in rats and dogs. The animals were exposed from 4 to 5 min/d, 5 d a week for 5 weeks. The 25-day cumulative dosage (Ct) to which the rats were exposed to was 91,000 mg·min/m^3 (3,640 mg·min/m^3 per day), whereas the dogs were exposed to a cumulative dosage of 17,000 mg·min/m^3 (680 mg·min/m^3 per day). No lethality occurred in the dogs, but the rats became hyperactive and aggressive, biting the noses and tails of other rats and scratching their own noses. No changes were found in blood values for sodium, potassium, protein, albumin, or creatinine throughout the tests. Five of the thirty exposed rats died, two following the cumulative dosage of 25,000 mg·min/m^3 and three after 68,000 mg·min/m^3. Gross pathological examination of the rats that died was negative, as were those of six other rats that were sacrificed after 5 weeks exposure. The exposed rats lost about 1% of body weight, whereas unexposed rats gained about 20% during the 5 weeks. There were no significant differences in organ-to-body weight ratios for heart, kidneys, lungs, liver, or spleen following the 5-week exposures. It was concluded

TABLE 21.1 Acute Inhalation Toxicity LCt_{50} (mg·min/m^3)

	CS Smoke	CS Aerosol
Guinea pig	35,800	67,000
Rabbit	63,600	54,090
Rat	69,800	88,480
Mouse	70,000	50,110

that repeated exposures did not make the animals more sensitive to the lethal effects of CS. The animals that died after exposure to CS showed increased numbers of goblet cells in the respiratory and gastrointestinal tracts and conjunctiva, as well as necrosis in the respiratory and gastrointestinal tracts, pulmonary edema, and occasionally hemorrhage in the adrenals. Death appeared to result from poor transfer of oxygen from the lungs to the blood stream, probably because of edema, hemorrhage in the lungs, and obstruction of the airways. Marrs and his coworkers (1983b) also studied the effects of repeated exposures to CS. Mice, rats, and guinea pigs were exposed to neat CS aerosols for 1 h/d, 5 d/week for 120 days. High concentrations of CS were fatal to the animals after only a few exposures, whereas mortality in the low and medium concentrations did not differ significantly from the controls. They concluded that CS concentrations below 30 mg/m^3 were without deleterious effects. Acute inhalation toxicity of CS was studied in several animal species generated in smoke by Ballantyne and Callaway (1972) and as an aerosol by Ballantyne and Swanston (1978). These data are presented in Table 21.1.

The effects of CS inhalation on embryonic development in rats and rabbits were studied at concentrations consistent with those expected in riot control situations (~10 mg/m^3). Although the concentrations were low and the duration of exposure (5 min) may not have been adequate to assess the fetotoxic and teratogenic potential of CS, no significant increase in the numbers of abnormal fetuses or resorptions were noted (Upshall, 1973). The mutagenic potential of CS and CS2 were studied in microbial and mammalian bioassays. CS was positive in the Ames Assay (von Daniken et al., 1981), whereas Zeiger et al. (1987) reported questionable genotoxicity for *Salmonella typhimurium*, and those of Reitveld et al. (1983) and Wild et al. (1983) reported CS negative when tested in *S. typhimurium* strains TA 98, TA 1535, and TA 1537 with and without metabolic activation (National Toxicology Program [NTP], 1990). The mutagenic potential for CS and CS2 was investigated in mammalian assays, such as the Chinese hamster ovary (CHO) test, for the induction sister chromatid exchange (SCE) and chromosomal aberration (CA) and the mouse lymphoma L5178Y assay for induction of trifluorothymidine (Tfi) resistance. The results of these assays indicated that CS2 induced sister chromatid exchange, chromosomal aberrations, and Tfi resistance (NTP, 1990, McGregor et al., 1988, Schmid et al., 1989). The Committee on Toxicology of the National Research Council (National Academy of Science, 1984) reported that, taken in their totality, the test of CS for gene mutation and chromosomal damage provides no clear evidence of mutagenicity. Although most of the evidence is consistent with nonmutagenicity, in the committee's judgment, it is unlikely that CS poses a mutagenic hazard to humans. CS2 was evaluated for carcinogenicity in the NTP (1990) 2-year rodent bioassay. Compound-related nonneoplastic lesions of the respiratory tract were observed. The pathological changes observed in the exposed rats included squamous metaplasia of the olfactory epithelium and hyperplasia and metaplasia of the respiratory epithelium. In mice, hyperplasia and squamous metaplasia of the respiratory epithelium was observed. Neoplastic effects were not observed in either rats or mice, and it was concluded that the findings suggest that CS2 is not carcinogenic to rats and mice (see also Chapter 9 by Weisburger and Hudson). McNamara et al. (1972) also tested CS in methylene chloride in mice and rats for carcinogenicity in a 2-year study. No tumorigenic effects were observed in the CS-exposed animals.

21.5.2 Metabolism

CS is absorbed very rapidly from the respiratory tract, and the half-lives of CS and its principal metabolic products are extremely short (Leadbeater, 1973). The disappearance of CS follows first-order kinetics over the dose range studied. CS spontaneously hydrolyzes to malononitrile (Patai and Rappaport, 1962), which is transformed to cyanide in animal tissues (Nash et al., 1950; Stern et al., 1952). Metabolically, CS undergoes conversion to 2-chlorobenzyl malononitrile (CSH2), 2-chlorobenzaldehyde (oCB), 2-chlorohippuric acid, and thiocyanate (Cucinell, 1971; Feinsilver et al., 1971; Leadbeater, 1973; Leadbeater et al., 1973; Ballantyne, 1977a; Paradowski, 1979). CS and its metabolites can be detected in the blood after inhalation exposure, but only after large doses. After inhalation exposure of CS in rodent and nonrodent species, CS and two of its metabolites, 2-chlorobenzaldehyde and 2-chlorobenzyl malononitrile, were detected in the blood (Leadbeater, 1973; Leadbeater et al., 1973). In the Leadbeater study (1973), with uptake by the respiratory tract in humans, only 2-chlorobenzyl malononitrile was detected in trace amounts in the blood. CS and 2-chlorobenzaldehyde were not detected, even after high doses of CS of up to 90 mg·min/m^3. This finding is consistent with the CS uptake studies in animals; and with the maximum tolerable concentration in humans, which is below 10 mg/m^3, it is unlikely that significant amounts of CS would be absorbed by the inhalation route at or near the tolerable concentrations. Experiments were conducted to determine the CS metabolite thiocyanate in humans exposed to amounts of CS that are intolerable. In their previous work, Cucinell et al. (1971) reported dogs exposed to 48,000 mg·min/m^3 of CS aerosol showed an unimpressive increase in plasma and urine thiocyanate concentration 24 h after exposure. These were lower than those observed in human subjects who smoked cigarettes. Smoking and nonsmoking human volunteers were exposed to doses up to 1.1 mg·min/m^3 of CS (intolerably CT). Plasma and urine levels were significantly higher in smokers than in nonsmokers, and exposure to CS did not cause any significant increases in plasma and urine thiocyanate levels. Plasma and urine thiocyanate levels were measured in human volunteers following exposure to intolerable airborne concentrations of CS. Because cigarette smoking also increases thiocyanate in body fluids, levels in nonsmokers, light smokers, and heavy smokers, before and after CS exposure were compared. There was no statistical difference in plasma or urine thiocyanate concentration between nonexposed and CS-exposed volunteers. However, concentrations were significantly higher for both light and heavy smokers than for nonsmokers. Thus it was concluded that plasma and urine levels of thiocyanate CS metabolite are not high enough to detect after human exposure to intolerable levels of CS.

21.5.3 Human Volunteer Studies

Human volunteers have been exposed to CS under varying conditions and concentrations to determine the dosage that will incapacitate 50% of the exposed population in 1 min (ICt_{50}). Because the toxicity in all animal species studied was low, and the potency as a sternutator and lacrimator was high, it was necessary to study effectiveness of CS in humans, whether CS could be a replacement for CN and DM as a riot control agent. Both healthy military and civilian personnel volunteered for these studies and, following preexposure medical evaluation, were placed in one of four groups to establish base lines for comparison with postexposure results. The first two groups were of untrained men, one group with masks available, one without; the other two groups were of trained men (previously exposed), with and without masks available. Special categories of subjects included those with hypertension, those liable to hay fever, drug sensitivity, or bronchial asthma, those with jaundice or hepatitis, those with a history of peptic ulcer unaccompanied by gastrointestinal bleeding, and those who were between 50 and 60 years of age. Prior to exposure all men received instructions in the use of the protective masks (M9A1 or M17). Four subjects at a time were exposed in a wind tunnel (eight cubic feet) at a fixed speed of 5 mph at temperatures from 0°F to 95°F.

CS was disseminated from a 10% solution in acetone, from a 10% solution in methylene dichloride, or from a miniature M18 thermal grenade. CS solution dissemination was accomplished by using spray nozzles (Spraying Co., type J) placed in the air intake. Airborne samples of CS were taken isokinetically from before dissemination to 1 min following the exposures. Particle-size determinations were made with a six-stage, modified cascade impactor, and airborne concentrations were estimated by ultraviolet light absorption of 260 μm using a Beckman DU spectrophotometer.

The mass median diameter of CS produced was 3.0 μm for the CS in acetone, 1.0 μm for the CS in methylene dichloride, and 0.5 μm for the miniature M18 CS thermal grenade. These studies revealed the following. Many untrained subjects were unable to don and retain their masks at low concentrations of CS, but at high concentrations they were able to mask well enough to remain in the contaminated atmosphere. When properly fitted these masks will fully protect against CS. In those who were unable to mask rapidly, panic was evident. Concentrations of 9 to 10 mg/m^3 forced 50% of the subjects to leave the chamber within 30 sec, and that 99% left at approximately 17 mg/m^3, and 100% left and were considered incapacitated at 40 mg/m^3 or greater. Persons who had been exposed previously to a high concentration developed a fear of the agent, and even though subsequently exposed to a lower concentration, the time to incapacitation for trained men was shorter than expected. There were no significant differences noted in the time to incapacitation in subjects exposed to CS at 0–95°F, although the subjects appeared unable to tolerate the agent as well as those exposed at ambient temperature. At 95°F and relative humidity (RH) of 35% and 97% the skin-burning effects were much more prominent, possibly because of the excessive diaphoresis. Hypertensive subjects reacted similarly to and tolerated CS as well as normotensive individuals. However, their blood pressure elevation was greater and lasted longer than in normotensive individuals, possibly because of the stress of exposure. The hypertensive subjects recovered as rapidly as the normotensive subjects. Subjects with a history of peptic ulcer, jaundice, or hepatitis and those between the ages of 50 to 60 years reacted similarly to healthy subjects. Persons with a history of drug allergy, hay fever, asthma, or drug sensitivity were able to tolerate CS exposure as well as the healthy subjects; however, a higher percentage of this group had more severe chest symptoms than the healthy subjects. Although many of these lay prostrate on the ground for several minutes, no wheezing or ronchi were heard on auscultation, and recovery time was as rapid as for any other group tested. Hyperventilating subjects were incapacitated at much lower concentrations than normally breathing subjects, and recovery time was slightly prolonged, but only by 1 to 2 min. Although not significantly different, subjects exposed to CS disseminated from methylene dichloride appeared to tolerate the agent for a slightly longer period that those subjected to CS in acetone solution, nor were there many differences from CS disseminated from the miniature M18 CS smoke grenade. A group was also exposed to a combination of CS and DM. The effects of DM were negligible when CS was effective within 30 sec (Gutentag et al., 1960)

Owens and Punte (1963) exposed only the eyes and respiratory tract of six human volunteers from a group of fifty, who were best able to tolerate airborne CS, to small and large particles of CS. The mass-median diameter (MMD) for the small particles was 0.9 μm (4% were larger than 10 μm) and for the large particles it was 60 μm (4 % were below 20 μm). The ability to tolerate and recover from concentrations of 85 and 94 mg/m^3, respectively, was determined. The small particle size was more effective in producing eye irritation. Only two of the five men exposed to the 0.9-μm aerosol were able to tolerate the CS for 60 sec, whereas all six men exposed to the large size aerosol remained in the cloud for at least 60 sec. Following exposure, all subjects had difficulty in seeing. Recovery times were based on the subject's ability to sort and arrange cards. Recovery following exposure of the eyes to small particles averaged 90 sec, but it took approximately 280 sec following exposure to the large particle. The respiratory effects of exposure of the small particles were more dramatic. None of the six men could tolerate the small particles for longer than 30 sec, whereas four of the six men tolerated the larger particle exposure for at least 60 sec.

21.5.4 Human Clinical Signs and Symptoms

In a group of seven volunteers given 10 exposures of from 1 to 13 mg/m^3 of CS in a period of 15 days revealed no clinical abnormalities. The dominant effect of the first exposure remained the dominant effect on subsequent exposures. None of the volunteers developed a tolerance to CS during the 10 exposures.

The immediate effects upon exposure to aerosols of CS were on the eyes and were demonstrated by severe conjunctivitis accompanied by a burning sensation and pain that persisted from 2 to 5 min and usually disappeared abruptly rather than gradually. The conjunctivitis remained intense for up to 25 or 30 min. Erythema of the eyelids was generally present, persisted for an hour, and was occasionally accompanied by blepharospasm. Lacrimation was invariably present and tended to be profuse and lingering for up to 12 to 15 min. The occasional "tired feeling" in their eyes lasted for about 24 h. Photophobia, which was quite marked in 5–10% of the volunteers remained for up to an hour. On repeated exposures, the eye effects were reproduced. Rhinorrhea and salivation were profuse and persisted for up to 12 h.

The effects on the respiratory system appeared to depend on the duration of exposure and the depth of respiration. The first symptom was usually a burning sensation beginning in the nares and throat and then progressing down the respiratory tract; it was sometimes associated with coughing. As the exposure continued, the burning became painful and was rapidly followed by a "constricting sensation" throughout the chest, which caused incapacitation for several minutes. Panic usually accompanied and accentuated this symptom, and these volunteers appeared unable to inhale or exhale. Fresh air and encouragement abated these effects. Auscultation of the chest immediately after exposure did not reveal wheezing, rales, or ronchi. Airway resistance measured by the Asthmometer showed no significant changes, and a portable breath-recording apparatus measured breathing patterns of exposed individuals. The patterns verified that when the aerosol was inhaled, the subjects involuntarily gasped, and then held their breath or breathed slowly and shallowly. This was followed by short paroxysms of coughing that forced the individual to exit the exposure. An irregular respiratory rhythm was noted for several minutes after exposure was terminated. Many of the exposed individuals were aphonic for 1 to 2 min postexposure, and several were hoarse for 24 h. The authors concluded that the incapacitation caused by CS was due to the effects on the eyes, respiratory tract, or both, but they regarded the effects on the respiratory system as potentially the most capable of causing incapacitation (Gutentag et al., 1960). This conclusion was followed up Craig et al. (1960), who also exposed a group of volunteers in the wind tunnel, wearing a self-contained remotely controlled breath-recording system. They were exposed to 5–150 μg of CS per liter for 110–120 sec. Although the breathing patterns were disrupted by the CS exposure, adequate ventilation of the lungs was maintained, so they concluded that incapacitation is attributed to the unpleasant sensations rather than to any degree of respiratory failure. The apnea and cardiovascular changes observed after inhalation exposure to CS are not inconsistent with the Kratschmer reflex. These investigators also reported that sneezing was common among the observers exposed to small concentrations of CS at some distance from the exposure chamber (Craig et al., 1960).

Inhalation toxicity studies of aerosol dispersions of melted agents sprayed in the molten form and of dry-powder dispersions, sprayed from solutions of acetone or methylene dichloride or dispersed from grenades by liberation of hot gases, have been performed since World War I. Before the research on CS in 1958 and 1959, no toxicity studies were performed with munitions. In 1965, munitions studies were conducted with CN and DM. All these studies demonstrated that munitions-dispersed agents were less toxic than dispersion by other methods. The human LD_{50} value, based on the combined animal species toxicity data, is 52,000 mg·min/m^3 for CS by molten dispersion and 61,000 mg·min/m^3 dispersed by the M7A3 grenade.

21.5.5 Human In-Use Exposures

Park and Giammona (1972) reported the first documented case of pneumonia in the pediatric age group following an exposure to tear gas. A healthy 4-month old white male infant was exposed to CS

gas for 2–3 h. He was in a house into which police fired CS tear gas canisters to subdue a disturbed adult. Immediately when taken from the house to the emergency room he was observed to have copious nasal and oral secretions, he sneezed and coughed frequently, and he required suction to relieve upper airway obstruction. The pneumonitis was treated aggressively and the patient was discharged from hospital on the 12th day because no growth was noted in his blood and urine cultures, his respiratory problems had subsided, and his temperature had also returned to normal. However, within 24 h the infant was returned to the emergency room and was rehospitalized. A repeat chest roentgenogram demonstrated a progression of the pulmonary infiltrates. Following treatment with antibiotics the chest roentgenogram was clear by the 17th day, and improvement continued and the patient was discharged after 28 days of hospitalization.

The next reported case of serious intoxication with CS tear gas was by Krapf and Thalmann (1981). Eleven days after a thorough internal medical examination that revealed no clinical or pathological findings, a 43-year-old male was in a room in a cloud of fumes from a CS canister that a friend had ignited as a joke. He immediately suffered from burning pains in the eyes and in his upper respiratory tract, lacrimation, and pains in his chest with dyspnea and coughing. This unusual exposure led to serious long-term complications such as toxic pulmonary edema, gastrointestinal difficulties, and indications of liver damage and transient right heart insufficiency. After 3 months hospitalization, all tests were negative, and the patient was discharged to his home in a condition capable of work.

Hill et al. (2000) described what they considered to be the first reported case of major hepatitis attributable to CS inhalation exposure. They described a case in which a 30-year-old incarcerated male was sprayed with CS and was hospitalized later for 8 days with erythroderma, wheezing, pneumonitis with hypoxemia, hepatitis with jaundice, and hypereosinophilia. For months he continued to suffer from generalized dermatitis, recurrent cough and wheezing consistent with reactive airways dysfunction syndrome (RADS), and eosinophilia. Systemic corticosteroids were successful, but abnormalities recurred off treatment. Although the dermatitis resolved gradually over 6–7 months, the asthma-like symptoms persisted for a year after exposure. Patch testing confirmed sensitization to CS. The mechanism of the prolonged reaction is unknown but may involve cell-mediated hypersensitivity, perhaps to ad\ducts of CS, or a metabolite, and tissue proteins. The investigators reported this as the first documented case in which CS apparently caused a severe, multisystem illness by hypersensitivity rather than direct tissue toxicity.

Hu et al. (1989) reported other human exposures. They reported on the alleged use of tear gas in almost every major city in South Korea in June of 1987 (*New York Times*, July 1, 1987, Sec. 1:8). There were more than 350,000 uses of CS tear gas by the government against civilians who exhibited cough and shortness of breath for several weeks. Hospitalized patients with asthma and chronic bronchitis, exposed to CS wafting through hospital wards through open windows, experienced deterioration in lung function. Persons close to the exploding tear gas canisters and grenades sustained penetrating trauma from plastic fragments that was exacerbated by the tear gas. The lack of information and objective and epidemiological studies was due to fear of serious government reprisals.

Hu et al. (1989) also refers to the allegations that exposures to tear gas in Gaza and the West Bank of Israel have been associated with increases in miscarriages and stillbirths. Inquiries, by groups such as Amnesty International and Physicians for Human Rights, prompted a Government Accounting Office (GAO) investigation requested by Congressman Ronald Dellums. The GAO Report (1989) concluded that the Physicians for Human Rights fact-finding trip could not confirm any deaths linked to tear gas inhalation, nor could they substantiate the rumors of increased miscarriages. There was also no verifiable evidence available linking tear gas exposure to fetal deaths. In addition, the U.S. State Department reported that they did not have any medical evidence to support a direct causation between tear gas inhalation and the number of deaths and miscarriages alleged. The exaggerated number of almost 400 deaths attributed to the use of tear gas by the Israeli Defense Forces (IDF) has also been repudiated by the State Department. They have concluded that at least four deaths had resulted from tear gas use in enclosed areas and that the IDF was using primarily CN at the time.

The use of CS by the U.S. forces in Vietnam in the years 1964–1972 was to flush the enemy from bunkers and tunnels, reduce the ability of the enemy to deliver aimed fire while attacking, and to deny fighting positions and infiltration routes for extended periods (Walker, 1970).

Interest and possible concern developed about the adverse effects of chemicals used in peacekeeping operations in the United Kingdom following the use of CS by the Ulster Constabulary in Londonderry, Northern Ireland on August 13 and 14, 1969. As a result of the first use of CS for crowd control, a three-man Committee of Inquiry was established to determine the medical effects, if any, in persons exposed to CS. Their report, published in 1969, recommended an expansion of the committee and the assessment of the evidence regarding CS in the widest possible way (Himsworth, 1969, 1971). They reported that on exposure to various concentrations of CS the effects vary from a slight prickly or peppery sensation in the eyes and nasal passages to the maximum symptoms of profuse lacrimation and salivation from the eyes and nose, spasm of the eyelids, retching and sometimes vomiting, burning of the mouth and throat, cough, and gripping pain in the chest. Even at low concentrations, the onset of symptoms is immediate, and they disappear when removed from the exposure (Himsworth, 1969, 1971). Of the many tens of thousands of military personnel in the United Kingdom who were exposed to CS in the course of their training, as well as those of the U.S. military who undergo similar training, the signs and symptoms were similar to those described above, and there were no significant after effects. Of all the U.S. military exposed to CS in training, Thomas et al. (2002) reported that approximately 200,000 U.S. Marines were exposed to CS from 1996 to 2002 under field conditions with similar effects. They also reported that a cluster of nine U.S. Marine Corps Amphibious Reconnaissance students required hospitalization with pulmonary edema after strenuous exercise following exposure to CS. These patients did not become symptomatic until 36–40 h after the CS exposure and did not demonstrate evidence of airway dysfunction. McDonald and Mahon (2002) proposed that these cases attributed to the acute pulmonary effects of CS more likely represented a cluster of incidents of either water aspiration or swimming-induced pulmonary edema (SIPE). Water aspiration is a well-described cause of pulmonary edema. No details were provided in the report by Thomas et al. (2002) as to whether the symptomatic marines aspirated pool or seawater or whether they were breath-hold diving, but all became symptomatic immediately after pool or open ocean 1000- to 1500-m swims. Even when patients do not recall specific aspiration incidents while in the water, pulmonary edema has been described in divers and swimmers who have been immersed in cold water, and strenuous swimming alone has been reported as a cause of pulmonary edema. McDonald and Mahon (2002) also reported that cases of pulmonary edema associated with immersion occurred at the U.S. Basic Underwater Demolition/Seal School (BUDS) and at the Israel Naval Medical Institute. The case definition of pulmonary edema associated with immersion includes hypoxemia and radiograph air space filling that occurs during or immediately after swimming, followed by resolution of symptoms or radiographic improvement by greater than 50% within 48 h. On exposure, the eyes are red, but the redness disappears on leaving the contaminated atmosphere. On the skin CS causes a burning sensation on the exposed parts that can be followed by redness or the appearance of small blisters or vesicles at the point of friction. These effects are more prevalent in fair-skinned persons especially if the skin is hot and moist. Infants asleep in rooms where CS entered via broken windows were sufficiently distressed to awaken themselves crying from sleep. On being snatched from the contaminated atmosphere, they quieted rapidly and required no hospitalization. The authors also found no special susceptibility to CS associated with old age. Human volunteers and members of the Himsworth committee over 50 years of age were exposed to 35 mg/m^3, and the symptoms experienced and the time to recover from these were no different from those in young adults. Exposure to CS was determined not to have had any effect on pregnancy because comparison of the 9 months following exposure compared with the 9 months of the previous year demonstrated no difference in abortions, stillbirths, or congenital abnormalities. Middle-aged and elderly people who had chronic bronchitis and had been significantly exposed to CS did not show exacerbation different than that caused by natural causes. Following the riots of 1969, no increase occurred

in the death rate from chronic bronchitis and asthma. Individuals with asthma, especially children who were exposed to CS, did not show any difference in the number of attacks from their experience prior to the exposure. The committee reported that there is ample evidence that if CS causes unconsciousness in humans, it can do so only rarely and that many, if not all of the cases reported are more probably the result of other conditions that occur in riot situations. In animals, unconsciousness does not occur after inhaling CS (Himsworth, 1971). The Himsworth reports (1969, 1971), considered to be the most extensive independent study of the use of CS agent on humans, by U.K. forces in Northern Ireland in the late 1960s, found that no deaths and no long-term injuries resulted from the widespread use of CS agent there (U.S. Congress, 1996).

On April 19, 1993, CS was used unsuccessfully to induce the residents to leave the structure when it was injected through the walls of the Davidian residence in Waco, Texas. Had the building been airtight, which was not the case, the total amount delivered into the building would have been 411.92 mg/m^3. This concentration is far below the 61,000 mg/m^3 projected to be lethal to 50% of a given population of humans. In reality, the concentration of CS inside the Branch Davidian residence did not reach even these levels, because the residence was poorly constructed and allowed for air to move in and out of the residence continuously. The total amount of methylene chloride mixed with the CS inserted into the building was 1,924.87 mg/m^3. Because a human lethal estimate apparently has not been published, this amount was compared with that for rats and found to be far below the estimated LCt_{50} of 2,640,000 $mg \cdot mim/m^3$ for rats. Thus the committee concluded that the methylene chloride could not have caused the death of any of the Davidians. During the insertion of the CS into the Davidian compound, their leader David Koresh did not advise his followers to leave, instead, they spread highly flammable liquids throughout the compound and set fire to the whole building. In the aftermath of the fire, the bodies of more than 70 Branch Davidians were recovered. According to the autopsy reports from the Tarrant County, Texas, Coroner, 30 people died of asphyxiation due to smoke inhalation, two people died of injuries resulting from blunt-force trauma, and 20 people including David Koresh and a 20-month-old infant, died of gunshot wounds inflicted at close range by themselves or others within the compound. Of the nine Branch Davidians who survived the fire, seven escaped through openings in the walls and windows of the compound created by the combat engineering vehicles (CEV). The shoes and clothing of several of those who escaped contained concentrations of gasoline, kerosene, and other flammable liquids. CS is not a chemical accelerant or a flammable agent but will support combustion if ignited. The methylene chloride and carbon dioxide used in dissemination of the CS will not burn and will actually inhibit fire ignition (Union Calender 395). On April 12, 1993, the Federal Bureau of Investigation (FBI) presented its tear gas plan to Attorney General Janet Reno. Between that date and April 17, she conducted at least eight meetings with military and civilian tear gas experts to debate the tear gas plan in a barricade situation, the properties of the tear gas chosen, and the scientific and medical information concerning the toxicity and flammability of the type of tear gas proposed, and the effect of tear gas on vulnerable populations such as children, the elderly, and pregnant women. On April 17, the attorney general approved the tear gas insertion plan and informed the president of her decision.

As a result of the activities at Waco, Texas, the U.S. Congress initiated an investigation by the Subcommittee on Crime of the House Committee on the Judiciary and the Subcommittee on National Security, International Affairs, and Criminal Justice of the House Committee on Government Reform and Oversight, on the actions of the federal agencies involved in law enforcement activities in late 1992 and early 1993. As part of the investigation 10 days of public hearings were held in which more than 100 witnesses appeared and provided testimony concerning all aspects of the governments actions. In addition, the subcommittees reviewed thousands of documents requested from and provided by the agencies involved in these actions. Included in these documents were many that are referenced in this chapter. In the report it is stated that all those consulted who had personal knowledge or professional expertise agreed that the use of tear gas was the only way to compel the Branch Davidians to leave the compound without the use of force or loss of life. Evidence and testimony during the hearing clearly indicated the CS tear gas was not the direct or proximate cause of the

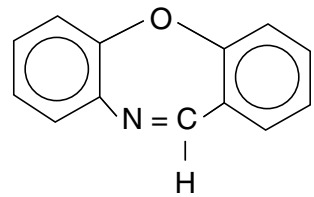
ignition or acceleration of the fire. As previously stated, CS is a common riot control agent used in the United States and Europe, the purpose of which is to cause irritation of the eyes, skin, and respiratory system sufficient to encourage an individual to leave the premises or any open area. CS is considered the least toxic agent in the family of chemical tear gas irritants. To reach a level that would be lethal to 50% of the population, CS must reach a concentration of 25,000 to 150,000 mg·min/m^3. The CS gas used was in a concentration that would only reach 16,000 mg·min/m^3, if all the CS used was released at the same time in a single closed room and if the residents were exposed continuously for 10 min. In reality, at Waco, the CS was released throughout different areas of the building while openings were created in the windows and walls. The CS was inserted for a total of 5 min over a 6-h period, and the wind velocity was 35 knots during the tear gas delivery. Therefore, given the amount of CS used, the presence of high winds, building ventilation, and the delivery of the gas at different areas of the compound, it is highly unlikely that anything close to the 50% lethality rate was reached. They further state that there are no documented cases in which the use of CS has caused death in humans, and the report that Amnesty International linked the use of CS to deaths of Palestinians in the West Bank and Gaza of Israel is an extremely biased reading of the report.

The report discussed the use of both CS and CN. CN has been reported to be lethal in closed quarters, whereas the overwhelming majority of evidence of ill effects of CS was anecdotal. Therefore, no reliable scientific data would lead to the conclusion that CS alone was implicated in any of the deaths. The Physicians for Human Rights also could not confirm the reports of deaths from CS inhalations. The Congressional Report also refers to the Himsworth Report issued by the British Government that found that there is no evidence of any special sensitivity of the elderly, children, or women. In addition, the Himsworth Commission chronicled the effects of CS exposure on an infant who, after being removed from the affected area, recovered rapidly. The Registrar General studied, under a pledge of medical secrecy, the individual health records of the employees who worked for at least 11 years at Nancekuke in Cornwall where CS is manufactured and compared them with a group of men of the same age from England and Wales. The results reported in Appendix 12 of the Himsworth Report (1971) indicate the workers may undergo multiple exposures to small doses of CS without their having any ill effect on them. There was no evidence of increased risk from any group of causes such as neoplasms, nervous system, circulatory system, respiratory system, digestive system, genitourinary system, accidents, poisoning, and violence as compared with the control group. From all causes there were only 41 deaths compared with the expected deaths of 48.3 over a 20-year period. For respiratory diseases and all the other causes except digestive, the mortality of workers at Nancekuke was somewhat less than for those in England and Wales.

Thirty-four young adults from 21 to 39 years of age were exposed to CS spray during an altercation with the police as they were trapped inside a 72-seat coach. They were exposed intermittently for up to 3 min and the whole group was out of the coach and into the fresh air within 10 min. Then some were sprayed directly on the face, others indirectly. All were interviewed a month after the exposure and asked about the adverse effects experienced 1 h postexposure. All but two recalled having respiratory (shortness of breath, chest tightness, cough, wheeze, exacerbation of asthma) and oral (pain, ulceration, excessive salivation) symptoms; and the symptoms were more severe in the directly exposed group. At 1 month, only oral symptoms were significantly more prevalent and at 8 to 10 months, symptoms were still reported, but there were no differences between the groups and clinical examinations revealed no specific abnormality (Karagama et al., 2003; Douglas, 2002). The authors felt that the retrospective reporting of symptoms present after 1 h after exposure were probably inaccurate and exaggerated in the hope of financial compensations. However, the data were reassuring in that there was no convincing evidence of long-term physical sequelae from exposure to CS spray in a confined space.

21.6 CR (DIBENZ (B,F) 1:4-OXAZEPINE)

The CAS number for CR is 257-07-8. It is a pale yellow solid with a pungent pepperlike odor and a molar mass of 195.3 corresponding to a molecular formula of $C_{13}H_9ON$. The molar solubility in



dibenz (b,f) – 1:4 – oxazepine (CR)

water at 20°C is 3.5×10^{-4} mol/l (= ~7 mg/100 ml). The melting and boiling points are 72°C and 335°C, respectively. The vapor is 6.7 times heavier than air, and the vapor pressure of the solid is 0.00059 mmHg at 20°C. CR is a stable chemical and may persist for prolonged periods in the environment. It was first synthesized in 1962 and is considered a potent sensory irritant of low toxicity.

21.6.1 Animal Toxicology

Ballantyne (1977) summarized the mammalian toxicology in various animal species. The acute toxicity of CR (LD_{50} and LCt_{50}) indicates that CR is less toxic than CS and CN by all routes of exposure. Animals exposed to CR exhibited ataxia or incoordination, spasms, convulsions, and tachypnea or rapid breathing. In the animals that survived, these effects gradually subsided over a period of 15–60 min. Increasing respiratory distress preceded death. The animals that died after intravenous and oral administration demonstrated congestion of liver sinusoids and alveolar capillaries. At necropsy, the surviving animals did not show any gross or histological abnormalities. The toxic signs following intraperitoneal administration included muscle weakness and heightened sensitivity to handling. These effects persisted throughout the first day of exposure. Some animals also exhibited central nervous system effects. Surviving animals did not exhibit any gross or histological abnormalities at necropsy. Several animal species were exposed to the acute inhalation of CR aerosols and smokes for various periods. Rats exposed to aerosol concentrations from 13,050 to 428,400 mg·min/m^3 manifested nasal secretions and blepharospasm or uncontrollable closure of the eyelids, which subsided within an hour after termination of the exposure. No deaths occurred during or after these exposures. There were also no deaths in rabbits, guinea pigs, or mice exposed to CR aerosols of up to 68,000 mg·min/m^3. Animals exposed to CR smoke generated pyrotechnically had alveolar capillary congestion and intra-alveolar hemorrhage, as well as kidney and liver congestion.

The potential of CR aerosols to produce physiological and ultrastructural changes in the lungs was evaluated by Pattle et al. (1974). Electron microscopy of rats exposed to CR aerosol of 115,000 mg·min/m^3 did not reveal any effects on organelles such as lamellated osmiophilic bodies. The studies by Colgrave et al. (1979) evaluated the lungs of animals exposed to aerosols of CR at dosages of 78,200, 140,900, and 161,300 mg·min/m^3 and found that they appeared normal on gross examination. On microscopic examination, however, the lungs revealed mild congestion, hemorrhage, and emphysema. Electron microscopy showed isolated swelling and thickening of the epithelium, as well as early capillary damage, as evidenced by ballooning of the endothelium. The authors concluded that these very high dosages of CR aerosols produced only minimal pulmonary damage.

In 1975, Lundy and McKay studied the cardiovascular effects of CR when administered intravenously. They found a dose-dependent increase in blood pressure of short duration and an increased heart rate and arterial catecholamines. The investigators postulated that the cardiovascular effects of CR were related to sympathetic nervous system effects as evidenced by the abolition of CR-induced presser effects by phentolamine and 6-hydroxydopamine.

Repeated inhalation exposures were conducted by Marrs et al. (1983a), who exposed mice and hamsters to concentrations of 204, 236, and 267 mg/m^3 CR for 5 d/week for 18 weeks. The high concentrations produced death in both species, but no single cause of death could be ascertained, although pneumonitis was present in many cases. Chronic inflammation of the larynx was observed in mice. Although alveologenic carcinoma was found in a single low-dose and a single high-dose group of mice, the findings and conclusions were questioned because the spontaneous occurrence of alveogenic carcinoma is high in many mouse strains (Grady and Stewart, 1940; Stewart et al., 1979). Further, this tumor type differs in many respects from human lung tumors. No lung tumors and no lesions were found in hamsters exposed to CR aerosols. Histopathology revealed hepatic lesions in mice, but these were of infectious origin and not CR related. The authors concluded that CR exposures at high concentrations reduced survivability and that CR produced minimal organ-specific toxicity at many times the intolerable human dose, which has been reported as 0.7 mg/m^3 (IC_{50}) within a minute (Ballantyne, 1977a) and 0.15 mg/m^3 (IC_{50}) within a minute (McNamara et al., 1972; Marrs et al., 1983).

Repeated cutaneous administration of CR was conducted in experimental animals by Marrs et al. (1982) who applied it to the skin 5 d/week for 12 weeks. The investigators concluded that repeated cutaneous applications of CR had little effect on the skin. They further postulated that in view of the absence of any specific organ effects, absorption of even substantial amounts of CR would have little effect.

Higgenbottom and Suschitzky (1962) were first to note the intense lacrimation and skin irritation caused by CR. Mild and transitory eye effects such as mild redness and mild chemosis were observed in rabbits and monkeys after a single dose of 1% CR solution. Multiple doses over a 5-d period of 1% CR solution to the eye produced only minimal effects. Biskup, et al. (1975) reported no signs of eye irritation in animals after single- or multiple-dose applications of 1% CR solutions. Moderate conjunctivitis following the application of 5% CR solution to the eyes of rabbits was reported by Rengstorff et al. (1975), although histological examination revealed normal corneal and eyelid tissues. Ballantyne and Swanston (1974) also studied the ocular irritancy of CR and arrived at a threshold concentration (TC_{50}) for blepharospasm in several species. Ballantyne et al. (1975) studied the effects of CR as a solid, as an aerosol, and as a solution in polyethylene glycol. Aerosol exposures of 10,800 and 17,130 mg·min/m^3 resulted in mild lacrimation and conjunctival injection that cleared in 1 h, but while in solution produced reversible dose-related increases in corneal thickness. The authors concluded that CR produced considerably less damage to the eye than CN and that a much greater degree of safety existed for CR than for CN.

The effects on skin were reported by Ballantyne (1977a) and by Holland (1974). They found that CR produced transient erythema but did not induce vesication or sensitization and did not delay the healing of skin injuries. The burning sensation on exposure to CR persists for 15–30 min, and the erythema may last for 1–2 h.

Upshall (1974) studied the reproductive and developmental effects of CR on rabbits and rats. They were exposed to inhalation of aerosolized CR at concentrations of 2, 20, and 200 mg/m^3 for 5 and 7 min. Groups of animals were also dosed intragastrically on days 6, 8, 10, 12, 14, 16, and 18 of pregnancy. No dose-related effects of CS were observed in any of the parameters measured and the number and types of malformations observed. No externally visible malformations were seen in any group and no dose-related effects of CR were noted in any of the fetuses in any group. Based on the overall observations, the author concluded that CR was neither teratogenic nor embryotoxic to rabbits and rats.

Only one study reported on the genotoxicity of CR. Colgrave et al. (1983) studied the mutagenic potential of technical grade CR and its precursor (2-aminodiphenyl ether) in the various strains of *S. typhimurium*, as well as in mammalian assay systems. CR and its precursor were negative in all the assays, suggesting that CR is not mutagenic. Further testing is required to exclude the genetic threat to humans and to determine the carcinogenic potential and its ability to cause other chronic health effects. Husain et al. (1991) studied the effects in rats of CR and CN aerosols on plasma glutamic

oxaloacetic transaminase (GOT), plasma glutamic pyruvic transaminase (GPT), acid phosphatase, and alkaline phosphatase. The rats exposed to CR exhibited no change in any of these parameters, whereas significant increases occurred in all these parameters in rats exposed to CN, suggesting that CN could cause tissue damage.

Upshall (1977) reported that CR aerosols are very quickly absorbed from the respiratory tract. Following inhalation, the plasma half-life ($t_{1/2}$) is about 5 min, which is about the same after intravenous administration. French et al. (1983) studied CR metabolism *in vitro* and *in vivo* and supported the previous conclusions that the major metabolic fate of CR in the rat is the oxidation to the lactam, subsequent ring hydroxylation, sulfate conjugation, and urinary excretion.

21.6.2 Human Volunteer Studies

Studies at Edgewood Arsenal and other research centers have been conducted to assess the effects of CR on humans following aerosol exposures, drenches, and local application (Ballantyne et al., 1973, 1976; Ballantyne and Swanston, 1974; Holland, 1974, Ashton et al., 1978). The human aerosol and cutaneous studies conducted at Edgewood Arsenal from 1963 to 1972 were summarized in a National Academy of Sciences Report (NAS) 1984 on irritants and vesicants. The respiratory effects following aerosol exposures included respiratory irritation with choking and difficulty in breathing or dyspnea, whereas the ocular effects consisted of lacrimation, irritation, and conjunctivitis. Ballantyne et al. (1973) reported the effects of dilute CR solution on humans following splash contamination of the face or facial drench. These exposures resulted in an immediate increase in blood pressure concomitant with decreased heart rate. In subsequent studies by Ballantyne et al. (1976), humans were exposed to whole-body drenches, which resulted in the same effects of immediate increase of blood pressure and bradycardia. The authors concluded that the cardiovascular effects in both studies were not due to the CR, because they theorized that there was insufficient CR uptake to produce the systemic effects on the heart. However, they did not provide an explanation for the cardiovascular changes. Lundy and McKay (1977) suggested that these cardiovascular changes resulted from the CR effects on the heart via the sympathetic nervous system. Ashton et al. (1978) exposed human subjects to a mean CR aerosol concentration of 0.25 mg/m^3 (particle size 1–2 μm) for 1 h. Expiratory flow rate was decreased approximately 20 min after the onset of exposure. The investigators theorized that CR stimulated the pulmonary irritant receptors to produce bronchoconstriction and increasing pulmonary blood volume by augmenting sympathetic tone.

The data for acute inhalation toxicity in various animal species from multiple sources for CR, CS, and CN generated pyrotechnically and also as an aerosol have been tabulated by Salem et al. (2001) and are presented in Table 21.2.

Estimates for lethality (LCt_{50}) and incapacitation (ICt_{50}) values in humans have been tabulated from many sources and reported by Salem et al. (2001). These data demonstrated the very high safety ratio and are presented in Table 21.3.

TABLE 21.2 Acute Animal Toxicity LCt_{50} (mg·min/m^3)

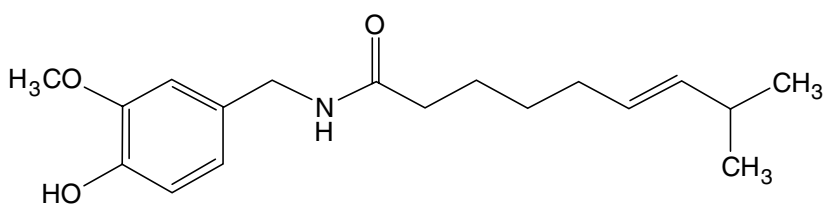
	Pyrotechnically Generated			Aerosol		
	CR	CS	CN	CR	CS	CN
Mouse	203,600	76,000	No data	169,500	67,200	18,200–73,500
Rat	139,000	68,000	23,000	428,400	88,460	3,700–18,800
Rabbit	160,000	63,000	15,800	169,000	54,100	5,840–11,480
Guinea pig	—	—	—	169,500	50,010	3,500–13,140

TABLE 21.3 Human Estimates (mg·min/m³)

Agent	LCt_{50}	ICt_{50}	LCt_{50}/ICt_{50} Safety Ratio
CN	8,500–25,000	20–50	425–500
CR	> 100,000	−1	100,000
CS	25,000–150,000	5	5,000–30,000
DM	11,000–35,000	20–150	550–233
BZ	200,000	112	1786

21.7 OC (OLEORESIN CAPSICUM) AND PAVA (NONIVAMIDE)

Oleoresin capsaicin is a reddish-brown, oily liquid obtained by extracting dried, ripe fruit of chili peppers, usually *Capsicum annuum* or *Capsicum fruitescenes*. The oleoresin is a mixture of many compounds. Its composition is variable and depends on factors such as maturity of the fruit, the environment in which the plants are grown, and the conditions of the extraction. More than 100 compounds have been identified in oleoresin capsicum. Among the branched- and straight-chain alkyl vanillylamides isolated from oleoresin capsicum, capsaicin is the major pungent component in many peppers, and it is particularly noted for its irritant properties. Depending on the variety of chili pepper, the oleoresin contains from 0.01 to 1.0% capsaicinoids on a dry mass basis. Some of the capsaicinoids found in the oleoresin are capsaicin (~70%) with a CAS number of 404-86-4, dihydrocapsaicin (~20%) with a CAS number of 19408-84-5, norhydrocapsaicin (~7%) with a CAS number of 28789-35-7, homocapsaicin (~1%) with a CAS number of 58493-48-4, and homodihydrocapsaicin (~1%) with a CAS number of 279-0605. Other components of the oleoresin such as phenolic compounds, acids, and esters, may also possess irritant properties.



Capsaicin

OC is considered a highly effective irritant that has received much attention as a less-than-lethal agent in civilian, governmental, and military sectors. OC spray or pepper spray has gained popularity as a law enforcement weapon in recent years. Because OC is a natural product, it is considered safe—a viewpoint that is not necessarily accurate. OC has been incorporated into a variety of formulations and marketed as "pepper gas," pepper mace, and pepper spray for self-defense, criminal incapacitation, law enforcement, and riot control purposes. OC exposure induces involuntary closing of the eyes and lacrimation. It also causes respiratory-related effects such as severe coughing and sneezing, nasal irritation, bronchoconstriction, and shortness of breath. It causes burning sensations of the skin and loss of motor control. As a result, many exposed individuals can be easily subdued. Acute effects of capsaicin and casaicinoids cause edema, hypertensive crisis, and hypothermia. Since 1990, there have been over 100 deaths reported after the use of OC spray. Although a causal relationship has not been established, most of the reported deaths had occurred within 1 h after exposure. Although there is an extensive database on capsaicin, very few data are available on the oleoresin capsicum. Studies reporting on capsaicin indicate that it is capable of producing mutagenic and carcinogenic effects, sensitization, cardiovascular and pulmonary toxicity, and neurotoxicity. It also has been reported to induce impairment in thermoregulation

against overheating (Obal et al., 1979, 1983; Monsereenusorn et al., 1982; Suzuki and Iwai, 1984; Buck and Burks, 1986; Fuller, 1990; Govindarajan and Sathyanarayana, 1991; O'Neill, 1991; Holzer, 1992). The causes of these in-custody deaths remain controversial, but the most common explanation is death from positional asphyxia. Other suggestions are excited delirium, heat prostration, drug interaction, cardiopulmonary sensitization, and the compromised Kratchmer reflex (Parenti, 1999).

Capsaicin was prepared and evaluated in humans as early as the 1920s. However, interest in its development waned when CS was synthesized and research efforts were redirected to the development of CS. Unlike other riot control agents such as CS, CR, and CN, which have definite chemical compositions, OC is a mixture of compounds containing capsaicinoids, various acids and esters, alcohols, aldehydes, ketones, and carotenoid pigments (Teranishni et al., 1980; Games et al., 1984; Govindarajan, 1986; Cordell and Araujo, 1993). Capsaicin as the major component is considered to be the active ingredient without consideration as to the activity of the other capsaicinoids. Although their activity is similar, they differ in potency (Cordell and Araujo, 1993).

21.7.1 Animal Toxicology

Not much is known about the toxicology of OC, but because it is a natural product and a much utilized food component, it is considered to be relatively safe, with a low order of toxicity (Clede, 1993). The pharmacology and toxicology of capsaicin, on the other hand, have been well characterized in both animal and human studies. The pharmacological actions of capsaicin and capsaicinoids were characterized in the 1950s (Issekutz et al., 1950; Toh et al., 1955). Glinsukon et al. (1980) studied the acute toxicity of capsaicin in several species and found that capsaicin is highly toxic by all routes of administration except gastric, rectal, and dermal. The intravenous doses of capsaicin caused convulsions within 5 sec and times to death were from 2 to 5 min. The toxic signs observed included excitement, convulsions with limbs extended, dyspnea, and death due to respiratory failure. We compared the acute intravenous LD_{50}s in mice with other well-known chemicals Table 21.4. This was done because there were no known comparative inhalation LD_{50}s in mice for all these chemicals, and the intravenous route has been considered very close to the inhalation route of exposure. This demonstrates that capsaicin's acute toxicity in mice is between that of nicotine and strychnine, two well-known potent poisons. Additionally, Glinsukon et al. (1980) also compared the intraperitoneal acute toxicity of capsaicin with the oleoresin capsicum in female mice and found the extract to be four times more toxic than the capsaicin with LD_{50}s of 1.51 and 6.50 mg/kg, respectively. Guinea pigs appeared to be more susceptible than mice and rats, whereas hamsters and rabbits were less vulnerable to the toxic actions of capsaicin.

The pulmonary pharmacology and toxicology of capsaicin has been studied in some detail. Inhalation of capsaicin is consistent with the induction of the Kratschmer reflex, which is apnea, bradycardia, and a biphasic fall and rise in aortic blood pressure. Exposure to capsaicin causes bronchoconstriction in animals and humans, the release of substance P, a neuropeptide, from sensory nerve terminals, and mucosal edema (Jansco et al., 1977; Russel and Lai-Fook, 1979; Davis et al., 1982; Fuller et al., 1985; Hathaway et al., 1993). The pulmonary effects of capsaicin appear to be species related. In guinea pigs, intravenous and intra-arterial administration causes bronchoconstriction (Biggs and Goel, 1985). The bronchoconstriction in the dog and cat after intravenous capsaicin depends on a vagal cholinergic reflex, as does the bronchoconstriction in the cat after aerosol exposure (Adcock and Smith, 1989). In guinea pigs, the bronchoconstriction following aerosol exposure suggests both a vagal-cholinergic and noncholinergic local axon reflex (Buchan and Adcock, 1992). The cardiorespiratory effects following intravenous administration resulted in a triphasic effect on blood pressure and altered cardiac parameters. The complex effects on the cardiovascular system consist of tachypnea, hypotension (Bezhold–Jarish reflex), bradycardia, and apnea (Chahl and Lynch, 1987; Porszasz and Szolesanyi, 1991/1992).

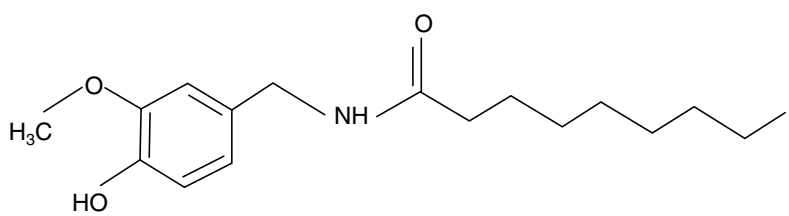
If capsaicin or pepper spray is the preferred agent of choice for self-defense or riot control, it has been suggested that much research is required, and preferably compared in parallel to CS and CR. Alternatively, capsaicin or an analog could be synthesized and evaluated as a single agent, in

TABLE 21.4 Mouse Intravenous LD_{50}s (mg/kg)[a]

Botulinum toxin	0.00001	Fentanyl[b]	11.2
Ricin[a]	0.005	Parathion	13
VX	0.012	DM	35
GB	0.10	CR	37
Nicotine	0.30	CS	48
Capsaicin	0.40	Caffeine	62
Strychnine	0.41	CN	81
Potassium cyanide	2.60	Cocaine	161
Mustard gas	3.30	Isopropyl alcohol	1509
Methamphetamine	10	Ethyl alcohol	1973

[a] Registry of Toxic Effects of Chemical Substances (RTECS).
[b] Gardocki and Yelnosky (1964).
[c] Patocka, available at www.asanltr.com/newsletter/01–4/articles Abrin& RicinRev.htm

comparison with CS and CR, rather than as a mixture with undetermined interactions of the multiple components. These components vary qualitatively and quantitatively depending on their natural origin (variety, species, maturity of fruit, hydrology, geology, and meteorology). Such a synthetic equivalent pelargonyl vanillamide (PAVA or Nonivamide), a potent sensory stimulant, has become available and is being used by police forces in the United Kingdom and other countries.



PAVA or Nonivamide

The available data have been reviewed by the U.K. Department of Health (2002) (COT Statement on the use of PAVA [nonivamide] as an Incapacitant Spray [COT/0202-April 2002] URL: http://www.doh.gov.uk/cotnonfood/pava.htm]. PAVA is also used as a food flavor in quantities up to 10 ppm, and in human medicine as a topically applied rubifacient, as is capsaicin. In the United States, PAVA has been given GRAS (generally recognized as safe) status by the Food and Drug Administration as a food flavor. Following a pilot exercise by the Sussex Police Force in the United Kingdom, they and the Northampton Police Force, as well as police forces in other European countries and in North America, are now using PAVA spray as an alternative chemical incapacitant to CS spray.

The spray used is a 0.3% solution of PAVA in 50% aqueous ethanol and is dispersed from handheld canisters by a nitrogen propellant. The coarse liquid stream spray pattern is considered to be directional and precise. The maximum effective range is 8–15 feet, aimed at the subject's face, especially the eyes. Users are cautioned not to use it at a distance of less than 3 feet to avoid pressure injury to the eyes. The particle size of the spray indicates that the bulk of the droplets are over 100 μm, but a small proportion of 1–2% are in the range of 2–10 μm, with trace amounts below 2 μm. Thus, it is

unlikely that large amounts of PAVA will reach the respiratory system. PAVA appears to be more toxic when administered parenterally, than when given orally, which suggests poor absorption. Topically applied PAVA in hydrophilic (oil-water emulsions) ointments for medical use have demonstrated from 50 to 70% absorption through rabbit skin in 14 h under occlusive dressing. Percutaneous absorption was also demonstrated from aqueous ethanol solution by using *in vitro* rat skin (Fang et al., 1996; Kasting et al., 1997). Once absorbed, PAVA is distributed throughout the body and is extensively metabolized and rapidly excreted within 24 h. The main route of metabolism is hydrolytic cleavage of the amide bond in the liver and other tissues including the skin (Kasting et al., 1997). Aliphatic hydroxylation may also occur as it does with capsaicin (Surh et al., 1995). The data on the toxicology of PAVA are limited. The data from the acute inhalation study in which rats were exposed to 3.6 mg/l for 4 h did not permit any conclusions to be drawn (Confarma AG. Report 21101618. Test of Inhalation Toxicity of Nonivamide/N only acid Capsaicin. Report for Swiss police Technical Commission, 1996).

No signs of skin irritancy were observed 3 d after exposure of 3.2% PAVA in polyethylene glycol on rabbits with occlusive dressing for 4 h. However, the in-use formulation of 0.3% in 50% aqueous ethanol did produce significant eye irritation in rabbits using the standard OECD procedure. Evidence of corneal opacity and damage to the iris were observed, but all eyes had recovered by 7 d postexposure. No data were available on the potential for skin sensitization.

21.7.2 Human Volunteer Studies

Volunteers were exposed to PAVA by inhalation to study the effects on the respiratory and cardiovascular systems. These studies included individuals with mild asthma. The aerosol was generated using a nebulizer to provide respiratory particles rather than the spray used by police. Ten healthy and 10 subject with mild asthma were exposed to a range of concentrations. The healthy subjects were exposed to aerosols generated from 0.3% PAVA in aqueous ethanol and the subject with asthma were exposed to aerosols generated from 0.1% solution. The healthy subjects experienced transient cough on exposure and minimal effects on FEV_1 (forced expiratory volume in one second [1% reduction]), heart rate (15% increase), and blood pressure (8% increase). Similar results were noted in the subjects with asthma who were exposed to 0.1% PAVA. These results were a 3% reduction in FEV_1, a 5% increase in heart rate, and a 5% increase in blood pressure. It was noted that in actual use subjects may experience a high level of stress that could lead to clinically significant bronchospasm.

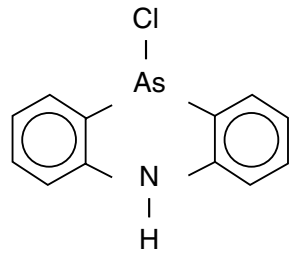
21.7.3 Human In-Use Exposures

No indication of significant adverse effects were observed, nor did there appear to be any persistent harm to skin or eyes in those exposed. However, based on animal experimentation, PAVA is an eye irritant and might cause marked effects in subjects wearing contact lenses. In view of the limited data available, a complete assessment of its adverse health effects is not possible.

21.8 DM (ADAMSITE)

DM (Adamsite) has a CAS Number of 579-94-9 and is also known as diphenylaminochloroarsine and phenarsazine chloride. It is a yellowish and odorless solid that is very stable in pure form. The melting point is 195°C and the vapor pressure is negligible (2×10^{-13} mmHg at 20°C). As a solid, the rate of hydrolysis is not significant, owing to the formation of any oxide coating. However, the rate of hydrolysis is rapid when it is an aerosol. DM has a molecular weight of 277.5 with the formula $C_6H_4(AsCl)(NH)\ C_6H_4$.

DM is among the group of compounds including diphenylchloroarsine (DA), diphenylcyanoarsine (DC), and chloropicrin, which are classified militarily as vomiting agents. DM has been characterized as both a vomiting agent and a sneezing agent (sternutator) and was used in World War 1. (Sidell [1997] has reported the estimated human LCt_{50} to be 11,000 mg·min/m^3.) DM effects, unlike those of CN, CS, and CR, have a slightly delayed onset and a relatively long recovery period. DM effects occur in about 3 min after inhalation of exposure and may last for several hours (British Ministry of Defense [BMOD],



10-chloro-5,10-diphenylarsazine (DM)

1972; Ballantyne, 1977a). Also unlike the tear agents, DM is more likely to cause prolonged systemic effects. Signs and symptoms of DM exposure include eye irritation, upper respiratory tract irritation, uncontrolled sneezing and coughing, choking, headache, acute pain, tightness in the chest, nausea, and vomiting as well as unsteady gait, weakness in the limbs, and trembling. Ballantyne (1977a) also indicated that mental depression might follow DM exposure. Exposures to high concentrations can result in serious illness as a result of pulmonary damage, edema, and death. (BMOD, 1972).

21.8.1 Animal Toxicology

The National Academy of Sciences Report (1984) reported on the toxicology of DM, based on the studies by Owens et al. (1967) and McNamara et al. (1969). Various animal species including monkeys have been exposed to DM. Following acute exposures the animals exhibited ocular and nasal irritation, hyperactivity, salivation, labored breathing, ataxia, and convulsions. Punte et al. (1962a) found these same effects at high concentration exposures for up to 90 min, as well as lethargy. Histopathology did not reveal any abnormalities at exposure dosages of below 500 mg·min/m^3. At higher dosages, animals that died or were killed demonstrated hyperemia of the trachea, pulmonary congestion and edema, and pneumonia. These effects were consistent to exposure to pulmonary irritants. DM toxicity values are presented in Table 21.5.

Striker et al. (1967) exposed monkeys to varying concentrations and durations. At a Ct dosage of 2565 mg·min/m^3, only one animal responded, and that was with oral and nasal discharge and diminished response to stimuli. A Ct of 8540 mg·min/m^3 resulted in ocular and nasal conjunctival congestion, facial erythema, and decreased responses, all of which were resolved within 24 h. Exposure to the high dosage of 28,765 mg·min/m^3 resulted in hyperactivity, copious nasal discharge, conjunctival congestion, marked respiratory distress, as well as gasping and gagging in all the exposed monkeys. Eight of these exposed monkeys died within 24 h of exposure. Necropsy of these animals revealed congestion and extremely edematous lungs. Microscopic examination revealed ulceration of the tracheobronchial tree and pulmonary edema. Studies were also conducted in which monkeys were exposed low-target concentrations of 100 and 300 mg/m^3 DM for 2–60 min and for 2–40 min, respectively. As the exposure duration increased toxic signs increased, which is characteristic of exposure to irritants. At the maximum dosage of 13,200 mg·min/m^3, the animals exhibited nausea and vomiting, oral and nasal discharge, and conjunctival congestion. Below 1296 mg·min/m^3 the only signs were blinking.

The effects of DM on the gastrointestinal tract were suggested as a possible cause of death. Dogs were given lethal doses of DM both intravenously and orally, while the following parameters were monitored: central venous pressure, right ventricular pressure, cortical electric activity, alveolar CO_2, respiratory rate, heart rate, electrocardiogram, and gastric activity. DM caused a marked elevation of both amplitude and rate of gastric activity for 15–20 min and then returning to normal. Pretreatment with trimethobenzamide, an effective antiemetic for peripheral and centrally acting emetics, did not prevent DM gastric activity, but chlorpromazine was effective. The authors concluded that DM

TABLE 21.5 DM Toxicity

Species	LCT_{5O} (mg·min/m³)	LD (mg/kg)[a]
Mice	22,400	17.9
Rats	3,700	14.1
Guinea pigs	7,900	2.4

[a] Theoretical dose calculated from respiratory volume, LCt_{50}, and estimated percent retention.

affects the stomach directly and that the primary cause of death following exposure to DM is its effects on the lungs as demonstrated in the studies of Striker et al. (1967).

The effects of DM on the eyes and skin of rabbits were studied with DM suspended in corn oil instilled into the eyes of rabbits in doses of 0.1, 0.2, 0.5, 1.0, and 5.0 mg. No effect was observed at 0.1 mg, but at 0.2 mg, mild conjunctivitis was observed. At 0.5 mg, mild blepharitis was also seen. Corneal opacity persisted over the 14-d observation period in rabbit eyes that were given 1.0 and 5.0 mg DM. Corn oil suspensions of DM (100 mg/ml) were placed on the clipped backs of rabbits at doses of 1, 10, 50, 75, and 100 mg. At 10 mg and higher, necrosis of the skin was observed. Rothberg (1969) studied the skin sensitization potential of riot control agents in guinea pigs. For DM, his results were negative, indicating that DM does not have the potential for skin sensitization.

21.8.2 Human Volunteer Studies

The human toxicology of DM was reviewed by Ballantyne (1977). He described the effects of human inhalation exposures to DM as beginning with acute pain in the nose and sinuses. This was followed by pain in the throat and chest, with sneezing and violent coughing, and also eye pain, lacrimation, blepharospasm, rhinorrhea, salivation, nausea, and vomiting. Recovery is usually complete in 1–2 h after exposure. The onset of signs and symptoms is delayed for several minutes, unlike the onset for CS and CN, which is almost immediate. The slow onset of DM allows for the absorption of much more DM before a warning is perceived. Threshold doses were estimated for irritation of the throat, lower respiratory tract, and initiation of the cough reflex to be 0.38, 0.5, and 0.75 mg/m³, respectively. Punte et al. (1962a) and Gongwer et al. (1958) studied the effects of varying concentrations of DM on human subjects and agreed that humans could tolerate concentrations of 22–92 mg/m³ for 1 min or more, and a concentration range of 22–220 mg/m³ would appear to be intolerable to 50% of a population for 1 min.

Although a dosage of 49 mg·min/m³ was sufficient to cause nausea and vomiting, based on studies in humans exposed to DM at Ct values between 7 and 236 mg·min/m³, high confidence is lacking because the estimate is founded on highly variable data. Ballantyne (1977a) estimated a dosage of 370 mg·min/m³ was required to cause nausea and vomiting. Inhalation of high concentrations of DM has resulted in severe pulmonary damage and death (BMOD, 1972). DM is less effective as a riot control or incapacitating agent than CS and CN, and it was conjectured that there might be greater differences among peoples' susceptibility to DM than to the other agents.

Castro (1968) found DM and CS to be cholinesterase inhibitors and suggested that this might explain their lacrimatory effect. Although DM has a direct effect on gastric activity, Striker et al. (1967) found evidence that the lethal effect of DM is respiratory.

Some physical characteristics of CS, CR, CN, DM, and Capsaicin are presented in Table 21.6.

21.9 FENTANYLS

During the Cold War (1945–1991), a great deal of research was directed to chemicals that were not necessarily lethal but would merely incapacitate enemy personnel. In particular, the United States and the former Soviet Union investigated a wide number of pharmacological agents for their potential

TABLE 21.6 Physical Properties of Selected Riot Control Agents

	CS	CR	CN	DM	Capsaicin
Molecular weight	188.5	195.3	154.5	277.5	305
Melting point, ºC	93	72	54	195	64
Vapor pressure, mmHg at 20ºC	0.00034	0.00059	0.0054	$2 \times 10 - 13$	0.011
Volatility, mg/m^3/ºC	0.71/25º	0.63/25º	1.06/52º	—	—
Solubility[a]	loc	loc	loc	lo	loc

[a] Solubility: l, limited in water; o, soluble in organics; e, soluble in chlorinated organics.

as incapacitants, such as depressants, hallucinogens, belladonna drugs, and opiate derivatives (Chemical Biological Weapons Nonproliferation Treaty [2002]).

A major breakthrough in opiate drugs for use in medicine was the synthesis of fentanyl in Belgium in the late 1950s; it was first patented by Janssen in France in 1963. Its primary use in medicine was alone or in combination for anesthesia. However, its major complication is respiratory depression, which can be monitored and reversed in an operating room but can be a problem if used operationally in the field. Since 1996, several different analogs of fentanyl have been introduced for use in anesthesia, such as carfentanil, sufentanil, alfentanil, and remifentanil. Their pharmacological activity is characteristic of opiates and they produce all the effects of heroin, including analgesia, euphoria, miosis, and respiratory depression. Because of their high lipid solubility, regardless of route of administration, the fentanyls reach the brain very quickly, thus providing a very fast onset of action. Some of the analogs have been synthesized specifically for sale as Persian white, China white, Mexican brown, and synthetic heroin, on the illicit drug market and to circumvent regulations on controlled substances. These illicit drugs are also called designer fentanyls and are used by abusers via intravenous injection, or they can be smoked or snorted.

Fentanyls are synthetic opiates recognized for their short acting and highly potent narcotic analgesic, anesthetic, and immobilizing properties in both animals and humans (Janssen, 1984; Hess and Knakel, 1985). Fentanyl or *N*-phenyl-*N*-[1-(2-phenylethyl)-4-piperidinyl] propanamide is a potent narcotic analgesic that has a molecular weight of 336.46. Its CAS Registry Number is 437-38-7. The citrate of fentanyl is a crystalline powder with a bitter taste and 1 g dissolves in about 40 ml of water. Fentanyl is also used as an adjunct to general anesthesia and as an anesthetic for induction and maintenance. It is primarily a mu-opioid agonist. Abuse of this drug leads to habituation or addiction. The CAS Registry numbers of some of the analogs of fentanyl are sufentanil, 56030-54-7; carfentanil, 59708-52-0, and remifentanil, 132875-61-7.

The feasibility of dissociating the respiratory depressant effect from the opiate-induced sedative activity of alfentanil and fentanyl with naloxone was studied in the rabbit by Brown and Pleuvry (1981). They found that naloxone was more effective as an antagonist to alfentanil than to fentanyl. Later studies by Mioduszewski and Reutter (1991) also suggested that in the rat and ferret dissociation of the opiate-induced sedation and respiratory depression was feasible. This was accomplished by coadministration of the opiate agonist with antagonists. The opiate-induced effects were akinesia, catalepsy, loss of righting reflex, light anesthesia, and apnea. The pharmacodynamic mechanism of the coadministration may involve competitive displacement of the opiate agonist by the antagonist at their common receptor sites within the central nervous system. A pharmacokinetic mechanism may also be involved such that the opiate uptake, distribution, and clearance are affected, either directly or indirectly, by the antagonist. Changes in respiratory frequency, oxygen consumption, and apnea were monitored in ferrets following the intravenous coadministration of the opiate agonist sufentanil and the antagonist nalmefene. These studies demonstrated a dissociation of the sufentanil-induced sedation/anesthesia and severe respiratory depression. Nalmefine coadministration shortened the duration but did not significantly delay the onset

of the opiate-induced sedative/anesthetic effect. Narcotic antagonists such as nalmefene, naltrexone, and naloxone have clinical application in the diagnosis of addiction, prophylactic treatment of narcotic abuse, and emergency treatment of narcotic overdosage. These antagonists displace either previously assimilated opiates from their receptor sites or, if administered prior to the narcotic, will preclude the narcotic agonist from acting at these sites (Langguth et al., 1990).

More recently, Manzke et al. (2003) reported that the serious adverse effects of opiate analgesia such as depression of breathing are caused by direct inhibition of rhythm-generating respiratory neurons in the Pre-Boetzinger complex (PBC) of the brain stem. Serotonin 4(a) or 5-HT4(a) receptors are strongly expressed in these neurons and their selective activation protects spontaneous respiratory activity. Rats treated with a 5-HT4 receptor specific agonist overcame the fentanyl-induced respiratory depression and reestablished stable respiratory rhythm without loss of fentanyl's analgesic effect.

Based on his studies of the dissociation of the sedative/anesthetic effects and respiratory depression of sufentanil coadministration with nalmefene in ferrets, Mioduszewski (1994) was awarded a U.S. patent for an opiate analgesic formulation with improved safety. The invention described is a homogeneous mixture of an opiate drug agonist and an opiate drug antagonist suitable for *in vivo* administration to a patient by intravenous injection or by inhalation as an aerosol. The opiate agonist of the fentanyl series (fentanyl, carfentanil, alfentanil, sufentanil), and the antagonist nalmefene, naloxone, or naltrexone, are in the ideal ratios so that the duration of agonist and antagonist effects are the same, and will not result in renarcotization and the associated respiratory depression if the agonist effects outlast those of the antagonist.

Opiate effects are mediated via multiple opioid receptors such as the mu, kappa, delta, and sigma (Martin, 1983). The mu receptors mediate analgesia, euphoria, physical dependence, and depression of ventilation, whereas kappa receptors mediate sedation and dieresis. Drugs may act at more than one opiate receptor with varying effect. Traditionally, narcotic antagonists such as naloxone and naltraxone have been used to reverse opioid agonists effects, whereas in these studies, naltraxone did not appear to have any effect on the potency and onset of carfentanil-induced sedative/hypnotic effects in the ferret. When used clinically, longer acting opioids such as fentanyl may produce renarcotization because of differences in the pharmacokinetics of agonists and antagonist (McGilliard and Takemori, 1978).

21.9.1 Human In-Use Exposures

Because fentanyl is not listed in any of the schedules of the Chemical Warfare Convention (CWC),and is traditionally characterized by the rapid onset and short duration of 15–30 min of analgesia, it can be legally considered a riot control agent according to the definition set forth in the CWC (Chemical Biological Weapons Non-proliferation Treaty [CBWNP], 2002).

On October 23, 2002, at least 129 of the almost 800 hostages died in the Moscow Dubrovka Theater Center when authorities subdued the hostage takers (the Chechnian terrorist group) there by pumping what many believe was fentanyl into the building (Couzin, 2003), while some believe that a mixture of fentanyl and halothane was used (CBWNP, 2002; Mercadente, 2003; Couzin, 2003; Miller and Lichtblau, 2003). It was also considered that the Russians might have used remifentanil because it is rather unique and extremely potent with relatively fast action and short duration. The chemical structure of remifentanil also allows the body to quickly metabolize the substance into nontoxic and water-soluble forms, thus minimizing risks for both hostages and hostage takers. Although Russian authorities insisted that emergency personnel were prepared with 1000 antidotes in anticipation of the raid, controversy still exists about whether local hospitals and physicians were adequately informed about the gas used during the operation (CBWNP, 2002; Glasser and Baker, 2002),

It has also been suggested that the Russian government revealed that a mixture of fentanyl and halothane was used to incapacitate the Chechnian terrorists in the attempt to liberate the hostages in Moscow. It was further suggested that it was likely that massive doses of carfentanil were used to saturate the theater so that the maximal effect by inhalation could be achieved. Carfentanil is a potent opioid used to rapidly immobilize large, wild animals, horses, and goats. It produces rapid catatonic

immobilization, characterized by limb and neck hyperextension. Adverse effects include muscle rigidity, bradypnea, and oxygen desaturation. Recycling and renarcotization have been reported as possible causes of death when low doses of antagonist are used (Miller et al., 1996).

Although there were naloxone syringes found in the theater, it is possible that the doses were insufficient to reverse the respiratory depression (Mercadante, 2003).

21.10 IN-USE RIOT CONTROL EFFECTS

The toxicological effects, which are actually the pharmacological effects, of exposure to riot control agents, but are perceived as adverse or toxicological, can be local or topical, as well as systemic following absorption. In addition the effects can be acute or long term. Also, the exposure can be either, acute, long, or repeated. The disposition of the agent in the exposed individual also needs to be considered. That is the absorption, distribution, metabolism (biotransformation), and excretion (ADME).

Riot control agents have been described as nonlethal or less-than-lethal agents. Exposures to these compounds involve ocular, cutaneous, and inhalation effects and, indirectly, oral or gastrointestinal effects. Their primary action is their local or topical effect on the eye, which appears to be the most sensitive target organ. They also act on the skin and respiratory tract. The immediate effects on exposure to irritants include intense irritation of the eyes, marked irritation of the nose, throat, and lungs, and irritation of the skin. The margin of safety between the dose eliciting the intolerable effect and the dose that causes serious adverse effects is large. For example, the estimated lethal human dose is 2600 times as great as the dose required to cause temporary disability and that of bromobenzyl cyanide is 3000 times as great. Riot control agents do not usually cause long-term or permanent toxic effects, although the risk for serious toxic effects, long-term sequelae, or even death increases with higher exposure concentration, with greater exposure duration, or in susceptible individuals. Overall, however, the toxicity of acute and short-term repeated exposures to riot control agents is well characterized.

21.10.1 Ocular

Exposure to riot control agents causes an immediate stinging sensation in the eyes and tearing, resulting in a temporary disabling effect. These effects are reversible and noninjurious at low concentrations. At high concentrations, however, some irritants can cause ocular damage. Moderate injury to the eye after exposure results in corneal edema, which is reversible. More serious injury may include corneal opacification, vascularization, scarring of the cornea, and corneal ulceration. The riot control agents that have been associated with ocular injury include chloroacetophenone (CN) and bromobenzyl cyanide (BBC). Ocular injuries are more prevalent following use of explosive or thermal type tear gas devices as contrasted with solvent spraytype devices. A description of these differences is provided by MacLeod (1969)

Ocular injuries have been reported by Macleod, 1969; Midtbo, 1964; Hoffman, 1965; Bregeat, 1968; Levine and Stahl, 1968; and Grant, 1986 (Table 21.7).

21.10.2 Cutaneous

Riot control agents at low concentrations also produce a tingling or burning sensation and transient erythema on the skin. At higher concentrations, agents such as CN, CS, and DM can cause edema and blistering. They can also induce an allergic contact dermatitis after an initial exposure. These effects are successfully treated with topical steroid preparations and oral administration of antihistamines for itching. Appropriate antibiotics can be administered to treat secondary infections.

21.10.3 CS

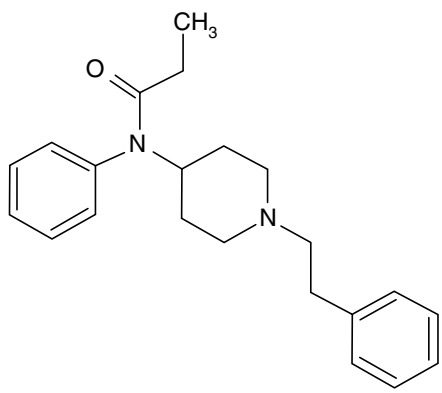
Exposure to CS is highly irritating to the mucous membranes that line the tissues of the eyes, nose, throat, and respiratory and gastrointestinal tracts. Irritation of the eyes may cause pain, excessive

TABLE 21.7 Human Ocular Irritancy

Compound	Onset/action	Threshold concentration (mg/m^3)	Intolerable concentration (mg/m^3)	10-min exposure lethal concentration (mg/m^3)
CN	Immediate	0.3	5–30	850
CR	Immediate	0.002	1	10,000
CS	Immediate	0.004	3	2,500
DM	Rapid	1	5	650
BBC	Rapid	0.15	0.8	350
Acrolein	Rapid	2–7	50	350
OC	Rapid	—	—	—

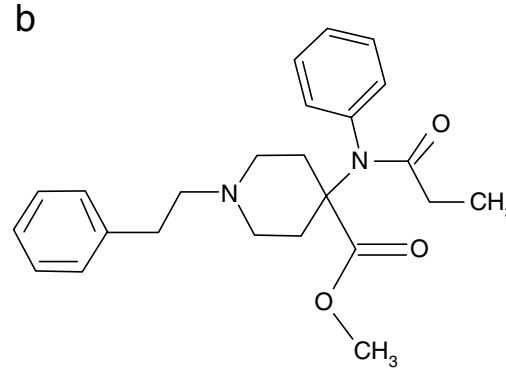
Source: Ballantyne and Swanston (1973, 1974) and Ballantyne (1977a).

a



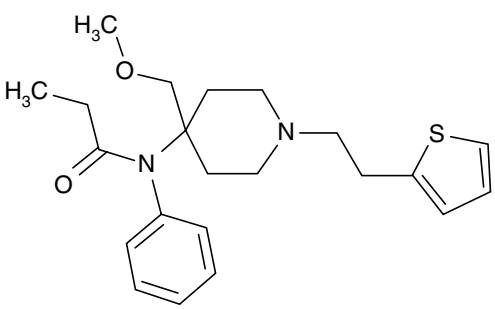
Fentany1
RN: 437-38-7

b



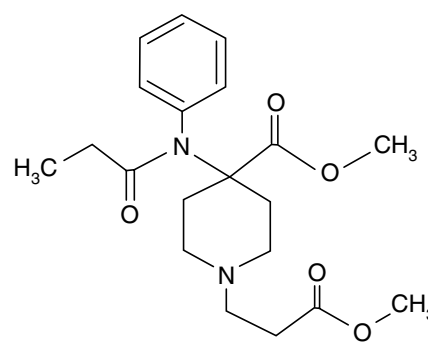
Carfentanil
RN: 59708-52-0

c



Sufentanil (USAN:BAN:INN)
RN: 56030-54-7

d



Remifentamil
RN: 132875-61-7

tearing, conjunctivitis, and blepharospasm (uncontrolled blinking). The nose and mouth may perceive a stinging or burning sensation with excessive rhinorhea or discharge of nasal mucus. Irritation of the respiratory tract may cause tightness of the chest, sneezing and coughing, and increased respiratory secretions. Severe lung injury and subsequent respiratory and circulatory failure characterize death in experimental animals following inhalation of very high dosages of CS. Irritation of the gastrointestinal tract may cause vomiting and/or diarrhea. After exposure of the skin, a burning sensation may be experienced, with subsequent inflammation and redness. Six minutes following exposure to CS, the irritation during exposure is so intense that the individual exposed seeks to escape. The lethal effects in animals following inhalation exposures is caused by lung damage leading to asphyxia and circulatory failure or bronchopneumonia secondary to respiratory tract injury. Pathology involving the liver and kidneys after inhalation of high dosages of CS is also secondary to respiratory and circulatory failure. The animal and human toxicological effects have been described by Ballantyne (1977a), Ballantyne and Callaway (1972), Ballantyne and Swanston (1978), Ballantyne et al. (1976), Beswick et al. (1972), Owens and Punte (1963), and Punte et al. (1962; 1963). Experimental studies included several animal species as well as human exposures to CS in actual use in Northern Ireland and in Waco, Texas, USA.

Various experimental animal species were exposed to aerosols of CS generated by various methods from exposure from 5 to 90 min. The toxic signs observed in mice, rats, guinea pigs, rabbits, dogs, and monkeys were immediate and included hyperactivity, followed by copious lacrimation and salivation within 30 sec of exposure in all species except the rabbit. The initial level of heightened activity subsided, and within 5–15 min after initiation of the exposure, exhibited lethargy and pulmonary stress, which continued for about an hour following cessation of the exposure. All other signs had disappeared within 5 min following removal from the exposure. When toxic signs were observed, they occurred after exposure by all the dispersion methods.

Lethality estimates were expressed by calculation of LCt_{50}s. From acute exposures to CS dispersed from a 10% CS in methylene dichloride the LCt_{50}s were as follows. Mice, 627,000 mg·min/m^3; rats, 1,004,000 mg·min/m^3; and guinea pigs, 46,000 mg·min/m^3. No deaths occurred in rabbits exposed to up to 47,000 mg·min/m^3. CS at dosages of up to 30,000 mg·min/m^3 did not cause any deaths in any of the monkeys with pulmonary tularemia. The combined LCt_{50} for mice, rats, guinea pigs, and rabbits was calculated to be 1,230,000 mg·min/m^3 for CS dispersed from methylene dichloride. Goats, pigs, and sheep did not exhibit hyperactivity on exposure to CS, and they were also resistant to its lethal effect. Therefore no LCt_{50} values could be calculated for goats, pigs, or sheep. However, a combined LCt_{50} was calculated for all the species tested; for mice, rats, guinea pigs, rabbits, dogs, monkey, goats, pigs, and sheep, the combined LCt_{50} was estimated to be 300,000 mg·min/m^3. LCt_{50}s were also calculated for CS dispersed from M18 and M7A3 thermal grenades. These values were 164,000 mg·min/m^3 for rats and 36,000 mg·min/m^3 for guinea pigs exposed to the M18 thermal grenade dissemination. For the M7A3 thermal grenade the values were as follows: rats, 94,000 mg·min/m^3; guinea pigs, 66,000 mg·min/m^3; rabbit, 38,000 mg·min/m^3; goat, 48,000 mg·min/m^3; pigs, 17,000 mg·min/m^3; dog, 30,000 mg·min/m^3; monkey, 120,000 mg·min/m^3. All the acute exposure results were combined and LCt_{50}s were calculated for all rodents to be 79,000 mg·min/m^3, for all nonrodent species tested to be 36,000 mg·min/m^3, and for all the species to be 61,000 mg·min/m^3. The LCt_{50}s for CS2 were also calculated. CS2 is 95% CS, 5% Cal-o-Sil R, and 1% hexamethyldisilazane, and the LCt_{50}s are the following: rats, 68,000 mg·min/m^3; guinea pigs, 49,000 mg·min/m^3; dogs, 70,000 mg·min/m^3; and monkeys, 74,000 mg·min/m^3 (Marrs et al., 1983b).

21.11 SUMMARY

Riot control agents are usually delivered by aerosol, vapor, or gas and belong to the following pharmacological classes: irritants, lacrimators, sternutators, emetics, sedatives, hypnotics, serotonin antagonists, hypotensives, thermoregulatory disruptors, neuromuscular blockers, centrally acting anesthetics, and malodorous substances. Of the many chemicals used as riot control agents, only

some of the most commonly used or those being investigated by law enforcement agencies and military around the world are reviewed in this chapter. These include CN, CS, CR, DM, OC and capsaicin, and PAVA, as well as the fentanyls. The historical use of irritant chemicals, going as far back as 400 BCE is also reviewed in this chapter.

Riot control agents are usually dispersed as aerosols, vapors, gases, or sprays, and in unprotected individuals, their first contact and effects are topical to the eyes and skin, and then inhaled if in the appropriate particle sizes. They induce intense irritation of the mucous membranes of the eyes and respiratory tract and the skin, especially in wet or sweaty areas. On the eyes, they produce irritation, blepharospasm, lacrimation, blurring of vision, conjunctivitis, and pain. In the nose and throat, they cause a tingling sensation, irritation, pain, and increased secretions, whereas in the respiratory tract they cause irritation, burning and pain, tightness of the chest, and respiratory reflexes. Occasionally, nausea, gagging, and vomiting may also occur. Most of the effects of riot control agents usually disappear within 20 min postexposure.

CN was used as a tear gas of choice for the three decades after its introduction toward the end of World War I. It was trademarked as MACE, but today Mace is used as the generic term for riot control agents. Because of its toxic profile, and the availability of CS, the use of CN has been markedly reduced. CS has largely replaced CN because it is considered at least as effective and safer. Although alleged, to date no human fatalities following exposure to CS has been validated. Extensive studies with CS have been conducted in experimental animals and human volunteers. In addition, many thousands of military troops have been exposed to CS during mask training, and there have been many human in-use exposures. Several overexposures in enclosed spaces have been reported, and although severe intoxication with respiratory problems resulted, with hospitalization and aggressive treatment, all patients recovered. There did not appear to be any increased sensitivity to what is usually considered susceptible populations (young, elderly, compromised [asthma, pregnancy]). Both CS and CN have been tested in the NCI Bioassay program and were not considered carcinogenic, except for CN for which the data from female rats were considered equivocal.

The 1990s found “pepper spray” (oleoresin capsicum) popular not only with the general public as a defensive spray, but also with law enforcement agencies and the military. This was in part because of its availability over the counter and because it was a natural product and therefore erroneously considered safe. Although capsaicin, considered the primary active ingredient in the oleoresin, has been studied extensively, there have been very few well-controlled and objective studies with oleoresin. As discussed earlier in this chapter, the U.S. military was evaluating capsaicin as a riot control agent in the 1920s, but redirected their efforts to the development of CS when it was synthesized in 1928. Since the 1990s over 100 in-custody deaths have been reported within an hour following exposure to OC spray. Although a direct cause- and-effect relationship has not been confirmed, more investigations are necessary. In addition, and unlike the other riot control agents that are defined chemicals, OC is a mixture of chemicals that qualitatively and quantitatively depend on many natural factors. The participation of these various components in the overall activity of the oleoresin spray has not been defined. A synthetic capsaicin equivalent, pelargonal vanillamide (PAVA or Nonivamide), a potent sensory stimulant, has become available and is being used by some police forces in several countries. Very few experimental data are available on this chemical.

DM (Adamsite), an arsenical vomiting agent, is also reviewed in this chapter. It is less effective as a riot control agent than CS and CN, and its effects are subject to more individual susceptibility.

CR is the most recently developed riot control agent that appears to be more potent and less toxic by all routes of exposure but does not have the extensive in-use exposures that CS and CN have.

During the Cold War, more research was directed at nonlethal or less- than-lethal chemicals that are capable of incapacitating enemy personnel and are nondestructive. This included the investigations of pharmacological agents with the potential to incapacitate or immobilize, such as depressants, hallucinogens, belladonna drugs, and opiate derivatives. The opiate derivatives used as short-acting anesthetics in surgery, such as the fentanyls, whose side effects include respiratory depression, and can be treated and reversed in an operating room, have also been considered.

Experimental studies in animals in the United States and the United Kingdom have been described in this chapter, as has the in-use exposure by the Russians against the Chechnian terrorists who held 800 hostages in the Moscow Dubrovka Theater Center in October 2002. Although the antidote was available, at least 129 hostages died.

REFERENCES

Adams, J.P., Fee, N., and Kenmore, P.I., Tear gas injuries, *J. Bone Jt. Surg.*, 48A, 436–442, 1966.

Adcock, J.J. and Smith, T.W., Inhibition of reflex bronchoconstriction by the opioid peptide BW 443C81 in the anaesthetized cat, *Br. J. Pharmacol.*, 96, 596, 1989.

Allen, W.F., Effect on respiration, blood pressure, and carotid pulse of various inhaled and insufflated vapors when stimulating one cranial nerve and various combinations of cranial nerves, *Am. J. Physiol.*, 87, 319–325, 1928–1929.

Anderson, P.J., Lau, G.S.N., Taylor, W.R.J., and Critchley, J.A., Acute Effects of the potent lacrimator o-chlorobenzylidene malononitrile (CS) tear gas, *Hum. Exp. Toxicol.*, 15, 461–465, 1996.

Ashton, I., Cotes, J.E., Holland, P., Johnson, G.R., Legg, S.J., Saunders, M.J., and White, R.G., Acute effect of dibenz (b,f)-1:4-oxazepine upon the lung function of healthy young men, *J. Physiol.*, 275, 85, 1978.

Aviado, D.M., Kratschmer reflex induced by inhalation of aerosol propellants, in *Conference on Toxic Hazards of Halocarbon Propellants*, U.S. Food and Drug Administration, Washington, DC, 1971, pp. 63–77.

Aviado, D.M. and Aviado, D.G., The Bezold-Jarsch reflex a historical perspective of cardiopulmonary reflexes, *Ann. N. Y. Acad. Sci.*, 940, 48–58, 2002.

Aviado, D.M. and Salem, H., Respiratory and bronchomotor reflexes in toxicity studies, in *Inhalation Toxicology*, Salem, H., Ed., Marcel Dekker, New York, 1987.

Aviado, D.M. and Salem, H., Acute effects of air pollutants on the lungs, *Arch. Environ. Health*, 16, 903–907, 1968.

Ballantyne, B., Riot-control agents (biomedical and health aspects of the use of chemicals in civil disturbances), in *Medical Annual*, Scott, R.B. and Frazer , J., Eds., Wright and Sons, Bristol, 1977a.

Ballantyne, B., The acute mammalian toxicology of dibenz (b,f)-1:4-oxazepine, *Toxicology*, 8, 347, 1977b.

Ballantyne, B., Beswick, F.W., and Thomas, D., The presentation and management of individuals contaminated with solutions of dibenzoxazepine (CR), *Med. Sci. Law*, 13, 265, 1973.

Ballantyne, B. and Callaway, S., Inhalation toxicology and pathology of animals exposed to o-chlorobenzylidene malononitrile (CS), *Med. Sci. Law*, 12, 43, 1972.

Ballantyne, B., Gall, D., and Robson, D.C., Effects on man of drenching with dilute solutions of o-chlorobenzylidene malononitrile (CS) and dibenz (b,f)-1:4-oxazepine (CR), *Med. Sci. Law*, 16, 159, 1976.

Ballantyne, B., Gazzard, M.F., Swanston, D.W., and Williams, P., The comparative ophthalmic toxicology of 1-chloroacetophenone (CN) and dibenz (b,f)-1:4-oxazepine (CR), *Arch. Toxicol.*, 34, 183, 1975.

Ballantyne, B. and Swanston, D.W., The irritant potential of dilute solutions of ortho-chlorobenzylidene malononitrile (CS) on the eye and tongue, *Acta Pharmacol. Toxicol.*, 32, 266, 1973.

Ballantyne, B. and Swanston, D.W., The irritant effects of dilute solutions of dibenzox-azepine (CR) on the eye and tongue, *Acta Pharmacol. Toxicol.*, 35, 412, 1974.

Ballantyne, B. and Swanston, D.W., The comparative acute mammalian toxicity of 1-chloroacetophenone (CN) and 2-chlorobenzylidene malononitrile (CS), *Arch. Toxicol.*, 40, 75, 1978.

Beswick, F.W., Holland, P., and Kemp, K.H., Acute effects of exposure to ortho-chlorobenzylidene malononitrile (CS) and the development of tolerance, *Br. J. Ind. Med.*, 29, 298, 1972.

Biggs, D.F. and Goel, V., Does capsaicin cause bronchospasm in guinea-pigs?, *Eur. J. Pharmacol.*, 115, 71, 1985.

Biskup, R.C., Swentzel, K.C., Lochner, Ma.A., and Fairchild, D.G., Toxicity of 1% CR in propylene glycol/water (80/20), Technical Report EB-TR-75009, May 1975, Edgewood Arsenal, Aberdeen Proving Ground, MD, 1975.

Boyd, K., Military Authorized to Use Riot Control Agents in Iraq, 2003. Available at www.armscontrol.org/act/2003_05/nonlethal_may03.asp.

Bregeat, P., Ocular injuries by lacrimogenic agents, *Bull. Soc. Opthalmol. (France)*, 68, 531, 1968.
(British) Ministry of Defence, Medical Manual of Defence against Chemical Agents, JSP 312, Her Majesty's Stationary Office, London, 1972.
Brown, J.H. and Pleury, B.J., Antagonism of the respiratory effects of alfentanil and fentanyl by natoxone in the conscious rabbit, *Br. J. Anaesth.*, 53, 1033–1037, 1981.
Buchan, P. and Adcock, J.J., Capsaicin-induced bronchoconstriction in the guinea pig: contribution of vagal cholinergic reflexes, local axon reflexes and their modulation by BW 443C81, *Br. J. Pharmacol.*, 1, 105, 448, 1992.
Buck, S.H. and Burks, T.F., The neuropharmacology of capsaicin: review of some recent observations, *Pharmacol. Rev.*, 38, 179, 1986.
Carson, B.B. and Stoughton, R.W., Reactions of alpha, beta unsaturated dinitriles, J. *Am. Chem. Soc.*, 50, 2825, 1928.
Castro, J.A., Effects of alkylating agents on human plasma cholinesterase, *Biochem. Pharmacol.*, 17, 295, 1968.
Chemical Biological Weapons Non-Proliferation Treaty, 2002. Available at http://cns.miis.edu/iiop/cnsdata?Action = 1&Concept = 0&Mime = 1&collection = CNS+Web…
Chahl, L.A. and Lynch, A.M., The acute effects of capsaicin on the cardiovascular system, *Acta Physiol. Hung.*, 69, 413, 1987.
CHPPM (Center for Health Promotion and Preventive Medicine), Detailed and General Facts about Chemical Agents, TG 218, U.S. Army Center for Health Promotion and Preventive Medicine, Aberdeen Proving Ground, MD, 1996.
Chung, C.W. and Giles, A.I., Sensitization of guinea pigs to alpha-chloracetphenone (CN) and ortho-chlorobenzylidene malononitrile (CS) tear gas chemicals, *J. Immunol.*, 109, 284, 1972.
Clede, B., Oleoresin capsicum, *Law Order*, March, 63, 1993.
Colgrave, H.F., Brown, R., and Cox., R.A., Ultrastructure of rat lungs following exposure to aerosols of dibenzoxazepine (CR), *Br. J. Exp. Pathol.*, 60, 130, 1979.
Colgrave, H.F., Lee, C.G., Marrs, T.C., and Morris, B., Repeated-dose inhalation toxicity and mutagencity status of CR (dibenz (b,f)-1:4-oxazepine), *Br. J. Pharmacol.*, 78, 169, 1983.
Cordell, G.A. and Araujo, D.E., Capsaicin: identification, nomenclature and pharmacology, *Ann. Pharmacother.*, 27, 330, 1993.
Couzin, J., A sigh of relief for painkillers, *Science*, 301, 150, 2003.
Craig, F.N., Blevins, W.V., and Cummings, E.G., Breathing Patterns During Human Exposure to CS, Report AD318487, Aberdeen Proving Ground, MD, 1960.
Cucinell, S.A., Swentzel, K.C., Biskup, R., Snodgrass, H., Lovre, S., Stark, W., Feinsilver, L., and Vocci, R., Biochemical interactions and metabolic fate of riot control agents, *Fed. Proc.*, 30, 86, 1971.
Davis, B., Roberts, A.M., Coleridge, H.M., and Coleridge, J.C.B., Reflex tracheal gland secretion evolved by stimulation of bronchal c-fiber in dogs, *J. Appl. Physiol.*, 53, 985, 1982.
Fang, J.F., Wu, P.C., Huang, T.B., and Tsai, Y.H., *In vivo* percutaneous absorption of capsaicin, nonivamide, and sodium nonivamide acetate from ointment bases: pharmacokinetic analysis in rabbits, *Int. J. Pharm.*, 128, 169–177, 1996.
Feinsilver, L., Chambers, H.A., Vocci, F.J., Daasch, L., and Berkowitz, L.M., Some metabolites of CS from rats, Edgewood Arsenal Technical Report, Series 4521, 1971.
French, M.C., Harrison, J.M., Inch, T.D., Leadbeater, L., Newman, J., and Upshall, D.G., The fate of dibenz (b,f)-1,4-oxazepine (CR) in the rat, rhesus monkey, and guinea pig, Part I, metabolism in vivo, *Xenobiotica*, 13, 345, 1983.
Fries, A.A. and West, C.J., The history of poison gases, in *Chemical Warfare*, McGraw-Hill Book Company, New York, 1921, pp. 1–9.
Fuller, R.W., The human pharmacology of capsaicin, *Arch. Int. Pharmacodyn.*, 303, 147, 1990.
Fuller R.W., Dixon, C.M.S., and Barnes, P.J., Bronchoconstrictor response to inhaled capsaicin in humans, *J. Appl. Physiol.*, 58, 1080, 1985.
Games, D.E., Alcock, N.J., van der Greef, J., Nyssen, L.M., Maarse, H., and Ten, M.C., Analysis of pepper and capsicum oleoresins by high performance liquid chromatography—mass spectrometry and field desorption mass spectrometry, *J. Chromatogr.*, 294, 269, 1984.
GAO Report, Report to the Honorable Ronald V. Dellums, House of Representatives, NSIAD-89–128, Israel's Use of Tear Gas, 1989.

Gardocki, J.F. and Yelnosky, J., A study of some of the pharmacologic actions of fentanyl citrate, *Toxicol. Appl. Pharmacol.*, 6, 48–62, 1964.

Garrett, B., Toxin of the Month: Capsaicin, 2000. Available at www.wgsiwg://main.188/http://chembio…/subscribe/features/capsaicin.html

Gaskins, J.R., Hehir, R.J., McCaulley, D.F., and Ligon, E.W., Lacrimating agents (CS and CN) in rats and rabbits—acute effects on mouth, eyes and skin, *Arch. Environ. Health*, 24, 449, 1972.

Glasser, S.B. and Baker, P., Russia Confirms Suspicions About Gas Used in Raid, Washington Post Foreign Service, Oct. 31, 2002, p. A15, 2002.

Glinsukon, T., Stitmunnaithum, V., Toskulkao, C., Buranawuti, T., and Tangkrisanavinont, V., Acute toxicity of capsaicin in several animal species, Toxicon., 18, 215, 1980.

Gongwer, L.E., Ballard, T.A., Gutentag, P.J., Punte, C.L., Owens, E.J., Wilding, J.L., and Hart, J.W., The comparative effectiveness of four riot-control agents, Technical Memorandum 24–18, U.S. Army Chemical Warfare Laboratories, Army Chemical Center, MD, 1958.

Govindarajan, V.S., Capsicum—production, technology, chemistry, and quality. Part II. Processed products, standards, world production and trade, *Crit. Rev. Food Sci. Nutr.*, 23, 207, 1986.

Govindarajan, V.S. and Sathyanarayana, M.N., Capsicum—production, technology, chemistry, and quality. Part V. Impact on physiology, pharmacology, nutrition and metabolism; structure pungency, pain and desensitization sequences, *Crit. Rev. Food Sci. Nutr.*, 29, 435, 1991.

Grady, H.G. and Stewart, H.L., Histogenesis of induced pulmonary tumors in strain A mice, *Am. J. Pathol.*, 16, 417, 1940.

Graebe, C., Chloracetophenone, 1869. Available at www.nti.org/e_research/profiles/NK/Chemical/1095.html and www.nadir.org/nadir/initiativ/sanis/archiv/allergie/kap_01.htm.

Graebe, C., *Berichte des Deutschem Chemisch Gesellscheft*, Commissionsverlog von R. Friedlander and Sohn, Berlin, 55, 35, 1881.

Grant, W.M., *Toxicology of the Eye*, 3rd ed., Charles C. Thomas Publishers, Springfield, IL, 1986, p. 882.

Gutentag, P.J., Hart, J., Owens, E.J., and Punte, C.L., The evaluation of CS aerosol as a riot control agent in man, Technical Report CWLR 2365, April 1960, U.S. Army Chemical Warfare Laboratories, Army Chemical Center, 1960.

Harris, B.L., Chemicals in war, in *Encyclopedia of Chemical Technology*, 4th ed., Vol. 5, John Wiley & Sons, New York, 1992.

Hathaway, T.J., Higenbottam, T.W., Morrison, J.F., Clelland, C.A., and Wallwork, J., Effects of inhaled capsaicin in heart-lung transplant patients and asthmatic subjects, *Am. Rev. Respir. Dis.*, 148, 1233, 1993.

Hess, L. and Knakel, J., First exposure with immobilization with carfentanil at Zoo Prague, *Gazella*, 3, 87–90, 1985.

Higginbottom, R. and Suschitzky, H., Synthesis of heterocyclic compounds. II. Cyclization of O-nitrophenyl oxygen, *J. Chem. Soc.*, 2367, 1962.

Hill, A.R., Silverberg, N.B., Mayorga, D., and Baldwin, H.E., Medical hazards of the tear gas CS, *Medicine*, 79, 234–240, 2000.

Himsworth, H., Thompson, R.H.S., and Dornhorst, A.C., Report of the Enquiry into the Medical and Toxicological Aspects of CS (Orthochlorobenzylidene Malononitrile). Part I. Enquiry into the Medical Situation following the Use of CS in Londonderry on 13th and 14th August, 1969, Cmnd 4173, H.M.S.O., London, 1969.

Himsworth, H., Black, D.A.K., Crawford, T., Dornhorst, A.C., Gilson, J.C., Neuberger, A., Paton, W.D.M., and Thompson., R.H.S., Report of the Enquiry into the Medical and Toxicological Aspects of CS (Orthochlorobenzylidene Malononitrile). Part II. Enquiry into Toxicological Aspects of CS and Its Use for Civil Purposes, Cmnd 4775, H.M.S.O., London, 1971.

Hoffman, D.H., Injuries of the eyes from short-range discharge of tear gas weapons, *Klin. Monatsbl. Augenheilkd.*, 147, 625, 1965.

Hoffman, D.H., Eye burns caused by tear gas, *Br. J. Opthalmol.*, 51, 265, 1967.

Holland, P., The cutaneous reactions produced by dibenzoxazepine (CR), *Br. J. Dermatol.*, 90, 657, 1974.

Holland, P. and White, R.G., The cutaneous reactions produced by o-chlorobenzylidene-malononitrile and w-chloroacetophenone when applied directly to the skin of human subjects, *Br. J. Dermatol.*, 86, 150, 1972.

Holzer, P., Capsaicin: selective toxicity for thin primary sensory neurons, in *Handb. Exp. Pharmacol.*, 102, 420, 1992.

Hu, H., Tear gas—harassing agent or toxic chemical weapon?, *JAMA*, 262, 660, 1989.

Hu, H., Toxicodynamics of riot-control agents (lacrimators), in *Chemical Warfare Agents*, Chap. 11, Somani, S.M., Ed., Academic Press, New York, 1992.

Husain, K., Kumar, P., and Malhotra, R.C., A comparative study of biochemical changes induced by inhalation of aerosols of o-chloroacetophenone and dibenz (b,f)-1,4-oxazepine in rats, *Indian J. Med. Res.*, 94, 76, 1991.

Issekutz, B., Lichtneckert, I., and Nagy, H., Effect of capsaicin and histamine on heat regulation, *Arch. Int. Pharmacodyn.*, 81, 35, 1950.

Jansco, G., Kiraly, E., and Jansco-Gabor, A., Pharmacologically-induced selective degeneration of chemosensitive primary sensory neurons, *Nature*, 270, 741, 1977.

Janssen, P., The development of new synthetic narcotics, in *Opioids in Anesthesia*, Esafenous, F.G., Ed., Butterworth Publishers, Boston, 1984.

Kasting, G.B., Francis, W.R., Bowman, L.A., and Kinnett, G.O., Percutaneous absorption of vanilloids: *in vivo* and *in vitro* studies, *J. Pharm. Sci*., 86, 142–146, 1997.

Krapf, R. and Thalmann, H., Akute exposition durch CS-rauchgas und linische beobachtungen, *Schweiz. Med. Wochenschr.*, 11, 2056, 1981.

Kratschmer, F., Uber Reflexe von der Nasenschleimhaut auf athmung und Kreislaub, *Sitzungsber. Akad. Wiss.*, 62, 147–170, 1870.

Kumar, P., Flora, S.J.S., Pant, S.C., Sachan, A.S., Saxena, S.P., and Das Gupta, S., Toxicological evaluation of 1-chloroacetophenone and dibenz (b,f)-1:4-oxazepine after repeated inhalation exposure in mice, *J. Appl. Toxicol.*, 14, 411, 1994.

Laibson, P.R. and Oconor, J., Explosive tear gas injuries of the eye, *Trans. Am. Acad. Ophthalmol. Otolaryngol.*, 74, 811–819, 1970.

Langguth, P., Khan, P.J., and Garrett, E.R., Pharmacokinetics of morphine and its surrogates. XI. Effects of simultaneously administered naltrexone and morphine on the pharmacokinetics and pharmacodynamics of each in the dog, *Biopharm. Drug Dispos.*, 11, 419–444, 1990.

Leadbeater, L., The absorption of ortho-chlorobenzylidenemalononitrile (CS) by the respiratory tract, *Toxicol. Appl. Pharmacol.*, 25, 101, 1973.

Leadbeater, L., Sainsbury, G.L., and Utley, D., o-Chlorobenzyl malononitrile, a metabolite formed from o-chlorobenzylidene malononitrile (CS), *Toxicol. Appl. Pharmacol.*, 25, 111, 1973.

Leopold, I.H. and Lieberman, T.W., Chemical injuries of the cornea, *Fed. Proc. Am. Soc. Exp. Biol.*, 30, 92, 1971.

Levine, R.A. and Stahl, C.J., Eye injury caused by tear-gas weapons, *Am. J. Ophthalmol.*, 65, 497–508, 1968.

Lundy, P.M. and McKay, D.H., Mechanism of the cardiovascular activity of dibenz [b,f][1,4] oxazepine (CR) in cats, Suffield Technical Paper 438, Defence Research Establishment, Ralston, Alberta, Canada, 1975.

MacLeod, I.F., Chemical mace: ocular effects in rabbits and monkeys, *J. Foren. Sci.*, 14, 34, 1969.

Macrae, W.G., Willinsky, M.D., and Basu, P.K., Corneal injury caused by aerosol irritant projectors, *Can. J. Ophthalmol.*, 5, 3, 1970.

Manzke, T., Guenther, U., Ponimaskin, E.G., Haller, M., Dutschmann, M., Schwarzacher, S., and Richter, D.W., 5-HT4(a) Receptors avert opioid-induced breathing depression without loss of analgesia, *Science*, 301, 226–229, 2003.

Marrs, T.C., Colgrave, H.F., and Cross, N.L., A repeated dose study of the toxicity of technical grade dibenz-(b,f)-1,4 oxazepine in mice and hamsters, *Toxicol. Lett.*, 17, 13, 1983a.

Marrs, T.C., Colgrave, H.V., Cross, N.L., Gazzard, M.F., and Brown, R.F.R., A repeated dose study of the toxicity of inhaled 2-chlorobenzylidene malononitrile (CS) aerosol in three species of laboratory animals, *Arch. Toxicol.*, 52, 183, 1983b.

Marrs, T.C., Gray, M.I., Colgrave, H.F., and Gall, D., A repeated dose study of the toxicity of CR applied to the skin of mice, *Toxicol. Lett.*, 13, 259, 1982.

Martin, W.R., Pharmacology of opioids, *Pharm. Rev.*, 35, 285–323, 1983.

McDonald, E.C. and Mahon, R.T., Letter to the Editor, *Mil. Med.*, 167, iii–iv, 2002.

McGilliard, K. and Takemori, A.E., Antagonism by naloxone of narcotic-induced respiratory depression and analgesia, *J. Pharmacol. Exp. Ther.*, 207, 454–503, 1978.

McGregor, D.B., Brown, A., Cattanach, P., Edwards, I., Mcbride, D., and Caspary, W.J., Responses of the L5178Y tk + /tk- mouse lymphoma cell forward mutation assay II: 18 coded chemicals, *Environ. Mol. Mutagen.*, 11, 91, 1988.

McNamara, B.P., Vocci, F.J., Owens, E.J., Ward, D.M., and Anson, N.M., The Search for an Effective Riot-Control Agent Solvent System for Large Volume Dispersers, Edgewood Arsenal Technical Report 4675, October 1972.

Mercadente, S., 2003. Available at http://bmjjournals.com/cgi/eletters/324/7333/356

Midtbo, A., Eye injury from tear gas, *Acta Ophthalmol.*, 42, 672, 1964.

Miller, J. and Lichtblau, E., U.S. Investigates Moscow Theatre Seige Seeking Qaeda Link, 2003. Available at wysiwyg://17/http://www.nytimes.com/200…DI.html?8br = &pagewanted = print&position =

Miller, M.W., Wild, M.A., and Lance, W.R., Efficacy & Safety of naltrexone hydrochloride for antagonizing carfentanil citrate immobilization in captive Rocky Mountain elk (Cervus elaphus nelsoni), *J. Wild Dis.*, 32, 234–239, 1996.

Mioduszewski, R. and Reutter, S., Dissociation of opiate-induced sedation and respiratory depression in ferrets by opiate antagonistic co administration: potential pharmacological mechanisms, in *Proceedings of the 1991 U.S. Army Chemical Research, Development and Engineering Center Scientific Conference on Chemical Defense Research*, 19–22 November 1991, AD-A269 546, pp. 691–698, ADE 479569, 1991.

Mioduszewski, R., U.S. Patent 5,834,477, 1994.

Monsereenusorn, Y., Kongasamut, S., and Pezalla, P.D., Capsaicin—a literature survey, *CRC Crit. Rev. Toxicol.*, 321, 1982.

Nash, J.B., Doucet, B.M., Ewing, P.L., and Emerson, G.A., Effects of cyanide antidotes and inanition on acute lethal toxicity of malononitrile in mice, *Arch. Int. Pharmacodyn.*, 84, 395, 1950.

National Academy of Sciences, *Possible Long-Term Health Effects of Short-Term Exposure to Chemical Agents*, Vol 2. *Cholinesterase Reactivators, Psychochemicals, and Irritants & Vesicants*, National Academy Press, Washington, DC, 1984.

National Institutes of Health (National Toxicology Program), Toxicology and Carcinogenesis Studies of 2-Chloroacetophenone (CAS 532–27–4) in F344/N Rats and B6C3F1 Mice (Inhalation Studies), NTP TR 379, March 1990, National Toxicology Program, Research Triangle Park, NC, 1990.

National Institutes of Health (National Toxicology Program), Toxicology and Carcinogenesis Studies of CS2 (94% o-chlorobenzalmalononitrile) (CAS 2698–41–1) in F344/N Rats and B6C3F1 Mice (Inhalation Studies), NTP TR 377, March 1990, National Toxicology Program, Research Triangle Park, NC, 1990.

Oaks, L.W., Dorman, J.E., and Petty, R.W., Tear gas burns of the eye, *Arch. Ophthalmol.*, 63, 698–706, 1960.

Obal, F., Jr., Benedek, G., Jansco-Gabor, A., and Obal, F., Salivary cooling, escape reaction and heat pain in capsaicin-desensitized rats, *Plugers Arch.*, 382, 249–254, 1979.

Obal, F., Jr., Obal, F., Benedek, G., and Jansco-Gabor, A., Central and peripheral impairment of thermoregulation after capsaicin treatment, *J. Therm. Biol.*, 8, 203–206, 1983.

Office of the Press Secretary (The White House), Press Release dated June 23, 1994 (Subject: Riot Control Agents), Washington, DC, 1994.

Oksala, A. and Salminen, L., Eye injuries caused by tear-gas hand weapons, *Acta Ophthalmol.*, 53, 908–913, 1975.

Olajos, E.J. and Salem, H., Riot control agents: pharmacology, toxicology, biochemistry and chemistry, *J. Appl. Toxicol.*, 21, 355–391, 2001.

O'Neill, T.P., Mechanism of capsaicin action: recent learnings, *Respir. Med.*, 85, 35, 1991.

Owens, E.J. and Punte, C.L., Human respiratory and ocular irritation studies utilizing *o*-chlorobenzylidene malononitrile aerosols, *Am. Ind. Hyg. Assoc. J.*, 24, 262–264, 1963.

Paradowski, M., Metabolism of toxic doses of *o*-chlorobenzylidene malononitrile (CS) in rabbits, *Polish J. Pharmacol. Pharm.*, 31, 563, 1979.

Parenti, C., Death in Custody from Excited Delirium, 1999. Available at www.salem.com/health/features/1999/69/29excited_delerium/print.html.

Park, S. and Giammona, S.T., Toxic effects of tear gas on an infant following prolonged exposure, *Am. J. Dis. Child.*, 123, 245, 1972.

Patai, S. and Rappaport, Z., Nucleophilic attacks on carbon-carbon double bonds. Part II Cleavage of arylmethylenemalonitriles by water in 95% ethanol, *J. Chem. Soc. (Lond.)*, Part 1 (71), 383,1962.

Pattle, R.E., Schock, C., Dirnhuber, P., and Creasy, J.M., Lung surfactant and organelles after an exposure to dibenzoxazepine (CR), *Br. J. Exp. Pathol.*, 55, 213, 1974.

Penneys, N.S., Israel, R.M., and Indgin, S.M., Contact dermatitis due to 1-chloroacetophenone and chemical mace, *N. Engl. J. Med.*, 281, 1969.

Porszasz, R. and Szolesanyi, J., Circulatory and respiratory effects of capsaicin and resiniferatoxin on guinea pigs, *Acta Biochim. Biophys. Hung.*, 26, 131, 1991/1992.

Punte, C.L., Gutentag, P.J., Owens, E.J., and Gongwer, L.E., Inhalation studies with chloroacetophenone, diphenylaminochloroarsine, and pelargonic morpholide. II. Human exposures, *Am. Ind. Hyg. Assoc. J.*, 23, 199, 1962a.

Punte, C.L., Owens, E.J., and Gutentag, P.J., Exposures to ortho-chlorobenzylidene malononitrile, *Arch. Environ. Health*, 6, 72, 1963.

Punte, C.L., Weimer, J.T., Ballard, T.A., and Wilding, J.L., Toxicologic studies on o-chlorobenzylidene malononitrile, *Toxicol. Appl. Pharmacol.*, 4, 656, 1962b.

Reitveld, E.C., Delbressine, L.P., Waegemaekers, T.H., and Seutter-Berlage, F., 2-Chlorobenzylmercapturic acid, a metabolite of the riot control agent 2-chlorobenzylidene malononitrile (CS) in the rat, *Arch. Toxicol.*, 54, 139, 1983.

Rengstorff, R.H., Petrali, J.P., Merson, M., and Sim, V.M., The effect of the riot control agent dibenz (b,f)-1:4-oxazepine (CR) in the rabbit eye, *Toxicol. Appl. Pharmacol.*, 34, 45, 1975.

Rothberg, S., Skin sensitization potential of the riot control agents CA, DM, CN, and CS in guinea pigs, Technical Report EATR 4219, U.S. Army Medical Research Laboratory, Edgewood Arsenal, MD,. 1969, 19 p.

Royer, J. and Gainet, F., Ocular effects of ethyl bromoacetate tear gas, *Bull. Soc. Ophthalmol. (France)*, 73, 1165, 1973.

Russel, J.A. and Lai-Fook, S.J., Reflex bronchoconstriction induced by capsaicin in the dog, *J. Appl. Physiol.*, 47, 961, 1979.

Salem, H., (2003), Issues in Chemical and Biological Terrorism, *Int. J. Tox* 22:465–471.

Salem, H., Olajos, E.J., and Katz, S.A., Riot control agents, in *Chemical Warfare Agents: Toxicity at Low Levels*, Somani, S.M. and Romano, Jr. J.A., Eds., CRC Press, Boca Raton, FL, 2001.

Schmid, E., Bauchinger, M., Ziegler-Skylakaksis, K., and Andrae, U., 2-Chloro-benzylidene malononitrile (CS) causes spindle disturbances in V79 Chinese hamster cells, *Mutat. Res.*, 226, 133, 1989.

Secretary of Defense (SECDEF), 2003. Available at www.armscontrol.org.act/2003_05/nonlethal_may03.asp

Sidell, F.R., Riot-control agents, in *Textbook of Military Medicine, Medical Aspects of Chemical and Biological Warfare*, Chap. 12,

Stern, J., Weil-Malherbe, H., and Green, R.H., The effects and the fate of malononitrile and related compounds in animal tissues, *Biochem. J.*, 52, 114, 1952.

Stewart, H.L., Dunn, T.B., Snell, K.C., and Deringer, M.K., Tumors of the respiratory tract, in *WHO Monographs on the Pathology of Laboratory Animals*, Vol 1. *The Mouse*, Turusov, S.U., Ed., International Association for Research on Cancer (IARC), Lyons, 1979, p. 251.

Striker, G.E., Streett, C.S., Ford, D.F., Herman, L.H., and Welland., D.R., A clinicopathological study of the effects of riot-control agents in monkeys. V. Low concentrations of diphenylaminochloroarsine (DM) or o-chlorobenzylidene malononitrile (CS) for extended periods, Technical Report 4072, U.S. Army Medical Research Laboratory, Edgewood Arsenal, MD, 1967.

Surh, Y.J. and Lee, S.S., Capsaicin, a double-edged sword; toxicity, metabolism, and disease preventive potential, *Life Sci*., 56, 1845, 1995.

Suzuki, T. and Iwai, K., Constituents of red pepper species: chemistry, biochemistry, pharmacology and food science of the pungent principal of capsicum species, in *The Alkaloids*, chap. 4, 23, Brossi, A., Ed., Academic Press, Orlando, FL, 1984.

Swearengen, T.F., *Tear Gas Munitions*, Charles C. Thomas, Springfield, IL, 1966.

Teranishni, R., Keller, U., Flath, R.A., and Mon, T.R., Comparison of batchwise and continuous steam distillation—solvent extraction recovery of volatiles from oleoresin capsicum, African type (capsicum frutesens), *J. Agric. Food Chem.*, 28, 156, 1980.

Thomas, R.J., Smith, P.A., Rascona, D.A., Louthan, J.D., and Gumpert, B., Acute pulmonary effects from o-chlorobenzylidenamalonitrile "tear gas": a unique exposure outcome unmasked by strenuous exercise after a military training event, *Mil. Med.*, 167, 136–139, 2002.

Toh, C.C., Lee, T.S., and Kiang, A.K., The pharmacological actions of capsaicin and analogues, *Br. J. Pharmacol.*, 10, 175, 1955.

U.K. Department of Health, COT Statement on the use of PAVA (nonivamide) as an Incapacitant Spray, COT/0202-April 2002. Available at www.doh.gov.uk/cotnonfood/pava.htm

Upshall, D.G., Effects of o-chlorobenzylidene malononitrile (CS) and the stress of aerosol inhalation upon rat and rabbit embryonic development, *Toxicol. Appl. Pharmacol.*, 24, 45–59, 1973.Upshall, D.G., The effects of dibenz (b,f)-1,4-oxazepine (CR) upon rat and rabbit embryonic development, *Toxicol. Appl. Pharmacol.*, 29, 301, 1974.

Upshall, D.G., Riot control smokes: lung adsorption and metabolism of peripheral sensory irritants, in *Clinical Toxicology*, Duncan, W.A. and Leonard, B.J., Eds., Excerpta Medica, Amsterdam, The Netherlands, 1977, p. 121.

USAMRICD, Medical Management of Chemical Casualties Handbook, 3rd Ed., MCMR-UV-ZM, Aberdeen Proving Ground, MD, 2000.

U.S. Congress, Investigation into the Activities of Federal Law Enforcement Agencies toward the Branch Davidians, Union Calendar 395, House of Representatives Report 104-749, U.S. Government Printing Office, Washington, DC, 1996.

U.S. General Accounting Office (GAO), Use of U.S. Manufactured Tear Gas in the Occupied Territories, GAO/NSIAD-89-128, Washington, DC, 1989.

Verwey, W.D., *Riot-Control Agents and Herbicides in War*, A.W. Sijthoff, Leyden, 1977.

Von Daniken, A., Friederich, U., Lutz, W.K., and Schlatter, C., Tests for mutagencity in salmonella and covalent binding to DNA and protein in the rat of the riot control agent o-chlorobenzylidene malononitrile (CS), *Arch. Toxicol.*, 49, 15, 1981.

Walker, P.H., Department of the Army, Letter of Instructions 23-70 Employment of Riot Control Agent CS, 1970. Available at http://66.102.7.104/search?q = cache:cREobU-AdEEj:25thaviation.org/history/id539.htm+CS+use+in+Viet…

West, C.J., The history of poison gases, *Science*, 49, 412–417, 1919.

Wild, D., Eckhardt, K., Harnasch, D., and King, M.T., Genotoxicity study of CS (ortho-chlorobenzylidene malononitrile) in salmonella, drosophila, and mice, *Arch. Toxicol.*, 54, 167, 1983.

World Health Organization (WHO), Health Aspects of Chemical and Biological Weapons, Report of a World Health Organization Group of Consultants, Geneva: WHO, 1970.

Zeiger, E., Anderson, B., Haworth, S., Lawlor, T., Mortelmans, K., and Speck, W., Salmonella mutagenicity test. III. Results from the testing of 255 chemicals, *Environ. Mutagen.*, 9 (Suppl. 9), 1, 1987.

22 Chemical Warfare Agents and Nuclear Weapons

Nabil M. Elsayed and Harry Salem

CONTENTS

22.1 INTRODUCTION

Chemical warfare agents (CWAs) are chemical substances that rely on their toxic properties to produce lethal, incapacitating, or damaging effects on humans, animals, and plants when suitably delivered. Since their introduction in World War I by the Germans, they have remained another form of weapons to be used by combatant armies. However in recent years, they have been used by armies

against rebellious noncombatant civilians and by terrorists against innocent, nonsuspecting citizens, as was the case in Japan. The risk of their deployment by terrorists increased significantly after the attack on the New York World Trade Center in September 11, 2001, and the anthrax incidents that followed. Although the United States and most European countries place greater emphasis and allocate most of their antiterrorist resources to prepare for potential chemical and biological terrorism, blasts from explosive detonation remain the single most frequent cause of human death and injury and property damage by terrorist attacks.

Nuclear weapons are a class of weapons that derive their enormous destructive power from energy produced by nuclear reactions that are not generally included when discussing CWAs. However, with increased concern of potential nuclear terrorism and the possible deployment of portable nuclear weapons or radiological dispersion devices (dirty bombs), it was necessary to include nuclear weapons and dirty bombs in the discussion of CWAs in this chapter. Unlike other CWAs, they are more destructive to life and property, and highly persistent in the environment long after the initial detonation. Nuclear weapons were used only twice on the battlefield by the United States against Japan during World War II.

22.2 BRIEF HISTORY OF CHEMICAL WARFARE

The use of chemical weapons dates back to ancient times, when the Chinese in 1000 BCE used arsenical smoke in battle. In 600 BCE, in Greece, Solon of Athens poisoned the water supply of the city during the siege of Kirrha. In 429 BCE the Spartans used noxious smoke and flame against the Athenian cities during the Peloponneisian War. Poison-tipped arrows were used during the Trojan War. In fact, the English word for toxin or poison comes from the Greek word *toxikon,* which in turn is derived from the Greek word *toxon* or arrow. In 184 BCE, Hannibal of Carthage hurled clay pots full of vipers onto the deck of enemy ships, and in 1100, Leonardo da Vinci proposed a powder of arsenic sulfide and verdigris as an antiship weapon.

Modern chemical warfare was first launched by Germany during World War I when on October 27, 1914, in breach of the 1899 International ban; the Germans fired 105-mm shells filled with the lung irritant dianisidine chlorosulfate against British troops near the town of Neuve-Chapelle. The attack was unsuccessful because the explosives of the shells destroyed the chemical. In November 1914 the Germans fired shells containing xylyl bromide against the Russian troops near Balimov, but the cold weather prevented vaporization of the chemical. Then on April 22, 1915, under the guidance of a renowned chemist, Fritz Haber, the Germans released 168 tons of chlorine from 5730 cylinders against French and Algerian troops defending Ypres, Belgium, and two days later the German launched another gas attack using 150–200 tons of chlorine gas against the Canadians. The results of these two days were estimated at 5000 dead and 10,000 disabled. The British Army responded with their first gas attack on September 25, 1915 and launched an attack at Loos, Belgium, also using chlorine gas. During the course of the war, the Germans developed and used phosgene, a more effective gas than chlorine and diphosgene. In response, the French used hydrogen cyanide, then cyanogen chloride (Smart, 1997). On July 17, 1917 the Germans launched an attack using a new chemical agent developed by Lommel and Steinkopt that caused skin blisters and death upon inhalation of high concentrations. The Germans named the chemical LoSt after the two chemists who developed it. However, the British called it mustard gas, although it is an oily liquid, because of its mustard or garlic smell and the yellow cross identification markings that were used on the canisters containing it. The French called it Yprite after the location where it was first used. The mustard gas attack resulted in 20,000 British casualties after 6 weeks of use. The British retaliated using a combination of high explosives and mustard gas. The British used mustard gas again after the war when they fired mustard-filled shells against the Bolshevik troops of the newly formed Soviet Union during the Russian Civil War of 1918–1921. At the end of World War I, the total number of casualties was over 83,000 dead and almost 1.2 million injured. Germany, Britain, and France each suffered

8000–9000 fatalities resulting from the gas war, whereas Russia's losses were the heaviest, 56,000 dead because they were late in deploying protective masks. The American Expeditionary Force suffered 71,345 chemical casualties and 1462 dead (Joy, 1997). After the war, mustard gas continued to be produced and was used in the 1920s and 1930s by the British in Afghanistan, the Italians in Ethiopia, and the Japanese in China (Table 22.1)

In December 1936, another German scientist, Gerhard Schrader, who was developing new pesticides, made an accidental discovery that, when he sprayed insects with a solution of one of his new chemicals diluted 1 to 200,000, he succeeded in killing them. In the course of his experiments, he was contaminated in a laboratory accident and developed side effects that included pinpoint-constricted pupils, sensitivity to light, shortness of breath, and giddiness. Unlike other gases that had to be inhaled to produce toxicity, this chemical was capable of inducing spasms and lethality after dermal exposure (U.S. Army Medical Research Institute of Chemical Defense [USAMRICD], 2000). The new chemical was later named Tabun (GA) and marked the introduction of the organophosphates as potent chemical nerve agents. Other G agents were later developed, including Sarin (GB) and Soman (GD). At the end of World War II, the Soviets captured 12,000 tons of Tabun and the processing plant that produced them, which was moved to Russia

TABLE 22.1 Chronology of Major Military User States of Chemical Weapons[a]

Period	User	Chemical(s) used
1000 BCE	Chinese	Arsenical smoke
Siege of Kiirha Greece (600 BCE)	Solon of Athens	Poisoned water supply
Peloponnesian War (429 BCE)	Spartan *Vs* Athens	Burning pitch and sulfur
Peloponnesian War (424 BCE)	Spartans	Burning pitch and sulfur
World War I (1914)	France *Vs* Germany	Tear gas
World War I (April 22, 1915)	Germany *Vs* France	Chlorine gas
World War I (September 25, 1915)	Britain *Vs* Germany	Chlorine gas
World War I (February 26, 1918)	Germany *Vs* US	Phosgene and Chloropicrin
World War I (June 1918)	US *Vs* Germany, and establishment of the Chemical Warfare Service	
Russian Civil War (1918–1921)	Britain *Vs* Russian Bolsheviks	Adamsite, and Mustard gas artillery shells
Spanish Civil War (1922–1927)	Spain *Vs* RIF Rebels in Spanish Morocco	
Abyssinia (Ethiopia) Conquest (1935)	Italy *Vs* Ethiopians	Mustard gas by aerial spray
China (1936)	Japan *Vs* China	Mustard gas, phosgene, and hydrogen cyanide
Vietnam, Cambodia, Laos (1962–1970)	US *Vs* Vietnamese	Defoliants (Agents Orange, Blue, Purple, and White), and tear gas
Yemen, civil war (1963–1967)	Egypt *Vs* Yemeni Royalists	Phosgene and Mustard gas
Afghanistan	Soviet Union *Vs* Afghanistan	Alleged use of Yellow Rain (mycotoxins)
Iran-Iraq War (1983–1988)	Iraq *Vs* Iran	Mustard gas, Tabun
Iraq Anfal campaign (1987–1988)	Iraq *Vs* Kurds	Hydrogen Cyanide, Mustard gas

[a] Adapted from Monterey Institute for International Studies (2002).

to form the basis of the Soviet chemical weapons program. After the war, the more potent V agents including VX, VE, VG, and VS were developed by both the Soviet Union and the United States. Table 22.1 presents a chronology of the major state use of chemical weapons in military conflicts.

22.3 CLASSIFICATION OF CHEMICAL WARFARE AGENTS

CWAs can be classified in two ways: first, operationally, based on their lethality and persistence in the environment as shown in Figure 22.1; second, categorized by classes according to their mechanism of action or the major target organ they attack. Table 22.2 lists the general characteristics of CWAs that are used either to inflict lethality, incapacitation, or distraction of the enemy in combat or for crowd control of civilians during riots. Agents used for incapacitation or distraction have also been classified as nonlethal or less than lethal. In general, these include agents used by law enforcement personnel, such as mace, which although ordinarily are nonlethal at effective concentrations, could be lethal, especially in confined spaces, at very high concentration or prolonged exposure.

22.3.1 Lethal Chemical War Agents

22.3.1.1 Pulmonary Agents

Pulmonary or choking agents are chemicals that exert their toxicity almost exclusively by inhalation, although they can also cause eye and skin irritation. They include chlorine (Cl), phosgene (CG), diphosgene (DP), chloropicrin (PS), and toxic pyrolysis products such as nitrogen oxides (NO_x), phosphorus oxides (PO_x), sulfur oxides (SO_x), and perfluoroisobutylene (PFIB). Inhaled pulmonary agents readily penetrate the respiratory system, reaching the deep lung (respiratory bronchioles

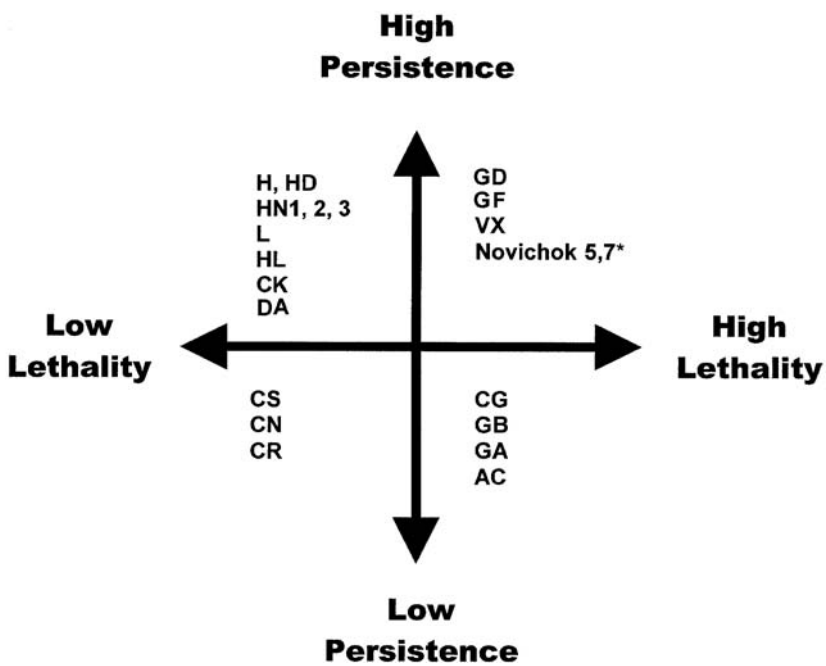


FIGURE 22.1 A system for classification of chemical warfare agents by persistence and lethality. Modified from Evison et al. (2000). *Novichoks are a class of nerve agents developed by the former Soviet Union with very little information available. They are believed to have high lethality and persistence.

TABLE 22.2 Characteristics of Some Lethal, Warfare and Nonlethal, Riot Control Chemical Agents[a,b]

Category/Name	Code	State (20°C)	Odor	Inhalation Lethality[b]	Action	Response
Warfare Agents (Lethal)						
Pulmonary (Choking)						
Chlorine	CL	Gas	Bleach	3,000	Rapid	
Phosgene	CG	Gas	New mown hay, green corn	3,200	Immediate	Lung damage and flooding
Diphosgene	DP	Liquid	New mown hay, green corn	3,200	Immediate	Lung damage and flooding
Chloropicrin	PS	Liquid	Stinging pungent	20,000	Rapid	Inhalation
Blister (Vesicants)						
Sulfur mustard, distilled	H, HD	Liquid	Garlic	1,500	Delayed	Blisters, tissue burns, blood vessels injury, necrosis
Nitrogen mustard	HN-1	Liquid	Fishy or musty	1,500	Delayed	Blisters, tissue burns, blood vessels injury, necrosis
Nitrogen mustard	HN-2	Liquid	Soapy to fruity	3,000		
Nitrogen mustard	HN-3	Liquid	None	1,500	Delayed	Blisters, tissue burns, blood vessels injury, necrosis
Phosgene oxime	CX	Solid or liquid	Sharp, penetrating	3,200 (Est)	Immediate	Violent irritation of mucus membranes of eyes, nose, lung
Lewisite	L	Liquid	Geranium	1,500	Rapid	Blisters, tissue burns, blood vessels injury, necrosis, systemic poisoning
Mustard-Lewisite	HL	Liquid	Garlic	1,500	Immediate stinging, delayed blistering	Blisters, tissue burns, blood vessels injury, necrosis, systemic poisoning
Phenyldichloroarsine	PD	Liquid	None	2,600	Immediate eye, slower skin effects	Irritation, nausea, vomiting, blisters
Ethyldichloroarsine	ED	Liquid	Fruity, biting; irritating	3,000–5,000	Immediate stinging, delayed blistering	Blisters, death

(*Continued*)

TABLE 22.2 Characteristics of Some Lethal, Warfare and Nonlethal, Riot Control Chemical Agents[a,b] (Continued)

Category/Name	Code	State (20°C)	Odor	Inhalation Lethality[b]	Action	Response
Methyldichloroarsine	MD	Liquid	None	3,000–5,000	Rapid	Blisters, skin, eyes, respiratory tract damage, systemic poisoning
Blood						
Hydrogen cyanide	AC	Gas or liquid	Bitter almond	2,500–5,000	Very rapid	Interfere with oxygen uptake, accelerate the rate of breathing
Cyanogen chloride	CK	Gas	Bitter almond	11,000		Chocking, irritating, slow breathing
Arsine	SA	Gas	Garlic	5,000	Delayed	Damage to blood, liver, and kidneys
Nerve (Organophosphates)						
Tabun	GA	Liquid	None	400	Very rapid	Cessation of breathing, death
Sarin	GB	Liquid	None	100	Very rapid	Cessation of breathing, death
Cyclosarin	GF	Liquid	None Sweet, musk, peach, shellac	35	Very rapid	Cessation of breathing, death
Soman	GD	Liquid	Camphor	70	Very rapid	Cessation of breathing, death
VX						
	VX	Liquid	None	50		
Novichok, 5, 7 (Russian nerve agent)	N/A	N/A	N/A	N/A	N/A	5-10 the potency of VX.
			Riot Control Agents (Non-lethal)			
Tear (Lacrimators)						
Chloracetophenone	CN	Solid	Apple blossom	14,000	Instantaneous	Lacrimation, respiratory tract irritation
Chloracetophenone and chloroform	CNC	Liquid	Chloroform	11,000 (Est)	Instantaneous	Lacrimation, respiratory tract irritation
Chloracetophenone in chloroform	CNS	Liquid	Flypaper	11,400	Instantaneous	Lacrimation, vomiting, and chocking

TABLE 22.2 Characteristics of Some Lethal, Warfare and Nonlethal, Riot Control Chemical Agents[a,b] (Continued)

Category/Name	Code	State (20°C)	Odor	Inhalation Lethality[b]	Action	Response
Chloracetophenone / benzene and carbon tetrachloride	CNB	Liquid	Benzene	11,000 (Est)	Instantaneous	Powerful Lacrimation
Bromobenzylcyanide	CA	Liquid	Sour fruit	8–11,000 (Est)	Instantaneous	Lacrimation, eye and respiratory tract irritation
o-chlorobenzylmalonnitrile	CS	Solid	Pepper	> 50,000	Instantaneous	Highly irritating, less toxic
Vomiting						
Diphenylchlorarsine	DA	Solid	None	15,000 (Est)	Very rapid	Cold-like symptoms, headache, vomiting, nausea
Adamsite	DM	Solid	None	15,000	Very rapid	Cold-like symptoms, headache, vomiting, nausea
Diphenylcyanoarsine	DC	Solid	Bitter almond / garlic	10,000 (Est)	Rapid	Cold-like symptoms, headache, vomiting, nausea
Incapacitating (Hallucinating)						
3-Quinuclidinyl benzilate	BZ	Solid	None	20,000	Delayed	Tachycardia, dizziness, vomiting, dry mouth, blurred vision, stupor, random activity

[a] *Sources*: Marrs et al. (1996); Ellison (2000); USAMRICD (2000).

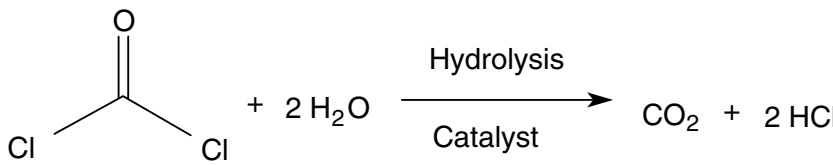
[b] N/A, not available; Est, estimated for a 70-kg man.

[c] Inhalation lethality expressed as the median lethal exposure (LCt_{50}, mg·min/m^3) in a 70-kg man.

and alveoli) and the peripheral compartments of the respiratory tree. They may cause nasopharyngeal, airway and laryngeal irritation, apnea, chest tightness, capillary and alveolar wall damage, delayed pulmonary edema, and extensive fluid buildup in the lungs that could interfere with oxygen uptake and leads to adult respiratory distress syndrome (ARDS) symptoms or noncardiogenic pulmonary edema (USAMRICD, 2000). Because of their reactivity, pulmonary agents are removed at the alveolar-capillary membrane surface or proximally in the respiratory tract and do not diffuse systemically to cause significant clinical response.

Chlorine is a lung irritant used during World War I. It was considered inefficient and was replaced with other more potent pulmonary gases. Exposure to chlorine results in both central airway damage and pulmonary edema. Inhalation of 1000 ppm of chlorine will incapacitate the target within few seconds and cause death in 2–3 min. The LCt_{50} for chlorine is 6000 ppm.

Phosgene is a more effective pulmonary toxicant than chlorine; it was first prepared by John Davy in 1812 and was developed as a chemical weapon by the Germans during World War I. It was first used against British troops near Ypres, Belgium on December 1915. Four thousand cylinders of phosgene released 88 tons, causing 1069 casualties and 120 deaths. Subsequently both sides used substantial amounts of phosgene, which accounted for approximately 85% of all chemical weapon deaths by the end of World War I. At present, the United States produces over a billion pounds of phosgene per year for industrial use. Inhalation of phosgene damages the lungs and produces pulmonary edema (Marrs et al., 1996). Hydrolysis of phosgene leads to HCl formation, which contributes to its toxicity (Equation 22.1).



(22.1)

The carbonyl group (C = O) of the phosgene molecule can undergo acylation reactions with several functional groups such as amino ($-NH_2$), sulfhydryl (–SH), and hydroxyl (–OH) groups, rendering them nonfunctional and thus contributing to the pathophysiological effects (Marrs et al, 2000; USAMRICD, 2000). No antidote for phosgene intoxication is available.

The LCt_{50} of phosgene is approximately 3200 mg·min/m^3, which is twice as toxic as chlorine. The former Soviet Union stockpiled phosgene, and it was used by Egypt against the Yemeni Royalists during the Yemeni Civil War, 1963–1967 (Table 22.1). The medical management regime includes O_2 therapy and high doses of steroids to prevent pulmonary edema. Treatment of pulmonary edema, if it occurs, is with pulmonary expiratory end pressure (PEEP) to maintain P_{AO_2} above 60 mmHg. For more detailed information on phosgene, please refer to Chapter 20.

Chloropicrin is a lung irritant and is also a soil fumigant used for its broad biocidal and fungicidal properties, mainly in high-value crops such as strawberries, tomatoes, tobacco, and flowers; it is used to treat timber as well. John Stenhouse first synthesized it in 1848. During World War I, chloropicrin was classified as a choking agent or was mixed with sulfur mustard to lower its freezing point. Currently, it is classified as a tear agent in the United States. *On April 9, 2004, the British Broadcasting Corporation (BBC) News reported a gas attack using chloropicrin in Sofia, Bulgaria in which 40 people were hurt, one of them seriously, which brings chloropicrin back as a potential terrorist chemical weapon.* Chloropicrin is a colorless to light-green oily liquid with an intense penetrating odor. Upon decomposition, chloropicrin can produce phosgene, nitrogen oxides, and chlorine compounds. The inhalation median lethal concentration (LC_{50}) for 10 min is 30 ppm, whereas bronchial or pulmonary lesions are produced at 20 ppm. Dermal and ocular contact with chloropicrin can lead to chemical burns or dermatitis and prolonged eye exposure can lead to blindness.

There is no antidote available for chloropicrin exposure, and in severe cases of respiratory compromise, if P_{AO_2} cannot be maintained above 60 mmHg, then PEEP is recommended to open the alveoli (Ellison, 2000).

22.3.1.2 Cyanide Agents (Blood Agents)

This class of agents was referred to earlier as "blood agents" because of the observed cyanide systemic effects compared with other agents, such as the blister agents, that were thought to produce only local effects. However, it has been shown that most chemical agents are equally capable of exerting systemic effects. The blood agents include hydrogen cyanide (AC), cyanogen chloride (CK), and arsine (SA). Liquid cyanide delivered in enclosed munitions rapidly vaporizes and is inhaled, forming the cyanide anion (CN^-), which is readily distributed to virtually every organ and tissue in the body.

During World War I, cyanide was used by the French who deployed almost 4000 tons without much success, possibly because of the small (1–2 lb) caliber munitions used to deliver it. Another potential reason for its ineffectiveness is its high volatility and quick dispersal before building up a sufficiently high concentration. Inhalation is the primary route for cyanide toxicity. Cyanides bind to the enzyme cytochrome oxidase as a ligand, blocking oxygen uptake and intracellular oxygen utilization, causing cyanosis and death. Hydrogen cyanide (HCN) was also used in the United States for execution of criminals in the "gas chambers." The medical management regime includes O_2 therapy and an antidote in a two-step process. In the first step, a methemoglobin-forming drug, such as amyl nitrite or sodium nitrite, is given to cyanotic patients to oxidize hemoglobin iron from Fe(II) to the Fe(III) state, which binds preferentially to cyanide (Equation 22.2). The second step is to provide a sulfur donor such as sodium hyposulfite to convert the toxic cyanide to nontoxic thiocyanate (Equation 22.3). A more effective antidote with fewer side effects is α-ketoglutaric acid. Decontamination of cyanides is done under basic conditions by using hypochlorite (Marrs et al., 1996). For more specific details see Chapter 30.

$$\text{CNHb} \xrightarrow[\text{Sodium nitrite (IV)}]{\text{Amyl nitrite (inhalation)}} \text{CNHb} \tag{22.2}$$

$$\text{CN}^- \xrightarrow[\text{Sulphur donor}]{\text{Sodium thiosulfate}} \text{SCN}^- \tag{22.3}$$

22.3.1.3 Blister Agents (Vesicants)

Vesicants were the major military threats during World War I. The most common effects of blister agents are dermal edema, erythema and blister formation, ocular irritation, conjunctivitis, corneal opacity and eye damage, and upper airway sloughing, pulmonary edema, metabolic failure, neutropenia, and sepsis. In addition, they can cause gastrointestinal effects and suppression of bone marrow stem cell production (USAMRICD, 2000). The major vesicants in this class include sulfur mustard (H), nitrogen mustards (HN1, HN2, and HN3), lewisite (L), mustard lewisite (HL), and phosgene oxime (CX). Sulfur mustard or mustard gas (bis dichloroethyl sulfide), which is actually a viscous liquid, has a long history of use in the battlefield as illustrated in Table 22.1. Mustard gas was first used by Germany against the Allies during World War I, by Britain against the Bolsheviks during the Russian revolution, by Italy in their campaign to occupy Ethiopia, by the Egyptians against the Yemeni Royalists, and by the Iraqis against Iran during the Iran-Iraq war, and again against the Kurds to quell their uprising. The exact mechanism of action of sulfur mustard injury has not been elucidated with a high degree of certainty, and several hypotheses have been proposed (Papirmeister et al., 1985, 1991; Somani and Babu, 1989). Figure 22.2 presents the current knowledge of the possible mechanism(s) of action of sulfur mustard toxicity (Sidell et al., 1997). It is possible that the application of modern biochemical tools such as genomics and proteomics may lead to a more concrete understanding of the process of sulfur mustard injury and repair. In recent years, however, numerous reports have been published suggesting that antioxidant therapy using *N*-acetylcysteine (NAC) and glutathione may potentially have a beneficial effect in the treatment of sulfur mustard injury or accelerating the process of healing (Elsayed et al., 1992, Gross et al., 1993; Amir et al., 1998; Atkins et al., 2000, Anderson et al., 2000, Kumar et al., 2001; Das et al., 2003; Elsayed and Omaye, 2004).

Because no antidote has yet been developed for sulfur mustard, medical management is limited to decontamination and supportive therapy. The standard decontamination procedure uses 0.5% household bleach (Smith and Dunn, 1991). Other vesicants in this class include the nitrogen mustards (HN1, HN2, and HN3). The "nitrogen mustard group," HN1 and HN3, is more likely to act as efficient vesicants (Mann, 1948), whereas mechlorethamine or mustine hydrochloride (HN2) has been used clinically for many years as a cancer chemotherapeutic agent. Exposure to the vapor of nitrogen vesicants for 10 min affects the skin at concentrations as low as 30 ppm, the eyes at 1 ppm. The LC_{50} for inhalation exposure is 18 ppm, and the median lethal dose (LD_{50}) for skin exposure

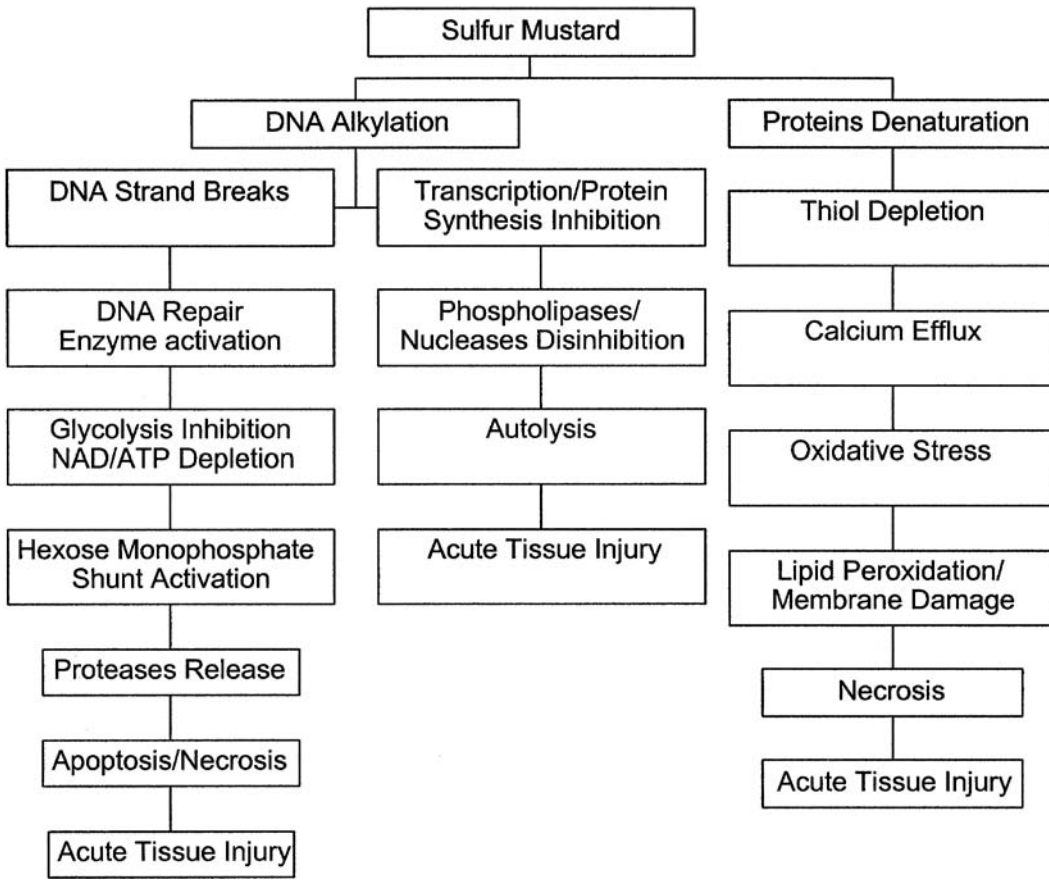


FIGURE 22.2 A diagram summarizing present knowledge of sulfur mustard mechanism(s) of action leading to acute tissue injury. Modified from Sidell et al., 1997.

is 700 mg. Nitrogen mustards are not detoxified by the body, i.e., the exposures are cumulative (Ellison, 2000). HN3 and sulfur mustard comprise another group, the "mustard gas group" because they produce similar vesicating effects (Mann, 1948).

Lewisite (L) was named after Dr. Wilford Lee Lewis who first synthesized it in 1918, but its production was late to join other CWAs used in World War I. Although it is used by itself, some countries such as the former Soviet Union combined it with mustard (HL) to lower its freezing point for ground dispersal and aerial spraying (USAMRICD, 2000). Inhalation of Lewisite has an LCt_{50} of 1500 mg·min/m^3, which is similar to that of mustard. Medical management of the exposure to Lewisite and the Lewisite/Mustard mixture includes intramuscular injection of British Anti-Lewisite (BAL) or Dimercaprol.

Phosgene oxime (CX) is not a true vesicant; it is rather an urticant or nettle agent that produces corrosive type lesions of the skin and tissues that may not fully heal for 2 months. Inhaled, CX vapor is extremely irritating and causes almost immediate tissue damage upon contact (USAMRICD, 2000). The exposure causes lung membranes to swell and pulmonary edema formation that can be lethal (Ellison, 2000). Medical management is limited to immediate decontamination of the exposed areas and symptomatic management of the lesions.

22.3.1.4 Nerve Agents (Organophosphates)

Nerve agents are the most toxic class of known chemical agents. They exhibit high lethality and low to high persistence as shown in Figure 22.1. Chemically, they are generally organic esters of phosphoric

acid, or organophosphate, pesticides. They were developed and stockpiled by Germany just before and during World War II, but were never deployed in the battlefield. The only known military use of nerve agents was in the Iran-Iraq war in the 1980s. The "G" agents are more volatile and tend to have high lethality and low persistence as illustrated in Figure 22.1. However, some G agents can be thickened using different substances to increase their persistence and percutaneous toxicity. The G agents are colorless to brownish liquids with a consistency ranging from water to light machine oil, and they are hazardous through inhalation, skin, eye, and ingestion exposures. The LC_{50} for the G series are estimated as low as 1 ppm for a 10-min exposure, and the percutaneous LD_{50} is estimated as 300 mg per individual. The "V" agents are less volatile and tend to posses both higher lethality and persistence than the G agents. They can be either solids or liquids and can be thickened to increase their persistence. The LC_{50} for inhaled V agents is as low as 0.3 ppm for a 10-min exposure, and the LD_{50} for skin exposure is estimated as 100 mg per individual. Another subclass of the G agents is the "GV" agents that are considered as intermediate volatility agents (IVAs). They combine the attributes of both the G and the V agents, i.e., they have greater percutaneous toxicity than the G agents and greater inhalation toxicity than the V agents, but they are not as stable as either series.

All series of nerve agents pose significant additional hazards upon hydrolysis or combustion because they produce hydrogen fluoride, nitrogen oxides, and phosphorous oxides as well as residual toxic organophosphates (Ellison, 2000).

Nerve agents are cholinesterase inhibitors that disrupt the functions of the nervous system by inhibiting the enzymes butyrylcholinesterase in the plasma and acetylcholinesterase in red blood cells at the cholinergic receptor sites in tissues and organs. These sites include smooth and skeletal muscles, central nervous system, and most exocrine glands (USAMRICD, 2000). The clinical effects of the nerve agents are due to accumulation of excess acetylcholine. A major characteristic associated with exposure to nerve gas vapor is bilateral miosis often accompanied by sharp or dull pain around the eyeballs, dim and blurred vision, nausea, and occasionally vomiting. Inhalation of the vapor results in rhinorrhea, bronchoconstriction, tightness of the chest, and apnea. The effects on the central nervous system include loss of consciousness, seizure, and apnea. Medical management includes decontamination, ventilation, administration of antidote, and supportive therapy. The antidotes available include atropine and pralidoxime chloride (2-PAMCI). In 2003, the U.S. Food and Drug Administration approved the use of pyridostigmine bromide as a pretreatment against the nerve agent Soman (GD).

Another class of nerve agents the "Novichok, 5, and 7" was developed by the former Soviet Union. Information about the composition, persistence, and human toxicity of this class of agents is very scarce. However, available information indicates that the Novichok agents are five to eight times as lethal as agent VX, a highly lethal agent in the U.S. arsenal (Ellison, 2000). Novichok agents can occur in binary form. These forms can be disguised as harmless agricultural products to evade international inspection and verification regimes.

22.3.2 Nonlethal, Riot Control Agents

Riot control agents or tear gases were the first CWAs deployed during World War I. The French police used them to control rioters before World War I and then the French military used them during the war with limited success. The use of riot control agents in the battlefield decreased as more lethal chemicals were developed. They are currently used mostly by law enforcement agencies. In addition to law enforcement application, tear agents are used in many countries for military training and were used extensively by the U.S. military in Vietnam for tunnel denial (TM-8-285, 1956; USAMRICD, 2000). For more detailed information, see Chapter 21.

22.3.2.1 Tear Agents (Lacrimators)

Tear agents are local irritants that cause transient discomfort and intense eye pain and discomfort, forcing closure of the eyes and leading to temporary inability to fight or resist arrest. They may

also irritate the respiratory tract, causing difficulty breathing. In high concentration they can irritate the skin, causing temporary burning and itching, nausea, and/or vomiting. These effects are transient, persisting no more than a few minutes after the exposure is ended (TM-8–285, 1956; USAMRICD, 2000). In an enclosure, very high concentration of tear agents can be lethal (Ellison, 2000). Lacrimators are mostly solids with low vapor pressure. They are colorless to yellow with a floral to a pepperlike smell. Tear agents dispersed as aerosols are not persistent. However, release of large amounts resulting in significant solid or liquid deposition can pose a persistent hazard.

Medical management is not required as casualties of tear agents usually recover within 15 min after removal from a contaminated environment. High-level exposures can produce dermatitis and superficial skin burns similar to thermal burns and are treated similarly.

22.3.2.2 Vomiting Agents

Vomiting agents were originally developed as sternutators (sneezing agents). They are nonlethal chemicals with an estimated inhalation LC_{50} of 1000 mg/m^3 for a 10-min exposure and a short persistence of approximately 30 min. They produce a pepperlike irritation of the upper respiratory tract and eye irritation and lacrimation. They cause coughing, sneezing, nausea, vomiting, and a general feeling of malaise. They can produce local inflammation of the upper respiratory tract, nasal accessory sinuses, and eyes. Vomiting agents can be dispersed as aerosols to produce their effect by inhalation or by direct action on the eyes (TM 8-285, 1956; USAMRICD, 2000; Ellison, 2000). Agents of this class are crystalline solids that are dispersed by heat as fine particulate smokes ranging from canary yellow to white which become colorless after dilution with air. Medical management is not required, and victims usually recover from exposure within 2 h after removal from the contaminated atmosphere. Duration of the symptoms can be shortened by vigorous exercise (Ellison, 2000).

22.3.2.3 Incapacitating Agents (Hallucinating Agents)

Incapacitating agents are nonlethal CWAs designed to create confusion or hallucinations. They are anticholinergic agents that act as competitive inhibitors of acetylcholine at postsynaptic and postjunctional muscarinic sites. 3-Quinuclidinyl benzilate (BZ) is a major example of this class of agents and is an odorless, nonirritating solid. It is extremely persistent in soils, water, and most other surfaces.

Exposure to BZ results in tachycardia, dry mouth and mucous membranes, flushing, delirium, and hypertension. Toxicity can occur after exposure to these agents following inhalation, ingestion, or absorption through the skin. Medical Management involves administering physostigmine salicylate and supportive care. (TM 8-285, 1956; USAMRICD, 2000; Ellison, 2000).

22.4 CWA STOCKPILES IN THE USA AND RUSSIAN FEDERATION

As stated earlier, knowledge about modern chemical warfare began during World War I when the Germans used chlorine gas in 1915. Many countries including the United States prohibited the use of CWA. For example, during the Civil War, the U.S. War Department issued General Order 100 in April 24, 1863, proclaiming that "the use of poison in any manner, be it to poison wells, or foods, or arms, is wholly excluded from modern warfare." In July 29, 1899, The Hague Convention (II) with Respect to the Laws and Customs of War on Land was signed and declared that "it is especially prohibited … to employ poison or poisoned arms." The Geneva protocol of 1925 (The Avalon Project, 1998) also prohibited the use of poison in warfare.

After World War I, many governments wished to ban the use of chemical weapons in wars because of the horrible means by which people were killed and injured. In 1925, at the League of Nations (the predecessor of the present United Nations) 38 nations signed the Geneva Protocol for "… the prohibition of the use in war of asphyxiating, poisonous, or other gases, and bacteriological methods of warfare"; the protocol has since been signed by over 130 nations. The protocol contained large loopholes, including that it does not prohibit the manufacture and threat of use of

chemical weapons; it is vague on the term "other gases," and it has no provisions for the punishment of countries that use such weapons illegally. A partial chronology of the major legal frameworks to control the use or prohibit the manufacture of CWAs is presented in Table 22.3.

During World War II, chemical weapons were not used in battle in the European theater of operations although Italy used CW in the invasion of Ethiopia in Africa, and Japan used chemical and biological agents in the invasion of China (Table 22.1). After World War II, it was discovered that Germany had developed a new class of chemicals (nerve agents) that included tabun, sarin, and soman. The Germans possessed large quantities (20,000–30,000 tons of tabun) that the Allies seized. However, the Russians took over most of the manufacturing plants and moved them to Volgograd in Russia. With the beginning of the Cold War, a new cycle of research and development of new CWAs was underway, and large quantities of chemical agents and weapons were stockpiled. The United States and the Russian Federation (former Soviet Union) were the two major producers of CWAs and possessed the largest stockpiles of chemicals. In 1992, the "Convention on the Prohibition of the Development, Production, Stockpiling, and Use of Chemical Weapons and their destruction" (Chemical Weapons Convention [CWC]) was approved by the United Nations and went into effect in 1997 Tables 22.4 and 22.5 show the CWA stockpiles of the United States and the Russian Federation after both countries agreed to stop CWA production and began destruction of existing stockpiles (Federation of American Scientists [FAS], 2000; Russian Munitions Agency, 2003; Harigel, 2003).

TABLE 22.3 Partial Chronological Listing of Major Events and Developments of Legal Frameworks to Control the Use of Chemical Weapons[a]

Date	Framework	Major Provisions/Achievement
April 24, 1863	The US War Department, General Order 100	"The use of poison in any manner, be it poison wells, or foods, or arms, is wholly excluded from modern warfare."
July 29, 1899	The Hague Convention (II) with Respect to the Laws and Customs of War on Land	"It is especially prohibited … to employ poison or poisoned arms."
June 17, 1925	Geneva Protocol for the Prohibition of the Use in War of Asphyxiating, poisonous, or Other Gases, and Bacteriological Methods of Warfare	The Protocol was not ratified by the US, but was not signed by Japan.
April 10, 1972	Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction.	Biological Weapon Convention Signed by the USA, Britain, and the Soviet Union.
January 22, 1975	Geneva Protocol (1925)	The US ratifies the Geneva Protocol, signed originally by the US on June 17, 1925.
September 3, 1992	The Convention on the Prohibition of the Development, Production, Stockpiling, and use of Chemical Weapons and their destruction, CWC	The convention was approved by the UN.
April 29, 1997	The Convention on the Prohibition of the Development, Production, Stockpiling, and use of Chemical Weapons and their destruction, CWC	The convention entered into force. As of November 1997, 165 nations had signed the CWC, of which 104 nations had ratified the treaty.

[a] Adapted from the Avalon Project (1998).

TABLE 22.4 Estimated Stockpiles of Chemical Weapons in the Russian Federation[a]

Chemical Weapons Category 1		Chemical Weapons Category 2		Chemical Weapons Category 3	
Nerve Agents (GA, GB, VX)	32.2[b]	Chocking agents (CG)	10[b]	NA	NA
Blister Agents (H, L, HL)	7.8 [b]	artillery shells (122 mm) filled with (CG)	3,844[c]	Inert chemical munitions, discontinuous and propellant charges	288,300[c]
Total[b]	40[b]		10[b]		0[d]

[a] Adapted from the Russian Munitions Agency (2003).
[b] Metric tons × 1000.
[c] Units × 1000.
[d] All stockpiles of category 3 chemical weapons have been destroyed as of April 2003.

TABLE 22.5 Estimated Stockpiles of Chemical Weapons in the USA[a]

Unitary Chemicals		Binary Chemicals	
Nerve Agents (GA, GB, VX, TGA, TGB)	13,352.63[b]	Methylphosphate diflouride (DF)	680.19[b]
		Isopropyl alcohol and isopropylamine (OPA)	
		Ethyl 2-diisopropylaminoethyl methylphosphate (QL)	
Blister Agents (H, HD, HT, L)	17,2469.948[b]		
Total	30,599.57[b]		680.19[b]

[a] Adapted from Harigel (2002).
[b] Metric tons.

22.5 NUCLEAR WEAPONS

Nuclear weapons (NWs) are the most destructive weapons produced by humans for use on the battlefield. Although NWs were used only twice during World War II, their acquisition served to deter aggression and coercion by other nations, and the threat of mutual assured destruction served as an instrument of policy and diplomacy for a long time. This class of weapons can produce large-scale destruction such as the destruction of Hiroshima and Nagasaki in Japan. In addition, the chemical species produced (fallout) from NW detonation can linger for many years in the environment, causing long-term adverse effects to all living systems and rendering large areas of land "off-limits" for use. With mounting global terrorism since the beginning of the twenty-first century, the concern of potential nuclear terrorist attacks also increased. Although sophisticated NWs may not be an available option to the terrorists for lack of technical expertise or manufacturing facilities, miniature nuclear devices as remnants of the cold war, could potentially be manufactured, purchased, or obtained through illegal means. It should be noted that it is possible for rudimentary electromagnetic weapons to be developed and used by terrorists determined to inflict maximum damage. To understand the potential risk from deployment of NWs or radiological devices, it was necessary to include a review of NWs.

22.5.1 Major Types of Nuclear Weapons

Nuclear weapons can be in the form of bombs, missile warheads, artillery shells, bombs, and other devices. This review is limited to bombs, since a potential nuclear terrorist attack, if it materializes, most likely will be in the form of a small bomb or an explosive radiological dispersal device because the technical expertise and manufacturing facilities are beyond the reach of terrorists. The major types of nuclear bombs include: fission bomb, fusion bombs, salted bombs, neutron bombs, and some other devices.

22.5.1.1 Fission Bombs

Fission bombs or atom bombs (A bombs) are weapons that produce energy from nuclear fission of heavy elements such as uranium or plutonium bombarded by neutrons, which results in lighter elements and more neutrons that can trigger chain reactions. Fission bombs are ignited by forcing masses of enriched uranium or plutonium atoms into each other to cause a critical reaction. This is generally accomplished by detonating a high-explosives "lens" that surrounds a "pit" of radioisotope or by firing a uranium "bullet" into a radioactive "pit." (FM 8-10-7, 1993).

22.5.1.2 Fusion Bombs

Fusion bombs or thermonuclear weapons are generally known as hydrogen bombs (H bomb). These weapons require an extremely high temperature such as a fission core (an A bomb) to trigger chain reactions that use lighter elements such as hydrogen or helium and produce heavier elements and large amounts of energy (Alt et al., 1989; Rhodes, 1995).

22.5.1.3 Salted Bombs

These are advanced thermonuclear weapons that apply advanced designs to produce more devastation. One such design is to vary the material used in the shell of a nuclear device. For example, a fusion bomb with an outer shell of neutral (unenriched) uranium upon detonation produces intense fast neutrons that can cause the neutral uranium in the shell to fission despite being unenriched, thus significantly increasing the explosive yield of the weapon. By using different elements in the shell of the nuclear weapon, different fallout durations can be achieved. For example, using cobalt in the shell provides long-term (years) fallout duration when the neutral cobalt is converted by the fusion-emitted neutrons to cobalt-60, a powerful long-term gamma ray emitter (years). Other shell types include zinc an intermediate (months) fallout emitter, and gold, a short-term (days) fallout emitter (Glasstone and Dolan, 1977).

22.5.1.4 Neutron Bombs

These are small, enhanced radiation thermonuclear weapons in which the neutrons generated by the fusion reaction are not absorbed within the weapon body. These weapons use X-ray mirrors and shells made of chromium or nickel to permit the neutron to escape, causing an intense burst of high-energy destructive neutrons, hence, the name neutron bomb. However, in contrast to the salted bombs, the burst of ionizing radiation occurs only at the time of detonation and is not accompanied by additional enhanced residual radiation (Glasstone and Dolan, 1977; Cohen, 1983).

22.5.2 Distribution of Energy from Nuclear Detonation

Nuclear detonation results in massive release of energy in different forms and magnitudes. The major forms of energy from a nuclear detonation are blast overpressure, thermal radiation, ionizing radiations (immediate), and residual radiation (delayed fallout), and electromagnetic pulse

(Glasstone and Dolan, 1977). The distribution of energy from a moderate size nuclear detonation (less than 10 kilotons) is shown in Table 22.6.

22.5.2.1 Blast Overpressure

Almost 50–60% of the energy produced by nuclear detonation is due to blast overpressure (BOP) and the resulting "blast wind." Blast overpressure is the abrupt change in atmospheric pressure above (positive) or below (negative) ambient pressure. Peak overpressure from a 1-kiloton (KT) detonation can reach 200–400 kilopascals (kPa); depending on the distance from the center of the detonation point, different rates of lethality can occur (Table 22.7). In air, the magnitude of the overpressure is proportional to the air density, i.e., the higher the air density, the greater the BOP (static overpressure). Another factor that contributes to the destructive power of a nuclear detonation is the drag exerted by the blast wind (dynamic pressure). Both of these factors contribute to the physical damage produced by a nuclear detonation (Glasstone and Dolan, 1977; FM 8-10-7, 1993; FM 8-9, 1996). The rapid compression/decompression cycle produced by the impacting BOP waves transmits energy through the human body or solid objects that can be destructive. Three major types of

TABLE 22.6 Distribution of Total Energy Produced by Nuclear Weapon Detonation[a]

Physical Effect	Contribution (%)	Description
Blast Overpressure (BOP)		Abrupt changes in atmospheric pressure (above or below) ambient level representing rapid compression / decompression waves causing death or non-lethal injuries
• Standard Fission/Fusion	50	
• Enhanced Radiation Weapon	40	
Thermal Radiation		Direct effect of exposure to thermal energy (flash burns)
• Standard Fission/Fusion	35	Indirect effect of exposure to environmental fires (flame burns)
• Enhanced Radiation Weapon	25	
Initial Radiation		Neutron, gamma rays, alpha and beta particles emitted within the first minute after detonation (immediate)
• Standard Fission/Fusion	5	
• Enhanced Radiation Weapon	30	
Residual (fallout) Radiation		Alpha, beta and gamma rays (delayed)
• Standard Fission/Fusion	10	
• Enhanced Radiation Weapon	5	
Electromagnetic Pulse (EMP)	1	Broadband, high-intensity, short-duration burst of electromagnetic energy caused by ionized air molecules.

[a] Adapted from Alt et al. (1989); FM 8-10-7 (1993).

TABLE 22.7 Range and Lethality of Exposure to Peak Overpressures from a 1-KT Nuclear Detonation[a]

Peak Overpressure (kPa (psi)	Distance from Detonation Point. Meters (feet)	Lethality (%)
233–294 (34-43)	150 (492)	1
294–415 (43-60)	123 (404)	50
>415 (60)	110 (361)	100

[a] Adapted from FM 8-10-7 (1993).

injuries can occur from exposure to BOP. First, exposure to the shock waves only results in primary blast injury, mostly to the hollow organ systems such as the respiratory, gastrointestinal, and auditory systems. The lung, however, is the organ most sensitive to damage, and this injury can be fatal. Second, exposure to flying missiles results in secondary blast injury, and third, displacement of the body by the incoming BOP waves impacting solid objects results in tertiary blast injury (Schardin, 1950; Rössle, 1950; White et al., 1971; Stuhmiller et al., 1991; Elsayed, 1997). These injuries are further affected by the magnitude and frequency of the shock wave, the position and distance of the body in relation to the incoming wave, and the potential magnification of the BOP wave in enclosures. A possible mechanism of injury from exposure to the blast was proposed in 1997. In that mechanism, incoming blast waves would damage red blood cells, releasing heme iron which would initiate free radical-mediated reactions that continue to propagate after the initial blast exposure, resulting in delayed death (Gorbounov et al., 1995, 1997; Elsayed et al., 1997).

22.5.2.2 Thermal Radiation

Surface nuclear detonation emits a large amount (30–40%) of electromagnetic radiation (Table 22.6) outward from the fireball that includes visible, infrared, and ultraviolet lights. Most of the damage from thermal radiation is associated with skin burns and eye injury from the intense light, which can even start fires spread by the blast wind further away from the fireball at the point of detonation (ground zero). Thermal radiation can cause burns either directly following absorption of the thermal energy by the exposed surface (flash burns) or indirectly by fires in the environment (flame burns) spread by the blast wind. Several factors modulate the energy available to cause flash burns, including detonation yield, distance from the fireball, height of burst, weather conditions, and type of surrounding environment. The degree of burn injury depends on the flux measured in calories per surface area of exposed skin (cal/cm^2) and duration of the thermal pulse. For example, thermal radiation from a 1-KT detonation would produce second-degree burns on exposed skin at a range of 0.78 km, a pulse duration of 0.12 sec, and a flux of 4.0 cal/cm^2 (Glasstone and Dolan, 1977; FM 8-10-7, 1993; FM 8–9, 1996).

22.5.2.3 Initial Ionizing Radiation

Between 4 and 5% of the energy released from a nuclear detonation is in the form of initial neutrons and gamma rays. Neutrons are released almost entirely from the fission or fusion reactions, whereas initial gamma rays are produced from the same reactions and from decay of short-lived fission products. Nuclear detonation results in four types of ionizing radiation within the first minute (immediate radiation) following detonation. They include neutrons and gamma, alpha, and beta radiation.

Neutrons are uncharged particles emitted in the first few seconds of detonation that do not ionize tissue directly and are not a fallout hazard. However, because of their significant mass, they can interact with other atomic nuclei, disrupting their atomic structure and potentially causing 20 times more damage than gamma rays. Neutron emission constitutes 5–20% of the total energy of a nuclear weapon. However, enhanced radiation thermonuclear weapons (neutron bombs) produce a much higher yield of neutrons to kill people with radiation rather than destroy properties through blast effects.

Gamma rays are emitted during detonation and in fallout residual nuclear radiation. They are short-wavelength, uncharged high-energy radiation similar to X rays. They are a hazard due to their high energy and penetrability, which can result in whole-body radiation.

Alpha particles are heavier (four times the neutrons mass), charged particles and a fallout hazard. Because of their size, they lack penetrability and can be stopped by dead layers of skin or by a military uniform. Alpha particles represent a negligible external hazard but can cause serious internal damage if inhaled or ingested.

Beta particles are very light, negatively charged particles found primarily in fallout residual radiation. They have weak penetrability and can travel a short distance in tissues. However, exposure

to large quantities of beta particles can produce damage (beta burn) to the basal stratum of the skin similar to thermal skin burns (Glasstone and Dolan, 1977; FM 8-10-7, 1993; FM 8-9, 1996).

22.5.2.4 Residual (Fallout) Radiation

The major hazard from residual nuclear radiation is radioactive fallout and the neutron-induced hazards.

22.5.2.4.1 Fission Products

The splitting of heavy uranium or plutonium nuclei in fission reactions produces a large number of fission products (approximately 60 g/KT yield) with different half-lives, and an estimated activity of 1.1×10^{21} Bq (equivalent to 30 million kg of radium) estimated at approximately 1 min after detonation. These products have different decay rates (emission of beta and gamma rays), i.e., environmental persistence ranging from very short (seconds) to long (months) to very long (years to many years). The fission products with the greatest concern from fallout include strontium-90 ($t_{1/2}$, 28 years), cesium-137 ($t_{1/2}$, 30 years), carbon-14 ($t_{1/2}$, 5800 years), strontium-89 ($t_{1/2}$, 51 days), and iodine-131 ($t_{1/2}$, 8 days).

Iodine-131 poses the highest risk of internal exposure because it is deposited on the surface of vegetation, eaten by meat animals, then secreted in the milk, and ingested by humans. Iodine-131 concentrates preferentially in the thyroid gland and destroys it.

Strontium-90 and strontium-89 are produced in large amounts in nuclear fallout. Similar to iodine-131, consumption of contaminated fruits, vegetables, and milk are major health hazards because strontium is readily incorporated in the bones.

22.5.2.4.2 Unfissioned Nuclear Material

Nuclear detonation does not consume all the enriched uranium or plutonium in the fission reactions, and much of it is dispersed unfissioned by the explosion that continues to decay slowly by emitting alpha particles.

22.5.2.4.3 Neutron-Induced Activity

When atomic nuclei exposed to a flux of neutrons capture a neutron, they become radioactive and continue to decay over a long period by emitting beta and gamma radiation that can activate other atoms in the environment, thus producing a hazardous area that becomes "off limits" for use by humans.

22.5.2.5 Electromagnetic Pulse

The electromagnetic pulse (EMP) is a form of radiation produced by nuclear detonation when gamma rays (primary photons) collide with electrons and transfer some of their energy to the electrons. They can interact with a secondary photon moving in a new direction of motion with respect to the primary photon. This results in a Compton interaction changing the direction or scattering of the gamma ray photon and in the degradation of its energy. The electron that collides with the primary photon recoils in such a manner as to conserve energy and momentum and is called a Compton-recoil electron (Glasstone and Dolan, 1977; Makoff and Tsipis, 1988). The EMP is broadband, high-intensity, short-duration burst of electromagnetic energy distributed throughout the lower frequency spectrum between 3 Hz and 30 kHz with no known biological effects. However, the resulting electric and magnetic fields could couple with electrical or electronic systems and produce current and voltage surges that damage electronic equipment, wires, antennas, and metal objects, interfere with radiofrequency links, and microcircuits, and could disable satellites. The strength of the EMP depends highly on the altitude at which it is released; it is strongest at altitudes above 30,000 feet. The existence of EMP has been known since the 1940s when nuclear weapons were developed and tested. However, their effect was not fully known until 1962 when the United States conducted a series of high-altitude atmospheric

tests and detonated a nuclear device in the Pacific Ocean 800 miles from Hawaii. The resulting EMP disrupted radio stations and electric equipment throughout Hawaii (Ricketts et al., 1976).

22.5.3 Biological Effects of Ionizing Radiation

Ionizing radiation interacting with atoms deposits energy that causes further ionization (electron excitation), which could involve critical molecules or structures in the cell and produce damage. Direct interaction of radiation with a particular cellular molecule could cause irreparable damage and lead to death or malfunction. Indirect interaction causing cell damage occurs when radiation interacts with water molecules in the body to create toxic molecules (free radicals) that could in turn affect neighboring sensitive molecules. The most radiosensitive organs in the body are the hematopoietic and gastrointestinal systems. Direct and indirect cellular radiation damage is a function of specific tissue sensitivity and radiation dose. Although high doses of radiation can cause cell death, lower doses can cause a variety of effects, including delays in the mitotic cycle phases, disruption of cell growth, and changes in permeability and motility. In general, radiosensitivity tends to be inversely related to the degree of cell differentiation. Prediction of radiation damage is very difficult because exposed organs are often unknown. The primary medical concern is severe radiation sickness caused by whole-body irradiation. The LD_{50} for exposed persons within a period of 60 days from radiation exposure ($LD_{50}/60$) is approximately 450 centigray (cGY).

Cellular recovery is possible if a sufficient proportion of the stem cell population remains undamaged after radiation injury. Although complete recovery from radiation injury seems to occur, late somatic relapses have a higher probability of occurring because of radiation-induced damage (Glasstone and Dolan, 1977; FM 8–10–7, 1993; FM 8–9, 1996).

22.6 RADIOLOGICAL DISPERSAL DEVICES (RDD) (DIRTY BOMBS)

Radiological dispersal devices (RDD) or "dirty bombs" as they are also known are weapons that combine conventional explosives, such as dynamite, trinitrotoluene (TNT), pentaerythritol (PETN), or plastic explosives (C-3 and C-4), with radioactive materials. The radioactivity is then dispersed through the initial blast of the conventional explosive producing airborne radiation and contamination. In general, the conventional explosive detonation associated with RDD produces greater lethality and damage than the radioactive material. Because the intent of using dirty weapons is to cause disruption of services and generate public fear and panic rather than cause heavy casualties, death, and destruction, such a low outcome fulfils the potential objectives of the terrorist. Dirty bombs can be small devices that fit in a suitcase or as big as a truck. A dirty bomb, however, is not a nuclear weapon, because it does not involve nuclear fission reactions or generate destructive blast waves or thermal and ionizing radiation.

Many types of radioactive materials with military, industrial, or medical application could be used to produce dirty weapons by encasing a highly radioactive material in lead and surrounding the device with conventional explosives. The resulting explosion, depending on atmospheric conditions, would disperse radiation over a wide area and could cause potential health problems. For example, weapon-grade uranium and plutonium or spent nuclear fuel could be used and would be the deadliest materials. However, they are also the most difficult to obtain and handle by a potential terrorist. In contrast, medical-grade chemicals used mostly in cancer treatment, such as radium and cesium isotopes, could be used with less risk in handling but also with reduced potency. Two nuclear disasters illustrate the potential health hazard of radiological explosions.

First is the Chernobyl nuclear disaster in 1986 when the graphite moderators in the core of the reactor overheated, causing the graphite to ignite in the air circulated as the coolant. The resulting fire was very hard to fight, spreading radioactive material carried off in the smoke over large areas (U.S. Naval Academy [USNA], 2001). The accident resulted in a series of explosions that totally destroyed one reactor and dispersed radioactive material that contaminated Northern Ukraine,

Belarus, Russia, and Scandinavia. Thirty-one were killed and 140 developed significant illnesses among the plant personnel and rescue workers. A 30-km exclusion zone was established immediately surrounding the site and farming was prohibited in parts of the radiation fallout area, even 18 years after the disaster.

The second incident occurred in Goiania, Brazil in 1987 when scrap metal scavengers broke into a radiological clinic and stole a capsule containing about an ounce of highly radioactive cesium-137. The capsule was broken into pieces and passed to friends and relatives. This resulted in 4 dead, 249 contaminated, 14 overexposed, and 114,000 persons subjected to continuous monitoring. In addition, 85 houses and truck loads of personal possessions had to be destroyed; large amounts of contaminated earth were removed.

According to the International Atomic Energy Agency, 370 instances of nuclear smuggling have been reported from 1993 to 2000. In April 2001, a truck carrying 10 lead-lined boxes filled with highly irradiated strontium-90 scrap metal was intercepted by customs officers in Uzbekistan. To prepare the public, several national and international organizations have issued information sheets and public guidance in the event of a dirty-bomb attack (Ford, 1998; USNRC, 2003; CDC, 2003; WHO, 2003; OSHA, 2004).

22.7 CONCLUSIONS

Almost 100 years have passed since the introduction of CWAs in World War I, and significant advances have been achieved in understanding the mechanism of action of most of them. However, no effective antidotes exist for several of these agents. For example, sulfur mustard continues to be produced, stockpiled, and even used by some nations albeit clandestinely, and it could be possessed and possibly used by terrorists. There is no antidote for sulfur mustard available, and only decontamination and supportive treatment are recommended for the victims. Another threat to both military and civilian personnel is blast overpressure injury resulting from explosive detonation. In recent years, blast research has become limited to the development and improvement of protective vests that were indispensable in Iraq and elsewhere, as well as the development of more accurate predictive models of blast injury. Current research on chemical and biological terrorism should also include the effects of blast injury. Detonation of an explosive device with or without chemicals or biologicals has the potential to cause internal primary blast injury, combined with penetrating wounds from secondary blast injury caused by shattered glass and flying missiles, in addition to the adverse effects of any chemical or biological agent that survives the explosion. The results of nuclear or radiological detonation are even more complicated. They could involve blast injury from the detonation, burn injury from thermal radiation, and internal injuries from nuclear radiation. All these adverse effects would be further complicated by inhalation injury from the smoke and combustion gases in case of a fire, not to mention the psychological stress in response to the unexpected attack. These events suggest that awareness and consideration should be given to the complex multiple injuries that may occur simultaneously, and that preparedness and response to potential terrorist attacks should include medical management of combined injuries.

REFERENCES

Alt, L.A., Forcino, C.D., and Walker, R.I., Nuclear events and their consequences, in *Textbook of Military Medicine*, Part 1. Vol. 2. *Medical Consequences of Nuclear Warfare*, Walker, R.I. and Cerveny T.J., Eds., Office of the Surgeon General, Department of the Army, Washington, DC, 1989. pp. 1–14.

Amir, A., Chapman, S., Gozes, Y., Sahar, R., and Allon, N., Protection by extracellular glutathione against sulfur mustard induced toxicity in vitro, *Hum. Exp. Toxicol.*, 17, 652–660, 1998.

Anderson, D.R., Byers, S.L., and Vesely, K.R., Treatment of sulfur mustard (HD)-induced lung injury, *J. Appl. Toxicol.*, 20, S129–S132, 2000.

Atkins, K.B., Lodhi, I.J., Hurley, L.L., and Hinshaw, D.B., N-acetylcysteine and endiothelial cell injury by sulfur mustard, *J. Appl. Toxicol.*, 20, S125–S128, 2000.

CDC (Centers for Disease Control and Prevention), Dirty Bombs. Fact Sheet. Centers for Disease Control and Prevention, Department of Health and Human Services, Atlanta, GA, 2003.

Cohen, S.T., *The Truth about the Neutron Bomb: The Inventor of the Bomb Speaks Out,* Morrow, New York, 1983.

Das, S.K., Mukherjee, S., Smith, M.G., AND Chatterjee, D., Prophylactic protection by N-acetylcysteine against the pulmonary injury induced by 2-chloroethyl ethyl sulfide, a mustard analogue, *J. Biochem. Mol. Toxicol.*,17, 177–184, 2003.

Ellison, H., *Handbook of Chemical and Biological Warfare Agents*, CRC Publishers, Boca Raton, FL, 2000.

Elsayed, N.M., Toxicology of blast overpressure, *Toxicology*, 121, 1–15, 1997.

Elsayed, N.M., Gorbunov, N.V., and Kagan, V.E., A proposed biochemical mechanism for blast overpressure induced hemorrhagic injury, *Toxicology*, 121, 81–90, 1997.

Elsayed, N.M. and Omaye, S.T., Biochemical changes in mouse lung after subcutaneous injection of the sulfur mustard 2-chloroethyl 4-chlorobutyl sulfide, *Toxicology*, 199, 195–206, 2004.

Elsayed, N.M., Omaye, S.T., Klain, G.J., and Korte, Jr., D.W., Free radical-mediated lung response to the monofunctional sulfur mustard butyl 2-chloroethy sulfide after subcutaneous injection, *Toxicology*, 72, 153–165, 1992.

Evison, D. and Hinsley, D., Chemical weapons, Br. Med. J., 324, 332–335, 2002.

Federation of American Scientists, Weapons of Mass Destruction Around the World: Chemical Weapons, Washington, DC, 2000. Available at www.fas.org.

FM 8-10-7 Field Manual: Health Service Support in a Nuclear, Biological and Chemical Environment, Department of the Army, Washington, DC, 1993.

FM 8-9, Virtual Naval Hospital–NATO Handbook on Medical Aspects of NBC Defensive Operations AMedP-6(B). (Part I: Nuclear), Departments of the Army, Navy, and Air Force, Washington, DC, 1996.

Ford, J.L., Radiological Dispersal Devices. Assessing the Transnational Threat. Strategic Forum, Institute for National strategic Studies, National Defense University. Washington DC, 1998.

Glasstone, S. and Dolan, P.J., Eds., *The Effects of Nuclear Weapons*, Department of Defense and Energy Research and Development Administration, Washington, DC, 1977.

Gorbounov, N.V., Elsayed, N.M., Kisin, E.R., Kozlov, A.V., and Kagan, V.E., Air blast overpressure induces oxidative stress in rat lungs: Interplay between hemoglobin, antioxidants, and lipid peroxidation, *Am. J. Physiol.*, 272, L320–L334, 1997.

Gorbounov, N.V., Osipov, A.N., Day, B.W., Zyas-Rivera, B., Kagan, V.E., and Elsayed, N.M., Reduction of ferrylmyoglobin and ferrylhemoglobin by nitric oxide: a protective mechanism against ferryl hemoprotein-induced oxidations, *Biochemistry*, 34, 6689–6699, 1995.

Gross, C.L., Innace, J.K., Hovatter, R.C., Meier, H.L., and Smith, W.J., Biochemical manipulation of intracellular glutathione levels influences cytotoxicity to isolated human lymphocytes by sulfur mustard, *Cell Biol. Toxicol.*, 9, 259–267, 1993.

Harigel, G.G., Chemical and Biological Weapons: Use in Warfare, Impact on Society and Environment, Nuclear Age Peace Foundation, Santa Barbara, CA, 2003, pp. 1–25. Available at www.wagingpeace.org.

Joy, R.J.T., Historical aspects of medical defense against chemical warfare, in *Textbook of Military Medicine*, Part I. *Medical Aspects of Chemical and Biological Warfare*, Sidell, F.R., Takafuji, E.T., and Franz, D.R., Eds., Walter Reed Army Medical Center, Washington, DC, 1997, pp. 87–109.

Kumar, O., Sugendran, K., and Vijayaraghavan, R., Protective effect of various antioxidants on the toxicity of sulphur mustard administered to mice by inhalation or percutaneous routes, *Chem. Biol. Interact.*, 134, 1–12, 2001.

Makoff, G. and Tsipis, K., The Nuclear Electromagnetic Pulse, Program in Science and Technology for International Security, MIT Report 19, Massachusetts Institute of Technology Cambridge MA, 1988.

Mann, I., Pirie, A., and Pullinger, B.D., An experimental and clinical study of the reaction of the anterior segment of the eye on chemical injury, with special reference to chemical warfare agents, *Br. J. Ophthalmol. Suppl.*, 12, 1–171, 1948.

Marrs, T.C., Maynard, R.L., and Sidell, F.R., *Chemical Warfare Agents. Toxicology and Treatment*, John Wiley & Sons, New York, 1996.

Monterey Institute for International Studies, Chronology of State Use and Biological and Chemical Weapons Control, 2002. Available at http://cns.miis.edu/.

OSHA (Occupational Safety and Health Administration), Radiological Dispersal Devices (RDD)/Dirty Bombs, Occupational Safety and Health Administration, Department of Labor, Washington, DC, 2004.

Papirmeister, B., Feister, A.J., Robinson, S.I., and Ford, R.D., *Medical Defense against Mustard Gas: Toxic Mechanisms and Pharmacological Implications*, CRC Press, Boca Raton, FL, 1991.

Papirmeister, B., Gross, C.L., Meir, H.L., Petrali, J.P., and Johnson, J.B., Molecular basis for mustard-induced vesication, *Fundam. Appl. Toxicol*., 5, S134–S149, 1985.

Phillips, Y.Y. and Richmond, D.R., Primary blast injury and basic research: a brief history, in *Textbook of Military Medicine*, Part 1. Vol. 5. *Conventional Warfare Ballistic, Blast, and Burn Injuries*, Bellamy, R. and Zajtchuk, R., Eds., Office of the Surgeon General, Department of the Army, Washington, DC, 1991, pp. 221–240.

Rhodes, R., *Dark Sun: The Making of the Hydrogen Bomb*, Simon and Schuster Publishers, New York, 1995.

Ricketts, L.W., Bridges, J.E., and Miletta, J., *EMP Radiation and Protective Techniques*, John Wiley & Sons Publishers. New York, 1976.

Rössle, R., Pathology of blast effects, in *German Aviation Medicine, World War II,* Vol. 2, US. Government Printing Office, Washington, DC, 1950, 1260–1273.

Russian Munitions Agency, Chemical Disarmament, Chemical Weapons. History of CW Development, 2003. Available at www.munition.gov.ru/eng/hstchw.html.

Schardin, H., The physical principles of the effects of a detonation, in *German Aviation Medicine, World War II*, Vol. 2, US. Government, Washington, DC, 1950.

Sidell, F.R., Urbanetti, J.S., and Smith, W.J., Vesicants, in *Textbook of Military Medicine*, Part I. *Medical Aspects of Chemical and Biological Warfare*, Sidell, F.R., Takafuji, E.T., and Franz, D.R., Eds., Walter Reed Army Medical Center, Washington, DC, 1997, pp. 197–228.

Smart, J.K., History of chemical and biological warfare: an american perspective, in *Textbook of Military Medicine*, Part I. *Medical Aspects of Chemical and Biological Warfare*, Sidell, F.R., Takafuji, E.T., and Franz, D.R., Eds., Walter Reed Army Medical Center, Washington, DC, 1997, pp. 9–86.

Smith, W.J. and Dunn, M.A., Medical defense against blistering chemical warfare agents,. *Arch. Dermatol.*, 127, 1207–1213, 1991.

Somani, S.M. and Babu, S.R., Toxicodynamics of sulfur mustard, *Int. J. Clin. Pharmacol. Ther. Toxicol*., 27, 419–435, 1989.

Stuhmiller, J.H., Phillips Y.Y., and Richmond, D.R., The physics and mechanisms of primary blast injury, in *Textbook of Military Medicine*, Part 1. Vol. 5. *Conventional Warfare, Ballistic, Blast, and Burn Injuries*, Bellamy, R. and Zajtchuk, R., Eds., Office of the Surgeon General, Department of the Army, Washington, DC, 1991, pp. 241–270.

The Avalon Project, Laws of War: Laws and Customs of War on Land (Hague II), July 29, 1899, Yale Law School, New Haven, CT, 1998. Available at www.yale.edu/lawweb/avalon/lawofwar/hague02.htm

TM 8-285, Technical Manual: Treatment of Chemical Warfare Casualties, Departments of the Army, Navy, and Air Force, Washington, DC, 1956.

USAMRICD (U.S. Army Medical Research Institute of Chemical Defense), Medical Management of Chemical Casualties Handbook. 3rd ed., Chemical Casualty Care Division, Aberdeen Proving Ground, MD, 2000.

USNA (U.S. Naval Academy), Explosives. Naval Applications of Chemistry. Chemistry Department. U.S. Naval Academy, Annapolis, MD, 2001.

USNRC (U.S. Nuclear Regulatory Commission), Fact Sheet on Dirty Bombs, March 2003. http://www.nrc.gov/reading-rm/doc-collections/fact-sheets/dirty-bombs.html

White, C.S., Jones, R.K., Damon, E.R., and Richmond, D.R., The Biodynamics of Airblast, Technical Report, DNA 2738-.Tl, Department of Defense, Washington, DC, 1971.

WHO (World Health Organization), Health Protection Guidance in the Event of a Nuclear Weapons Explosion, WHO/RAD Information Sheet, Radiation and Environmental Health Unit, World Health Organization, Geneva, Switzerland, 2003.

23 The Inhalation Toxicology of Chromium Compounds

Sidney A. Katz, Bryan Ballantyne, and Harry Salem

CONTENTS

23.1 INTRODUCTION

Exposure to chromium compounds occurs by ingestion of food and water, by inhalation of airborne particulates and by contact with numerous manufactured items containing chromium or its compounds. The World Health Organization (WHO) (2000) has estimated that daily chromium intake by humans is $<$200 μg from foodstuff, 0.8–16 μg from drinking water, and $<$1 μg from ambient air. Gauglhofer (1991) has reviewed the metabolism of chromium. The absorption, transport, distribution, and elimination of chromium compounds in humans and animals depends on factors such as the solubility and oxidation state of the chromium compound, the route and site by which the victim or subject is exposed to the chromium-containing material, and the particle size of the chromium-containing material when the route of exposure is by inhalation. The bronchial tree is the primary target organ for the carcinogenic effects associated with the inhalation of hexavalent chromium.

23.2 INHALATION EXPOSURE

Inhalation of chromium-containing aerosols is of major concern in the exposure of humans to chromium compounds. The retention of chromium compounds from inhalation, based on a 24-h respiratory volume of 20 m^3 in urban areas with an average chromium concentration of 50 ng/m^3 has been estimated to be between 3 and 300 ng (WHO, 2000). Individual uptake may vary depending on concomitant exposures to other relevant factors such as tobacco smoking and on the distribution of the particle sizes in the inhaled aerosol.

Chromium absorption in the occupational/industrial environment is usually by inhalation of mists, fumes, or airborne particulates containing chromium compounds. Airborne particles vary greatly in size. Particles larger than 10 μm rarely enter the respiratory tract. When they do, they are deposited in the nose and eliminated by sneezing, blowing, and/or wiping. When airborne particles having mean aerodynamic diameters ranging from 0.1 to 10 μm are inhaled, approximately 50% are

Table 23.1 Predicted Lung Penetrations of Airborne Particulates

Impactor	Dimensions (μm)	Penetration
Stage 0	11 and above	—
Stage 1	7–11	—
Stage 2	4.7–7	Pharyngeals
Stage 3	3.3–4.7	Tracheal/primary bronchial
Stage 4	2.1–3.3	Secondary bronchial
Stage 5	1.1–2.1	Terminal bronchial
Stage 6	0.65–1.1	Alveolar
Stage 7	0.43–0.65	Alveolar

deposited in the upper respiratory tract, approximately 25% are deposited in the lower respiratory tract, and approximately 25% are exhaled. Particles smaller than 1 μm are either aspirated into the mucociliary escalator or engulfed by phagocytes. The phagocytes either migrate to or enter the distal end of the mucociliary escalator, or they are absorbed into the lymphatics of the lungs. Particles deposited in the upper respiratory tract may be carried by the cilia to the pharynx where they are coughed up or swallowed along with the particles inhaled through the mouth. Soluble particles may be absorbed through the epithelium into the blood (Lu, 1985). Absorption from the respiratory tract usually takes place at the alveoli. Migration through the alveolar cell membranes is by passive diffusion. The rate of absorption depends on solubility and concentration.

Chromium is among the elements concentrated in/on the smaller (<1 μm) particles collected from ambient air. Particles having mean aerodynamic diameters smaller than 1 μm are deposited predominantly in the alveolar regions of the lungs where absorption efficiency for most elements ranges from 50 to 80%. Larger particles do not enter the lungs but pass by cilial action to the stomach where absorption efficiency commonly ranges from 5 to 15% for most elements. Lung penetration can be predicted from cascade impactor data, which classify the collected particles according to their aerodynamic dimensions (Bloom and Noller, 1977) as tabulated in Table 23.1.

The highest concentrations of chromium in fly ash from coal-fired power stations was found in the 1- to 2-μm size fraction, and most of the dust particles from processing chromite ore had diameters in the 0.32- to 0.37-μm range (U.S. EPA, 1984).

23.3 EPIDEMIOLOGY

Epidemiological studies have played a key role in identifying some hexavalent chromium compounds as respiratory carcinogens. As early as 1890, Newman (1890) described an adenocarcinoma of the inferior turbinate in the nose of a Scottish chromate pigment production worker. Subsequently, Pfeil (1935) reported lung cancers among German workers who used dichromates to oxidize anthracene and its derivatives for the production of alizarin dyes in 1935, and Alwens and Jonas (1938) reported 20 cases of lung cancer among the workers at a chromate-producing facility at Griesheim near Frankfurt in 1936. After the Second World War, several retrospective mortality studies were reported for workers with histories of occupational exposures to chromium and its compounds. Among the industries included in these epidemiological studies were those producing chromium chemicals, those producing and/or using chromium pigments, those electroplating with chromium on to other metals, those welding stainless steel and other alloys containing chromium, those producing ferrochrome, and those using chromium compounds in the tanning of leather.

In response to the early reports associating the increased incidences of lung cancer with occupational exposures in the German chromate production industry, Machle and Gregoris (1948) used insurance company records to compare the 1930–1947 lung cancer mortality rates of nearly 1500 American chromate workers with those of a control group consisting of American oil refinery workers who died between 1933 and 1948. Of the 156 deaths reported among chromate workers, 32 were attributed to lung cancer. In the control populations, 10 of the 733 deaths were attributed to lung cancer. Baetjer (1950a, 1950b) used Baltimore hospital records to evaluate the 1925–1948 lung cancer mortality rates of chromate production workers. She found that the incidence of lung cancer mortality was significantly greater among the chromate production workers than it was among the members of her control group. Bidstrup (1951) and Mancuso and Heuper (1951) also reported higher-than-expected incidences of lung cancer mortalities among chromate production workers in the English Midlands and in (Painesville) Ohio, respectively, during the second quarter of the twentieth century. Later epidemiological studies (Hayes et al., 1979; Hill and Ferguson, 1979) confirmed that the incidence of lung cancer mortalities was increased among chromate production workers and suggested the risk of lung cancer appeared to decrease as the hygienic standards of the occupational environment improved. More recently, Hayes (1988) cited the lack of specific evidence confirming that the risks of respiratory cancer among chromate production workers had been reduced from 10-fold to 3-fold greater than those for non-chromate production workers as a result of improved industrial hygiene practices. Davies et al. (1991) reported the mortality rates for chromate production workers employed after changes both in the production process and in the industrial hygiene practices failed to show excess deaths. Among the changes in the production process was the elimination of lime additions to the chromite ore. The changes in the industrial hygiene practices included improvements to the kiln and flu gas ventilation system and enclosure of the raw materials and finished product-handling systems.

Proctor et al. (2002) stated, "Studies of chromate production industry workers may provide the best evidence, because (1) very few other confounding exposures to chemical carcinogens are expected in this industry; (2) exposures to sparingly soluble calcium chromates occurred during the process, and because of low water solubility, it is expected that these particles would be better transported from the respiratory system to the gastrointestinal tract than freely soluble Cr(VI) compounds; (3) historical exposures in these plants were generally measured and known to be substantial; (4) there is clear and consistent evidence of a dose-dependent increase in lung cancer rates among these workers; (5) many studies provide data for workers with both long exposure durations and long follow-up periods . . . ." The data from the original 1930s cohort of chromate production workers at Painesville (Ohio) have been reevaluated (Proctor et al., 2003) with inclusion of 800 recently discovered measurements of airborne hexavalent chromium concentrations. These measurements allowed the reconstruction of workers exposed at this facility between 1940 and 1972 and provided the basis for an improved cancer risk assessment.

Gibb et al. (2000a) reported that 60% of a cohort of 2357 chromate production workers showed clinical signs of nasal ulceration and nasal irritation. Gibb et al. (2000b) also reported cumulative hexavalent chromium exposure showed a strong dose–response relationship for lung cancer. Clinical signs of irritation, trivalent chromium exposure, and duration of employment were not found to be associated with a risk of lung cancer when included in a proportional hazards model with cumulative hexavalent chromium exposure and smoking. Smoking status did not confound the excess risk of lung cancer associated with cumulative hexavalent chromium exposure. Whole-body exposure of rats to cigarette smoke for 18 days coupled with daily intratracheal instillations of hexavalent chromium resulted in enhanced frequency of micronuclei in bone marrow polychromatic erythrocytes (Balansky et al., 2000). Individual exposures to cigarette smoke and hexavalent chromium enhanced the frequency of micronuclei and multiple nuclei in pulmonary alveolar macrophages. The combined exposure produced less than additive clastogenic effects. These findings were interpreted as ruling out any occurrence of synergism between cigarette smoke and hexavalent chromium in the carcinogenic process. However, measurement of point mutations at critical positions of codons 12,

13, and 61 of the *Ha-ras* and *Ki-ras* oncogenes in 38 lung cancer specimens from former Japanese chromate workers demonstrated that such activations are rare events (Ewis et al., 2001). These demonstrations led to the conclusion that activation of *ras* oncogenes does not have a major role in the tumorgenic process of chromium-related lung cancer.

Subsequently, Park et al. (2003) reviewed the 122 deaths caused by lung cancer in this cohort of 2357 chromate production workers. Their analysis included airborne hexavalent chromium concentrations and pack-years of smoking among the variables. A linear relative rate model gave a good and readily interpretable fit to the data. The estimated rate ratio for 1 mg/m^3-y in cumulative exposure to hexavalent chromium as CrO_3 was 2.44 (1.39 with a lag of 5 years). The excess lifetime risk for exposure to respirable hexavalent chromium at the current Occupational Safety and Health Administration (OSHA) permissible exposure limit (PEL), 0.1 mg/m^3, is 255 (116 of 1000). The current occupational standards for exposure to hexavalent chromium permit lifetime risks of lung cancer in excess of one in a thousand. This perspective is consistent with the findings of Lurie and Wolfe (2002) who reviewed 813 measurements of hexavalent chromium exposure made in the course of OSHA inspections conducted between 1990 and 2000. They found that the number of measurements actually made declined from 127 in 1990 to 67 in 2000, that neither the median time-weighted average (TWA) nor the median ceiling exposure declined during the study period, and that more than a fifth of the measurements exceeded the OSHA PEL. From their study, they concluded, "U.S. workers continue to be exposed to dangerously high hexavalent chromium levels, but . . ." However, Matos et al. (2000) observed only a small, nonsignificant increased risk of lung cancer after long-term exposure to chromium among 199 occupationally exposed men compared with 393 control subjects in Buenos Aires. The odds ratio for workers in the chemical and plastic manufacturing industries was 1.9 compared with 4.8 for workers in saw mills and wood mills.

An excess of lung cancer deaths has also been reported for workers in the chromium pigments industries. In addition to Newman's (1890) report of more than a hundred years ago, Langard and Norseth (1975) reported three cases of bronchial carcinoma among 24 workers at a small Norwegian factory that produced lead and zinc chromate pigments in 1975. On the basis of Norwegian cancer registry data, only 0.079 cases of lung cancer should have been observed in this group of workers. Davies (1979) reported 18 lung cancer deaths among 175 workers hired at an English zinc chromate–lead chromate pigment plant from 1932 to 1954. The expected incidence of lung cancer for 175 Englishmen was 8.17 during this period. No lung cancers were observed among 62 workers hired after 1955 when the plant underwent extensive renovations to improve the industrial hygiene conditions. The expected cancer incidence among these 62 workers was 1.14. As was suggested in the case of the chromate production workers, there may have been a relationship between the incidences of lung cancer and the efficiencies of the industrial hygiene practices.

Commercial-scale electroplating of chromium has been a British industry since 1925. The plating baths typically contained from 15 to 45% CrO_3 and a few percent sulfuric acid. During the plating process, both hydrogen ions and dichromate ions were reduced to the elemental states. The bursting of the hydrogen gas bubbles generated a chromic acid spray at the surface of the plating bath. The addition of fluorocarbon or organosilicon surfactants to the plating baths and/or improvements to the ventilation systems has been successful in reducing the potential inhalation hazards of this spray.

In 1975, Royle (1975) reported on a retrospective study of mortality among 1238 chromium platers compared with 1284 gender- and age-matched controls employed in and residing in the West Riding of Yorkshire between February 20, 1969 and May 31, 1972. Some of the results of his study are summarized in (Table 23.2). Subsequent studies by Silverstein et al. (1981), Franchini et al. (1983), and Sorahan et al. (1987) also indicated somewhat increased incidences of cancer death among chromium electroplaters. Subsequently, Sorahan and Harrington (2000) compared the mortality from lung cancer in 1163 Yorkshire chrome platers with that in gender- and age-matched industrial workers whose histories showed no occupational exposures to chromium. Although Sorahan and Harrington (2000) reported that confident interpretation was not possible, it appeared

Table 23.2 Chromium Carcinogenicity to Electroplaters

	Electroplaters	Controls
Number of subjects	1238	1284
Total number of deaths	109	85
Cardiovascular deaths	52	51
Total cancer deaths	39	21
Lung cancer deaths	17	10

that occupational exposure to hexavalent chromium might well be involved in the increased mortality from lung cancer found in this cohort of chrome platers. Actual mortality from cancer of the lung and bronchus was 60 in this cohort of chrome platers. The expected mortality was 32.5. The corresponding actual and expected mortalities for the control workers were 47 and 36.9, respectively.

Trivalent and hexavalent chromium compounds have been identified in the complex mixture of particles, fumes, and chemicals resulting from the manufacture of ferrochrome alloys and stainless steels (Koponen et al., 1981; Roberts, 1965a, 1965b). Langard (1980) reported on the incidences of lung cancer for workers in a Norwegian ferrochrome factory where the workplace air contained from 0.01 to 1.34 mg Cr/m^3 of which from 11 to 33% was in the hexavalent form in some places. Of 325 workers hired before 1960, seven died of lung cancer between 1928 and 1977. The expected national and regional incidences of lung cancer were 3.1 and 1.8, respectively, during this time. Axelsson et al. (1980), on the other hand, reported no excess lung cancer deaths among 1876 Swedish ferrochrome workers from 1930 to 1975. Airborne chromium concentrations in the Swedish factory were estimated to be from 0 to 2.5 mg/m^3 of which as much as 10% could have been in the hexavalent form. Moulin et al. (1990) studied mortality among 2269 workers employed for at least one year between January 1, 1952 and December 31, 1982 at a French factory producing ferrochrome and stainless steel. This factory was opened in 1952, and its production of ferrochromium continued until 1982. The production of stainless steel, which began in 1958 at this factory, continued to the present. For the period from 1952 to 1982, the mortality study demonstrated "... a statistically significant excess of deaths from lung cancer among workers in the production of ferrochromium and stainless steel considered together. No dose response relationship was seen with the duration of exposure, nor with time elapsed since first exposure. The risk of lung cancer, however, seemed to be attributable to earlier exposures. An additional case-control study conducted within the cohort showed that this risk of lung cancer was related to ferrochromium producing workshops. On the other hand, risks seen for the stainless steel production workers were not statistically significant." Moulin et al. (2000) studied the mortality of workers involved in stainless steel production from 1968 to 1992. Moulin et al. (2000) reported no lung cancer excess was observed in a cohort of 4894 workers exposed to chromium.

The high temperatures associated with the welding process vaporize significant quantities of metal, which then condense in the plumes of heated air to produce complex mixtures of vapors, and particulates, the compositions and chemistries of which are highly variable. Among the parameters associated with these variabilities are (1) the composition and characteristics of the alloy being welded, (2) the voltage, current, polarity, and arc time of the welding process, and (3) the ventilation and other industrial hygiene and occupational safety practices of the workplace. Stern (1982) has reviewed these parameters.

In reviewing mortality data for a group of 234 Swedish welders, Sjogren (1980, 1985) observed five lung cancer deaths where only two were expected, but the difference was not statistically significant. Becker et al. (1985) reported indications for excess risk of lung cancer among German welders, but no definitive relationships were demonstrated. Stern (1983) suggested the slight excess

Table 23.3 Incidence of Lung Cancer among Welders

Populations	Observations
Western Washington (USA): 3247 welders between 1950 and 1973 compared with nonwelder workers[a]	Deaths after more than 20 years work activity showed 74% excess for lung cancer among the welders[a]
Germany 1221 welders in 25 factories compared with 1694 nonwelders[b]	No excess in lung cancer incidence among welders[b]
New Mexico (USA): 506 patients with lung cancer compared with 771 controls[c]	Increased odds for lung cancer among welders[c]
California (USA): welders[d]	Welders had 33% excess mortality from lung cancer[d]

[a] Beaumont and Weiss (1981).
[b] Becker et al. (1985).
[c] Lerchen et al. (1987).
[d] Singleton and Beaumont (1989).

incidences of lung cancers among all welders might reflect a high incidence of lung cancer among a subgroup of welders who worked primarily with stainless steel. In addition to describing the various welding processes, Sferlazza and Beckett (1991) reviewed the respiratory effects of acute and chronic exposures to welding fumes. Some of their citations of studies on the incidence of lung cancer among welders are summarized in Table 23.3.

In a follow-up to their earlier study in 1985, Becker et al. (1991) found an increased overall cancer mortality, but they concluded, "The risk for lung cancer was not significantly raised, but increased with duration of exposure. Mortality from lung cancer only weakly increased after the removal of the effects of smoking and a part of the increase must clearly be attributed to exposure to asbestos. Hence the increased risk of lung cancer identified in this study is smaller than in the international study." The international study they referred to involved 11,092 welders employed by 135 companies in nine European countries (Simonato et al., 1991). One of the outcomes of this international study was identification of a statistically significant excess lung cancer mortality among welders—116 observed deaths versus 86.8 expected deaths. Another outcome from the pattern of the lung cancer mortalities suggested that the risk was greater for stainless steel welders than it was for mild steel welders.

23.4 CHROMIUM IN CANCEROUS TISSUES

Chromium concentrations in the lung may have a special significance because occupational exposures to some chromium compounds in a number of industrial settings have been associated with increased risks of respiratory system cancers.

To study possible relationships between pulmonary chromium contents and lung cancers, Anttila et al. (1989) measured the concentrations of chromium "… in 53 consecutive male patients operated on (f)or lung cancer and in 43 controls, and compared with the smoking habits, grade of pulmonary emphysema and occupation." The controls were selected from cancer-free male corpses with documentable smoking habits and work histories. They found the chromium concentration in the apicoposterior segments of the upper lobes of the lungs from the cancer patients was 6.4 ± 4.3 mg/kg and that from the controls was 4.0 ± 4.0 mg/kg on a dry weight basis with the results for nonsmoker controls excluded. The concentrations of chromium in other pulmonary segments from the cancer patients did not differ significantly from those from the smoker controls. The chromium concentration in the apicoposterior segments of the upper lobes of lungs from the nonsmoker controls was 2.0 ± 2.0 mg/kg.

Akslen et al. (1990) compared the distributions of chromium in the lung tissues, bronchial tissues, and hilar lymph nodes collected from 20 cancer surgery patients with those collected from the control group which consisted of 21 cancer-free cadavers representing similar age distributions and domiciles. The concentration of chromium in lung tissues from the cancer patients was more than twice that from the controls; the median values on a dry weight basis were 3.424 mg/kg and 1.682 mg/kg, respectively. No significant differences were found between the chromium concentrations in the bronchial or in the lymphoid tissues from these two groups.

The concentrations of nine metals including chromium were determined by Aduchi et al. (1991) in 1939 autopsy specimens of lung tissue, which included 224 specimens from victims of lung cancer. The chromium concentrations in the specimens from 174 male cancer victims were greater than those in the other 1094 specimens from control males. The mean values were 4.74 ± 9.33 and 3.47 ± 5.41 mg/kg, respectively, on a dry weight basis. The difference between the mean chromium concentration for lung tissue specimens from 50 female victims and those for 621 other specimens from females was not significant. The respective values for the lung tissue specimens from the female populations were 1.93 ± 1.92 and 2.11 ± 2.42 mg/kg.

Chromium was among the nine metals Takemoto et al. (1991) determined in 2274 postmortem lung tissue specimens. The cause of death was "malignant neoplasms" in 1396 cases. For chromium, the range of concentrations found in the lung was from 0 to 731 mg/kg on a dry weight basis. The mean value was 4.46 ± 26.75 mg/kg, the mode was 0.25 mg/kg, and the median was 1.49 mg/kg. Mean chromium concentration in the lung tissue increased with the age at the time of death and with the degree of pulmonary emphysema. Others (Anttila et al., 1989; Akslen et al., 1990; Aduchi et al., 1991) also reported positive correlations between chromium concentration in the lung and severity of emphysema and/or smoking habits. The concentrations of chromium and eight other elements were determined in lung tissue of 85 deceased smelter workers. All concentrations were significantly higher when compared with rural references (Gerhardsson and Nordberg, 1993). Ishikawa et al. (1994a) measured the chromium concentrations in 50 tissue specimens from bronchial bifurcations and adjacent regions obtained at autopsy from nine chromate workers. The chromium concentrations ranged from 0.04 to 39×10^{-10} g/μm tissue thickness/mm^2. In 80% of the cases, the chromium concentrations were greater at the bifurcations than at neighboring epithelial tissue. These results demonstrate the long-term retention of chromium in the bronchi of chromate workers. Ishikawa et al. (1994b) also compared the lung chromium burden of chromate workers with tumors to those without. For the former, Ishikaea et al. (1994b) reported from 40 to 15,800 μg of Cr/g dry tissue. The range reported for the latter was from 8 to 28 μg of Cr/g dry tissue.

Vanoeteren et al. (1982) investigated the distribution patterns for 23 elements in six sets of lungs collected at autopsy. On a wet weight basis, the mean chromium concentrations for the six sets of lungs were 0.255, 0.032, 0.128, 0.173, 0.062, and 0.087 mg/kg, but the distributions of chromium within the lungs were highly heterogeneous. Within a given lung, point-to-point chromium concentrations differed by a factor of 21/2. These differences were attributed to the presence of localizations of inhaled airborne particulates. With Christian and Feldman's (1970) value of 78.7 as the water content of adult human lung tissue, the chromium concentrations determined by Vanoeteren et al. (1982) correspond to the approximate range of 0.15 to 1.06 mg/kg dry mass. Koyama (1995) reported chromium (and nickel) concentrations were significantly higher in lung tissue specimens from 255 cancer patients than in those from 68 noncancer controls. Most of the specimens showing elevated chromium (or nickel) came from men who worked in heavy industry and had been diagnosed as having squamous cell carcinoma. Tsuchiyama et al. (1997) reported chromium concentrations from 0.41 to 12.7 μg/g (dry mass) in specimens of lung from autopsied urban dwellers. The concentrations of chromium were higher in the lungs of men than in women, of smokers than in nonsmokers, and in those with occupational exposures than in the unexposed. In his two-part review of chromium as an industrial carcinogen, Mancuso (1997a, 1997b) cited analytical data for chromium in the tissues of three chromate production workers who died of lung cancer. The results for the lungs are summarized in Table 23.4.

Table 23.4 Chromium Concentrations in the Lungs of Lung Cancer Victims

	Chromiumπ (μg/10 g)		
Tissue	**Case 1**	**Case 2**	**Case 3**
Left lung, left side, upper lobe	380 ± 10	30 ± 2	
Right lung, upper lobe	—	—	1480
Left lung, right side, upper lobe	456 ± 10	84 ± 9	—
Left lung, lower lobe	330 ± 10	87 ± 9	—
Right lung, lower lobe	—	—	1920
Tumor in left lung	26 ± 0.2	178 ± 9	—
Tumor in right lung	—	—	865

Table 23.5 Chromium Concentrations in Welders' Urine, Blood, and Semen

	Concentrations		
Subjects[a]	**Urine (nmol Cr/mmol creatinine)**	**Blood (nmol/l)**	**Semen (nmol/l)[b]**
Nonwelders	0.72 ± 0.54	8.47 ± 3.55	22
Mild steel welders	1.33 ± 0.53	14.5 ± 10.5	22
Stainless steel welders			
TIG	2.07 ± 1.3	17.3 ± 10.4	18
MMA/MAG	1.38 ± 1.12	17.3 ± 11.9	260

[a] TIG, tungsten inert gas; MMA, manual metal arc; MAG, metal active gas.
[b] Median.

23.5 INDUSTRIAL HYGIENE

Italian chromium chemical workers exposed to airborne hexavalent chromium concentrations as high as 200 μg/m^3 were reported to have elevated urinary chromium levels, and the chromium concentrations in the urines of workers exposed to (presumably airborne) chromates in two Indian paint factories were found to be 10-fold higher than those of the control population (International Agency for Research on Cancer [IARC], 1990). The inhalation of mists, fumes, and/or particles containing chromium is probably the major contributor to chromium exposure in the industrial/occupational setting. Oláh and Tölgyessy (1985) reported high concentrations of chromium in the aerosols produced during electric arc welding, and Iqbal et al. (1993) cited airborne chromium concentrations of 15,645 μg/m^3 in the fumes from mild steel welding electrodes and from 54 to 1158 μg/m^3 in fumes during stainless steel welding. Bonde and Christensen (1991) measured chromium concentrations in urine, whole blood, and seminal fluid collected from stainless steel welders using either the tungsten inert gas (TIG) or the manual metal arc (MMA) and the metal active gas (MAG) methods, from mild steel welders, and from metal workers who had never been welders. The median TWA exposure to chromium in the welding fume was 11 ± 11 μg/m^3 for the stainless steel welders using the TIG method. For the mild steel welders, the median TWA exposure was 3 ± 8 μg/m^3. In both cases, approximately one third of the airborne chromium was found to be in the hexavalent state. The results of these measurements are summarized in Table 23.5.

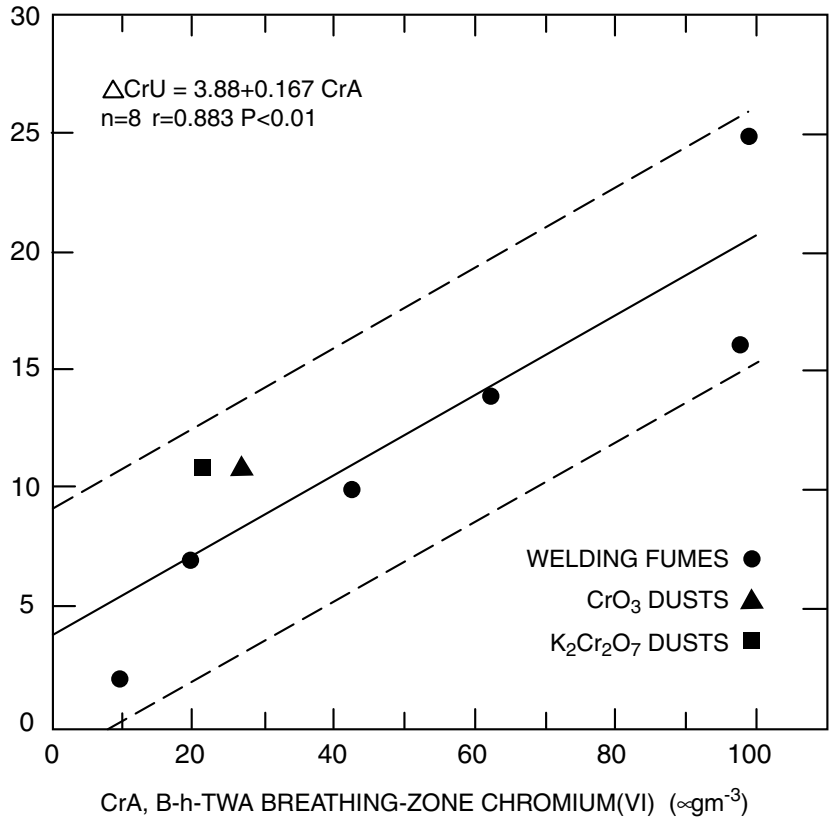


FIGURE 23.1 Urinary chromium–airborne chromium regressions.

23.6 ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Elevated urinary chromium concentrations have also been reported for electroplaters and welders exposed to airborne hexavalent chromium (Källiomäki et al., 1981; Sjogren, et al., 1983; Lindberg and Vesterberg, 1983; Welinder et al., 1983). Mutti et al. (1985) correlated differences between start-of-shift and end-of-shift urinary chromium levels (ΔCrU) with the concentrations of soluble hexavalent chromium in the workplace atmosphere (CrA). The correlation coefficient was 0.883 with $p < 0.01$. No correlations between ΔCrU and the concentrations of insoluble hexavalent chromium or of trivalent chromium in the air were found. The regressions for the ΔCrU-CrA data are presented in Figure 23.1. There is ample evidence for the absorption of soluble hexavalent chromium from the respiratory tract in the human. Biomonitoring of occupationally exposed workers has been used successfully to assess high-level inhalation exposures in the workplace, but monitoring low-level environmental exposures by measuring urinary chromium concentrations has been problematic (Paustenbach et al., 1997).

The analysis of autopsy/necropsy specimens as well as animal experiments have been used to demonstrate the absorption, distribution, metabolism, and elimination of chromium compounds.

Bragt and van Dura (1983) reported biphasic blood clearance patterns and biphasic whole-body elimination patterns of ^{51}Cr in rats that received intratracheal doses of either $Na_2{}^{51}CrO_4$, $Zn^{51}CrO_4$, or $Pb^{51}CrO_4$. The half-times for the fast-phase and slow-phase eliminations of chromium from sodium chromate were 5.0 and 71.1 d, respectively. For zinc chromate, the results were 2.4 and 30.9 d, respectively. For lead chromate, the corresponding half-times were 1.9 and 94.8 d. The half-times for

Table 23.6 Chromium Concentrations in Rat Tissues 50 d Postinstillation with $Na_2{}^{51}CrO_4$, $Zn^{51}CrO_4$, or $Pb^{51}CrO_4$

Tissue	Chromium Content (ng/g wet tissue)		
	$Na_2{}^{51}CrO_4$	$Zn^{51}CrO_4$	$Pb^{51}CrO_4$
Lung	1664 ± 556	2060 ± 543	4231 ± 2654
Spleen	385 ± 91	717 ± 217	19 ± 3
Kidneys	69 ± 18	68 ± 16	9 ± 4
Liver	20 ± 1	19 ± 3	2 ± 1
Blood	15 ± 1	112 ± 20	4 ± 1

fast-phase blood clearance were 4.6, 6.3, and 2.4 d for the rats that received the $Na_2{}^{51}CrO_4$, $Zn^{51}CrO_4$, and the $Pb^{51}CrO_4$, respectively. The numerical analysis of the whole-body chromium elimination was performed by assuming a two-compartment open kinetic model. The biphasic clearance and elimination patterns suggest the existence of slow-releasing storage compartments. Evaluation of chromium distribution to and from the compartments can be complicated by the possibility of different transport mechanisms for hexavalent and trivalent chromium and by the potential for reduction of the former to the latter. The ^{51}Cr tissue concentrations at 50 d postinstillation are summarized in Table 23.6. These data reflect both retention of chromium at the site of instillation and a relation between this retention of chromium and solubility of the compound instilled. If it is assumed that there is a common product from the biological reduction of hexavalent chromium, this latter aspect is important in that it may suggest the long-term stability of hexavalent chromium *in vivo.* The question of how and why more chromium was retained in the lungs of rats instilled with lead chromate than was retained in the lungs of the rats instilled with more soluble chromium compounds warrants further consideration. In these experiments, the blood, spleen, liver, and kidney were among the compartments involved in the metabolism and excretion of chromium absorbed from the lung.

Salem and Katz (1989) reported on the distribution of chromium in the rat after the intratracheal instillation of Whetlerite dust, a material containing both hexavalent and trivalent chromium. Their results indicated that the blood, spleen, liver, and kidneys are involved in the metabolism and elimination of chromium absorbed from the lung. Hilaski et al. (1992) did not observe elevations of the chromium concentrations in the livers and kidneys of rats 14, 28, and 180 d after 4-h inhalation exposures to atmospheres containing 5 mg/l of 3-μm Whetlerite dust particles. Reasons for this finding could include: (1) absorption, metabolism, and elimination of the inhaled chromium compounds at rates too rapid to leave significant chromium residuals in the organs on which the measurements were made beginning 2 weeks postexposure, and (2) residual chromium concentrations at or below the detection limits of the atomic absorption spectrometric techniques they used.

Wiegand et al. (1987) studied the distribution and metabolism both of hexavalent and of trivalent chromium absorbed from the lungs of rabbits after the intratracheal instillation of either $^{51}CrCl_3$ or $Na_2{}^{51}CrO_4$. The ^{51}Cr in blood samples from rabbits treated with trivalent chromium showed maximum concentrations 20 min postinstillation, and radioactivity was found to be "… exclusively confined to the plasma compartment." Four hours postinstillation, 85% of the radioactivity associated with the $^{51}CrCl_3$ dose was retained in the lungs, and 8% was excreted in the urine. By contrast, most of the ^{51}Cr radioactivity in the blood of the rabbits intratracheally instilled with $Na_2{}^{51}CrO_4$ was found to be in the red blood cells, and the percentages of the intratracheally instilled dose of $Na_2{}^{51}CrO_4$ retained in the lungs and excreted in the urine were 47 and 15.5%, respectively. The finding that most of the ^{51}Cr radioactivity in the blood of the rabbits intratracheally instilled with the $Na_2{}^{51}CrO_4$ indicates that chromium from the lungs entered the blood in the hexavalent form. This is suggestive of long-term, *in vivo* stability of hexavalent chromium. The balance of the $Na_2{}^{51}CrO_4$ dose was

Table 23.7 Distribution of ^{51}Cr in the Rabbit from the Lung 4-h Postinstillation of 0.5 mg Dose of $Na_2{}^{51}CrO_4$

Compartment	% Dose
Lung retention	47
Urinary excretion	15.5
Blood	6
Liver	11
Kidneys	13

distributed in the livers, kidneys, and other tissues as shown in Table 23.7. O'Flaherty (1993, 1996) developed a pharmacokinetic model for chromium compounds. In this model, she considered that the reduction rate for hexavalent chromium in the lung was different from that in the gastrointestinal tract, and she considered the differences in absorption, transport, and distribution for the trivalent and the hexavalent chromium. Her model generated "... the observed distributions of chromium between plasma and erythrocytes in rats given Cr(VI) intragastrically, intraduedenally, and intratracheally."

23.7 ANIMAL STUDIES OF CARCINOGENICITY

To complement her (Baetjer, 1950a, 1950b) earlier epidemiological findings of increased incidences of lung cancer among chromate production workers, Baetjer et al. (1959) conducted inhalation experiments in which 125 mice were exposed in chambers to the airborne dust from a chromate production factory. This dust contained both trivalent (4.7%) and hexavalent (7.1%) chromium compounds and the oxides of aluminum, calcium, iron, and magnesium. The mice were exposed to the airborne dust at concentrations of 1–2 mg/m^3 for 40 weeks, 5 d per week, 4 h/d. Baetjer et al. (1959) reported no increased incidence of lung cancer in the exposed rats relative to the control rats during the following 60 weeks. Subsequently, a large number of animal studies were conducted to evaluate the carcinogenicity of chromium and its compounds. In these studies, many chemical forms of chromium were administered by various routes to a variety of laboratory animals. These experiments and their results are summarized in Table 23.8. The tabulated data are partially responsible for the indictment of $CaCrO_4$, $SrCrO_4$, and $PbCrO_4$ as carcinogens. Aitio and Tomales (1991) have also tabulated the numbers of tumors produced in experimental animals after exposures to chromium compounds by intrabronchial implantations (INB). Their tabulation reflects high incidences of pulmonary carcinogenic responses to $CaCrO_4$ (25 of 100) and to $SrCrO_4$ (105 of 199). Their tabulated responses to some trivalent chromium compounds were 0 of 100 for $CrCl_3{\cdot}6H_2O$, 0 of 100 for Cr_2O_3, and 0 of 100 for $Cr(OH)(SO_4){\cdot}xH_2O$. These data have led to a generalization ascribing carcinogenic properties to those hexavalent chromium compounds having moderate or limited solubilities in water.

Tadao et al. (1999) reported the production of granulomas in the entire airways with progressive fibrotic changes as well as mobilization and destructive changes of macrophages and foamy cells in rats following intratrachael instillation of welding fumes containing both hexavalent and trivalent chromium. After a 13-week, nose-only inhalation study with two compounds of trivalent chromium, Cr_2O_3 and $Cr(OH)(SO_4)$, Dereanko et al. (1999) observed that the principle effects of both materials were primarily to the respiratory tract. Cr_2O_3 caused pathological changes in the bronchial and mediastinal lymphatic tissue and lungs consisting of the presence of pigment-laden macrophages, lymphoid and septal hyperplasia, and interstitial inflammation similar to that observed with other inert dusts. $Cr(OH)(SO_4)$ produced more severe and widespread effects in the nasal cavity, larynx, lungs, and mediastinal lymph nodes. The accumulation of foreign material, infiltration of alveolar

Table 23.8 Carcinogenicity of Chromium Compounds to Laboratory Animals

Chemical and Dose	Number of Animals	Route[a]	Cancer Incidence[b]
Factory dust, 1–2 mg/m^3	125 mice	INH	Same as control[c]
Factory dust	75 rats	INH	Same as control[d]
$CaCrO_4$, 13 mg/m^3	136 mice	INH	14/372 vs. 5/371[e]
$Na_2Cr_2O_7$, 100 μg/m^3	20 rats	INH	3/19 vs. 0/37[f]
CrO_3, 7 mg/m^3	50 mice	INH	1/19 vs. 2/10[g]
CrO_3, 3.5 mg/m^3	43 mice	INH	1/20 vs. 0/20[h]
CrO_2, 25 mg/m^3	500 rats	INH	2/214 vs. 0/212[i]
Production residue, 4 mg	100 rats	INB	1/93 vs. 0/24[j]
Production residue, 2 mg	100 rats	INB	1/99 vs. 0/100[k]
$SrCrO_4$, 2 mg	200 rats	INB	107/198 vs. 0/100[k]
$BaCrO_4$, 2 mg	100 rats	INB	0/101 vs.0/100[k]
$PbCrO_4$, 2 mg	100 rats	INB	1/98 vs. 0/100[k]
$CaCrO_4$, 4 mg	100 rats	INB	8/100 vs. 0/24[j]
$CaCrO_4$, 2 mg	100 rats	INB	25/100 vs. 0/100[i]
CrO_3 2, mg	100 rats	INB	2/100 vs. 0/100[i]
Na_2CrO_4, 2 mg	100 rats	INB	0/89 vs. 0/100[i]
$Na_2Cr_2O_7$, 2 mg	100 rats	INB	1/100 vs. 0/100[l]
Chromite roast, 2 mg	100 rats	INB	0/100 vs. 0/100[l]
Cr_2O_3, 2 mg	100 rats	INB	0/100 vs. 0/100[m]
Cr_2O_3, 4 mg	98 rats	INB	0/98 vs. 0/24[j]
Roasted ore, 25 mg	35 rats	INP	3/35 vs. 0/35[m]
Roasted ore, 25 mg	25 rats	INP	2/24 vs. 0/25[n]
$SrCrO_4$	35 rats	INP	17/28 vs. 0/34[o]
$BaCrO_4$	35 rats	INP	1/31 vs. 0/34[o]
$PbCrO_4$	35 rats	INP	3/34 vs. 0/34[o]
$Na_2Cr_2O_7$	35 rats	INP	0/26 vs. 0/34[o]
$Cr(CH_3COO)_3$	35 rats	INP	1/34 vs. 0/34[o]
$CaCrO_4$, 50 μg	80 rats	INT	6/80 vs. 0/80[p]
$CaCrO_4$, 250 μg	80 rats	INT	13/80 vs. 0/80[p]
$Na_2Cr_2O_7$, 50 μg	80 rats	INT	1/80 vs. 0/80[p]
$Na_2Cr_2O_7$, 250 μg	80 rats	INT	14/80 vs. 0/80[p]
Roasted ore, 25 mg	35 rats	IM	1/34 vs. 0/32[o]
$SrCrO_4$, 25 mg	35 rats	IM	15/33 vs. 0/32[o]
$BaCrO_4$, 25 mg	35 rats	IM	0/34 vs. 0/32[o]
$PbCrO_4$, 25 mg	35 rats	IM	1/33 vs. 0/32[o]
$Na_2Cr_2O_7$, 25 mg	35 rats	IM	0/33 vs. 0/32[o]
$CaCrO_4$, 25 mg	35 rats	IM	9/32 vs. 0/32[o]
$Cr(CH_3COO)_3$, 25 mg	35 rats	IM	1/34 vs. 0/32[o]

Table 23.8 Carcinogenicity of Chromium Compounds to Laboratory Animals (Continued)

$PbCrO_4$, 30 mg	40 rats	SC	26/40 vs. 0/60[q]
$CaCrO_4$ 10 mg	52 mice	SC	0/31 vs. 0/52[r]

[a] INH, inhalation; INB, intrabronchial implantation; INT, intrapleural administration; INT, intratracheal instillation; IM, intramuscular injection; SC, subcutaneous injection.

[b] Experimental vs. control.

[c] Beatjer et al. (1959).

[d] Steffee and Beatjer (1965).

[e] Nettesheim et al. (1971).

[f] Glaser et al. (1986).

[g] Aduchi et al. (1986a).

[h] Aduchi et al. (1986b).

[i] Lee et al. (1988).

[j] Laskin et al. (1970).

[k] Levy et al. (1986).

[l] Levy and Venett (1986).

[m] Payne (1960a).

[n] Hueper (1958).

[o] Hueper (1961).

[p] Steinhoff et al. (1986).

[q] Maltoni et al. (1981).

[r] Payne (1960b).

macrophages, septal cell hyperplasia, and granulomatous and chronic inflammation characterized these effects.

The short-term treatment of rats by intratracheal administration of 100 μl of $Na_2Cr_2O_7 \cdot 2H_2O$ solution of a concentration corresponding to 0.25 mg/kg triggered a variety of defense processes in the lung. The expression of 56 of 216 genes tested was increased two to three times in the lungs as an early response to hexavalent chromium administration. The altered genes are involved in the metabolic reduction of hexavalent chromium and in a variety of functions such as multidrug resistance and stress response, protein and DNA repair mechanisms, apoptosis, and cell cycle modulation (Izzotti et al., 2002). Intratracheal administration of $Na_2Cr_2O_7 \cdot 2H_2O$ solution corresponding to a dose of 0.25 mg/kg in the rat caused increased apoptotic indices for both bronchial epithelium and lung parenchyma. The expression of 13 of 18 apoptosis-related genes evaluated by cDNA array analysis was enhanced. These observations led to the proposal that apoptosis may provide a protective mechanism at the postgenotoxic stage of hexavalent chromium carcinogenesis (D'Agostini et al., 2002).

23.8 BIOCHEMISTRY OF CHROMIUM CARCINOGENICITY

About 50 years ago, Grogan and Oppenheimer (1955) sought to clarify the role of "... form, valency, and solubility of such a chromium containing etiologic factor, ..." as it related to "... the facts that

workers employed in the handling and processing of chromite ore, chromates and chromium pigments have a highly excessive liability to cancer of the lung; . . . ." Observing that hexavalent chromium compounds were more mutagenic and carcinogenic than were those of trivalent chromium, Kortenkamp et al. (1991) investigated the binding of chromium from solutions of chromium (III) glutathione complex and from solutions of sodium chromate either in the presence or absence of reduced glutathione by calf thymus cell nuclei. They found that the greatest binding took place from the solutions containing sodium chromate and reduced glutathione, and they concluded that the reduction of chromate is a prerequisite to the formation of chromium adducts with nuclear constituents. The involvement of glutathione in the reduction of hexavalent chromium may offer an alternative explanation to Valentine's (1964) observation of glutathione reductase inhibition; the loss of substrate by oxidation could explain the observations. In addition to glutathione (GSH), ascorbic acid (AsA) has been associated with the intra- and intercellular reduction of hexavalent chromium (Suzuki and Fukuda, 1990; Suzuki, 1990; Lewalter and Korallus, 1989). Wetterhahn's (Wetterhahn and Hamilton, 1989) uptake-reduction model (Figure 23.2) for the carcinogenicity of hexavalent chromium considered glutathione among the cellular components involved in the formation of reactive intermediates. The reduction-uptake model postulated the following: (1) hexavalent chromium penetrated cells more readily than did trivalent chromium, (2) hexavalent chromium was reduced to trivalent chromium intracellularly by various enzymatic and nonenzymatic factors, and (3) reactive intermediates generated during the reduction rather than the hexa- or the trivalent chromium alone caused DNA damage that resulted in carcinogenic transformations of the cells (Stabdever and Wetterhahn, 1991). The hydroxyl radical and pentavalent chromium complexes have been identified by electron paramagnetic resonance spectroscopy as reactive intermediates in the reductions of hexavalent chromium with hydrogen peroxide and with glutathione, respectively, and these reactive intermediates have been proposed as determinant factors in the carcinogenic transformation of cells (Aiyer et al., 1991). Suzuki et al. (1992) isolated, purified, and characterized a Cr(VI) reductase. This enzyme reduced hexavalent chromium to the trivalent state via a pentavalent intermediate with NADH in a 1:3 ratio. The relationship of the pentavalent intermediate to the reactive intermediates formed in the nonenzymatic reduction of hexavalent chromium was not established. Shainken–Kestenbaum et al. (1991) proposed that oxygen free radicals (OFR) were responsible for carcinogenic transformations, and they attributed the carcinogenicity of hexavalent chromium to its inhibition of superoxide dismutase. Popper and Woldrich (1991) also proposed that the formation of oxygen free radicals was involved in the mechanism of chromium toxicity. Popper et al. (1993) subsequently suggested the possibility of an unidentified second pathway of chromium toxicity that did not involve oxygen radicals. Sugiyama (1992) suggested that "… physiological antioxidants may play an important role in the induction of biological effects of chromium (VI) compounds, presumably by effecting the generation of chromium (V) or (III) and active oxygen radicals during reduction of chromium (VI)." Shi and Dalal (1990a, 1990b) proposed hydroxyl radicals as the key species in the mechanism of hexavalent chromium toxicity, and they demonstrated the production of hydroxyl radicals from hydrogen peroxide and the pentavalent chromium complexes formed when hexavalent chromium was reduced by cellular reductants such as fructose, glucose, ribose, GSH, AsA, or nicotinamide adenine dinucleotide phosphate (NADPH).

In human and other mammalian systems, the primary transport of chromium is in the blood as complexes of the trivalent form with a variety of "biocarriers." Transferrin (siderophilin) has been identified as the primary serum protein carrier for trivalent chromium. Hexavalent chromium that escapes reduction in the plasma is able to penetrate erythrocytes (RBC) where subsequent reduction, binding, and transport may take place. Agents such as the reduced forms of GSH, triphosphopyridine nucleotide (TPNH), diphosphopyridine nucleotide (DPNH), NADPH, and nicotinamide adenine dinucleotide (NADH), and AsA have been identified as active in the reduction of hexavalent chromium to the trivalent form. De Flora et al. (1997) reported quantitative estimates of hexavalent chromium reduction in several human body compartments. Some of their values for the adult human male are tabulated in Table 23.9. Dillon et al. (1997) demonstrated the reduction of hexavalent and pentavalent chromium complexes to the trivalent state by V79 Chinese hamster lung cells.

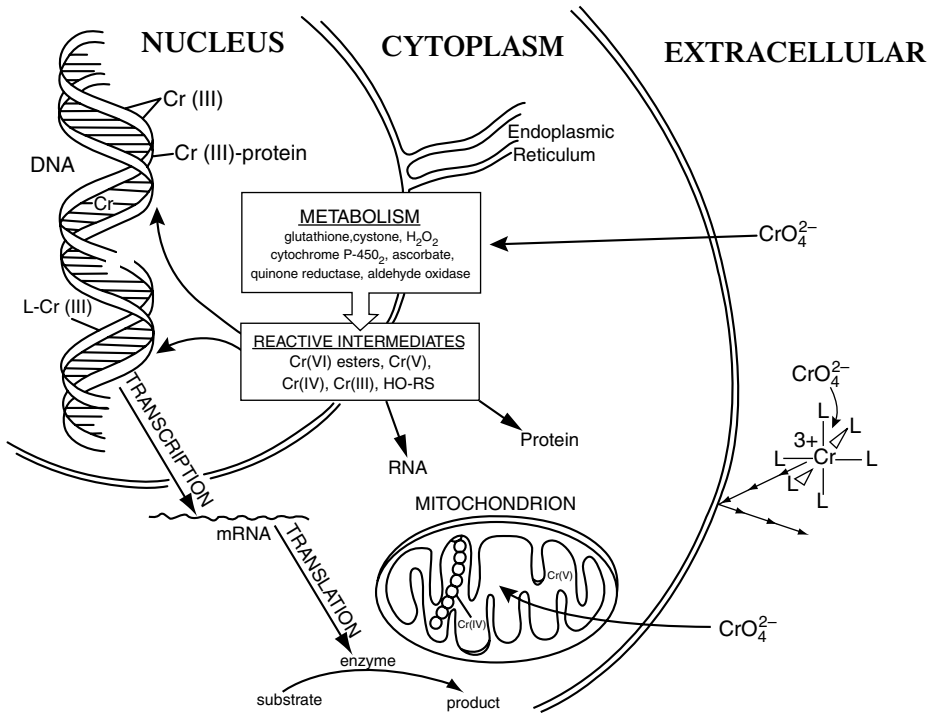


FIGURE 23.2 Reduction-uptake model.

Table 23.9 Estimated Hexavalent Chromium-Reducing Capacities in Some Human Body Compartments

Compartment	Hexavalent Chromium-Reducing Capacity
Saliva	0.7–2.1 mg/day
Gastric juice	>84–88 mg/day
Intestinal bacteria	11–24 mg daily fecal elimination
Liver	3300 mg
Whole blood	234 mg
Red blood cells	128 mg
Epithelial lining fluid	0.9–1.8 mg
Pulmonary alveolar macrophage	136 mg
Peripheral lung parenchyma	260 mg

In addition to studying the distribution of intratracheally instilled chromium as a function of its oxidation state, Wiegand et al. (1987) investigated the reduction of hexavalent chromium by GSH *in vitro*. Continuous measurements on the 370-nm absorbance of 100 μ*M* chromate at 37°C in pH 7.4 media containing either 100- or 1000-fold molar excesses of GSH showed two consecutive first-order processes. The half-times for these two processes were 0.7 and 12 min with 100-fold molar excess of GSH, and they were 0.15 and 1.1 min with 1000-fold excess of GSH. The stoichiometry of reaction for chromate reduction by GSH *in vitro* was determined to be 1 to 3 from measurements of the GSH concentrations in the reaction systems. Obviously, no enzymes are involved in this *in vitro* reduction of hexavalent chromium.

Table 23.10 Distribution of Chromium in the Rat from the Lungs after Intratracheal Administration of 1.2 μmol Na_2CrO_4

Postadministration (min)	Lungs (μmol)	Organs[a] (μmol)	Blood (mmol)
4	1.13	0.105	0.0072
10	1.07	0.130	0.0069
18	0.95	0.132	0.0044

[a] Total chromium recovered from liver, kidney, and spleen.

Suzuki and Fukuda (1990) conducted *in vitro* and *in vivo* experiments on the reduction of hexavalent chromium by GSH and AsA in the lung of the rat. Like Wiegand et al. (1987) they (Suzuki and Fukuda, 1990) found the *in vitro* reduction of hexavalent chromium by GSH took place by a two-phase process. Suzuki and Fukuda (1990) also found the *in vitro* redox reaction between AsA and sodium chromate was pseudo first order with respect to the hexavalent chromium concentration. In addition, Suzuki and Fukuda (1990) reported on a markedly pH-dependent *in vitro* reduction of hexavalent chromium with rat bronchoalveolar lavage fluid. The *in vivo* reduction of hexavalent chromium was evaluated from measurements on AsA, GSH, Cr (IV), and total chromium in the lung, liver, kidney, spleen, and blood of rats 4, 10, and 18 min after intratracheal administration of 1.2 μmol of sodium chromate in 0.6 ml of pH 7.4 saline. The biological half-time for "fast-phase" reduction of hexavalent chromium in the rat lung was found to be about 2 min, but 20% of the dose remained unreduced in the rat lung after 18 min. Most of the hexavalent chromium was quickly reduced. Only small amounts of chromium were released from the lungs. The amounts of chromium deposited in the kidney, liver, and spleen were only 10% of the dose administered to the lungs. One of the conclusions drawn from these observations proposed that extracellular AsA in the alveolar lining played an important role in the antioxidant defense against inhaled hexavalent chromium compounds. The distribution of chromium from the lungs to the blood and organs of the rat are summarized in Table 23.10.

In further studies on the reduction of hexavalent chromium by GSH and AsA at 37°C and pH 7.4, Suzuki (1990) observed a synergistic effect that was more conspicuous in systems containing 2 m*M* GSH and from 0.02 to 0.2 m*M* AsA. The apparent importance of AsA to the *in vivo* reduction of hexavalent chromium prompted Suzuki (1990) to suggest, "Sufficient intake of this vitamin would ensure the health maintenance of workers facing the risk of chromium (VI) inhalation." More than a quarter century ago, Samitz et al. (1965, 1968) recommended the use of AsA for protection against the inhalation of chromic acid mist, and they proposed a mechanism for the inactivation of hexavalent chromium by AsA in which the products of the *in vitro* reduction were a polynuclear Cr(III)-AsA complex and dehydroascorbic acid. From their considerations of intoxication by hexavalent chromium in welding fume and chromate dust, Lewalter and Korallus (1989) supported high doses of AsA as the therapeutic choice. GSH was not recommended because of its short biological half-life in plasma and because "… its capacity of intermediary Cr(VI)-stabilization a supportive therapy by GHS is even contraindicated."

Dayan and Paine (2001) reviewed the literature on the carcinogenicity of chromium compounds published between 1985 and 2000. According to their considerations of the pathogenic mechanisms involved, hexavalent chromium appears to be involved in the toxic mechanisms, but the underlying molecular damage may be due to the short-lived, highly reactive pentavalent species formed during its intercellular reduction. Point mutations in DNA and chromosomal damage and oxidative changes in protein and adduct formation are possible biochemical consequences of exposure to hexavalent chromium. The importance of these reaction products and the free radicals

they may generate in the formation of malignant tumors remains to be demonstrated. Dayan and Paine (2001) are of the opinion that the biochemical studies on the pathogenesis of carcinogenic properties of hexavalent chromium have not kept up with advances in understanding the molecular basis for other carcinogens.

23.9 SUMMARY AND CONCLUSIONS

The primary route of occupational exposure to chromium is by inhalation. Absorption from the lungs is indicated by elevated concentrations of chromium in urine, serum, and tissues of humans occupationally exposed to trivalent and hexavalent compounds in air. The absorption of inhaled chromium compounds is influenced by biological, chemical, and physical properties such as alveolar macrophage activity, oxidation state, solubility, and particle size. Results from the chemical analysis of autopsy specimens show the hilar lymph nodes, lungs, spleens, livers, kidneys, and hearts from workers exposed to chromium have elevated chromium concentrations when compared with unexposed controls. Lung chromium concentrations increase with age.

An increased incidence of lung cancer has been observed among workers both in the chromate-producing industry and in the manufacture of chromate pigments. Evidence of a similar risk exists among chrome platers, chromium alloy workers, and welders of stainless steel. Current occupational standards for hexavalent chromium exposure permit risks of lung cancer in excess of that usually considered acceptable, less than one in a thousand.

The numbers of tumors produced in experimental animals after exposures to chromium compounds reflect high incidences of pulmonary carcinogenic responses to $CaCrO_4$ (25 of 100) and to $SrCrO_4$ (105 of 199). The responses to some trivalent chromium compounds were 0 of 100 for $CrCl_3 \cdot 6H_2O$, 0 of 100 for Cr_2O_3, and 0 of 100 for $Cr(OH)(SO_4) \cdot xH_2O$. These data have led to a generalization ascribing carcinogenic properties to those hexavalent chromium compounds having moderate or limited solubilities in water.

The reduction-uptake model postulates the following: (1) hexavalent chromium penetrates cells more readily than does trivalent chromium, (2) hexavalent chromium is reduced to trivalent chromium intracellularly by various enzymatic and nonenzymatic factors, and (3) reactive intermediates generated during the reduction rather than the hexa- or the trivalent chromium alone cause DNA damage that results in carcinogenic transformations of the cells.

REFERENCES

Aduchi, S., Effect of chromium compounds on the respiratory system. Part 5. Long term inhalation of chromic acid mist in electroplating by C57BL female mice and recapitulation of our experimental data, *Jpn. J. Ind. Health*, 29, 17–33, 1987.

Aduchi, S., Takemoto, K., Ohshima, S., Shimizu, Y., and Takahama, M., Metal concentrations in lung tissues of subjects suffering from lung cancer, *Int. Arch. Occup. Environ. Health*, 63, 193–197, 1991.

Aduchi, S., Yoshimura, H., Katayama, H., and Takemoto, K., Effect of chromium compounds on the respiratory system. Part 4. Long term inhalation of chromic acid mist in electroplating to ICR female mice, *Jpn. J. Ind. Health*, 28, 283–287, 1986.

Aioto, A. and Tomatis, L., On the carcinogenicity of nickel and chromium and their compounds, in *Trace Elements in Health and Disease,* Aitio, A., Aro, A., Järvisalo, J., and Vainio, H., Eds., Royal Chemistry Society, London, 1991, pp. 159–168.

Aiyar, J., Berkovits, J., Floyd, R.A., and Wetterhahn, K.A., Reaction of chromium (VI) with glutathione or with hydrogen peroxide: Identification of reactive intermediates and their role in chromium (VI)-induced DNA damage, *Environ. Health Perspect*., 92, 53–62, 1991.

Akslen, L.A., Myking, A.O., Mørkve, Gulsvik, A., Raithel, H.J., and Schaller, K.H., Increased content of chromium and nickel in lung tissues from patients with bronchial carcinoma, *Pathol. Res. Pract.,* 186, 717–722, 1990.

Alwens, W. and Jonas, W., Der Chromat Lunenkrebs, *Acta Unio Int. Contra Cancrum*, 3, 103, 1938.

Anttila, S., Kokkonen, P., Pääkkö, P., Rainio, P., Kalliomäki, P-L., Pallon, J., Malmqvist, K., Pakarinen, P., Näntö, V., and Sutinen, S., High concentrations of chromium in lung tissue from lung cancer patients, *Cancer*, 63, 467–473, 1989.

Axelsson, G., Rylander, R., and Schmidt, A., Mortality and incidence of tumors among ferrochromium workers, *Br. J. Ind. Med*., 37, 121–127, 1980.

Baetjer, A.M., Pulmonary carcinoma in chromate workers, I. A review of the literature and report of cases, *Arch. Ind. Hyg. Occup. Med*., 2, 487–504, 1950a.

Baetjer, A.M., Pulmonary carcinoma in chromate workers, II. Incidence on basis of hospital records, *Arch. Ind. Hyg. Occup. Med*., 2, 505–516, 1950b.

Baetjer, A.M., Lowney, J.F., Steffee, H., and Budacz, V., Effect of chromium on incidence of lung tumors in mice and rats, *Arch. Ind. Health*, 20, 124–135, 1959.

Balansky, R.M., D'Agostini, F., Izzotti, A., and DeFlora, S., Less than additive interaction between cigarette smoke and chromium (VI) in inducing clastogenic damage in rodents, *Carcinogenesis*, 21, 1677–1682, 2000.

Beaumont, J.J. and Weiss, N.S., Lung cancer among welders, *J. Occup. Med*., 23, 839–844, 1981.

Becker, N., Chang-Claude, L., and Frantzel-Beyme, R., Risk of cancer for arc welders in the Federal Republic of Germany: Results of a second follow up (1983–8), *Br. J. Ind. Med*., 48, 675–673, 1991.

Becker, N., Claude, J., and Frentzel-Beyme, R., Cancer risk of arc welders exposed to fumes containing chromium and nickel, *Scand. J. Work Environ*., 11, 75–82, 1985.

Bidstrup, P.L., Carcinoma of the lung in chromate workers, *Br. J. Ind. Med*., 8, 302–305, 1951.

Bloom, H. and Noller, B.N., Application of trace analysis techniques to the study of atmospheric metal particulates, in *Symposium on Analytical Techniques for the Determination of Air Pollutants*, Clean Air Society of Australia and New Zealand, Melbourne, 23–27 May 1977, pp. 99–110.

Bonde, J.P. and Christensen, J.M., Chromium from biological samples from low-level exposed stainless steel and mild steel welders, *Arch. Environ. Health*, 46, 225–229, 1991.

Bragt, P.C. and van Dura, E.A., Toxicokinetics of hexavalent chromium in the rat after intratracheal administration of chromates of different solubilities, *Ann. Occup. Hyg*., 27, 315–322, 1983.

Christian, G.D. and Feldman, F.J., *Atomic Absorption Spectroscopy: Applications in Agriculture, Biology, and Medicine*, John Wiley & Sons, New York, 1970, p. 210.

D'Agostini, F., Izzotti, A., Bennicelli, C., Camoirano, A., Tampa, E., and De Flora, S., Induction of apoptosis in the lung but not in the liver of rats receiving intratracheal instrllations of chromium (VI), *Carcinogenesis*, 23, 587–593, 2002.

Davies, J.M., Lung cancer mortality in workers in chromate pigment manufacture: an epidemiological study, *J. Oil Chem. Assoc*., 62, 157–163, 1979.

Davies, J.M., Easton, D.F., and Bidstrup, P.L., Mortality from respiratory cancer and other causes in United Kingdom chromate production workers, *Br. J. Ind. Med.*, 48, 299–313, 1991.

Dayan, A.D. and Paine, A.J., Mechanisms of Chromium Toxicity, Carcinogenicity and Allergenicity: Review of the Literature from 1985 to 2000, *Hum. Exp. Toxicol*., 20, 439–451, 2001.

De Flora, S., Camoirano, A., Bagnasco, M., Bennicelli, C., Corbett, G.E., and Kereger, B., Estimates of the chromium (VI) reducing capacity in human body compartments as a mechanism for attenuating its potential toxicity and carcinogenicity, *Carcinogenesis*, 18, 531–537, 1997.

De Flora, S. and Wetterhahn. K.E., Mechanisms of chromium metabolism and genotoxicity, *Life Chem. Rep.*, 7, 169–244, 1989.

Dereanko, M.J., Rinehart, W.E., Hilaski, R.J., Thompson, R.B., Löser, E., Thirteen-week subchronic inhalation toxicity study with a recovery phase of trivalent chromium compounds, chromic oxide and basic chromium sulfate, *Toxicol. Sci.*, 52, 278–288, 1999.

Dillon, C.T., Lay, P.A., Cholewa, M., Legge, G.J.F., Bonin, A.W., Collins, T.J., Kostka, L., Shea-McCartth, G., Microprobe X-ray absorption spectroscopic determination of the oxidation state of chromium following exposure of V79 Chinese hamster lung cells to genotoxic chromium complexes, *Chem. Res. Toxicol*., 10, 533–535, 1997.

Ewis, A., Kondo, K., Lee, J., Tsuyuguchi, M., Hashimoto, M., Yokose, T., Mukai, K., Kodama, T., Shinka, T., Monden, Y., and Nakahori, Y., Occupational cancer genetics: infrequent *ras* oncogenes point mutations in lung cancer samples from chromate workers, *Am. J. Ind. Med.*, 40, 92–97, 2001.

Franchini, I., Magnani, F., and Mutti, A., Mortality experience among chrome plating workers. Initial findings, *Scand. J. Work Environ. Health*, 9, 247–252, 1983.

Gauglhofer, J., 11.7 Chromium, in *Metals and Their Compounds in the Environment: Occurrence, Analysis, and Biological Relevance,* Merian, E., Ed., VCH Publishers, Weinheim, 1991, pp. 855–878.

Gerhardsson, L. and Nordberg, G.F., Lung cancer in smelter workers: interactions of metals as indicated by tissue analysis, *Scand. J. Work Environ. Health*, 19, 90–94, 1993.

Gibb, H.J., Lees, P.S.J., Pinsky, P., and Rooney, B.C., Clinical findings of irritation among chromium chemical production workers, *Am. J. Ind. Med*., 38, 127–131, 2000a.

Gibb, H.J., Lees, P.S.J., Pinsky, P., Rooney, B.C., Lung cancer among workers in chromium chemical production, *Am. J. Ind. Med*., 38, 115–126, 2000b.

Glaser, U., Hochrainer, D., Klöppel, H., and Oldiges, H., Carcinogenicity of sodium dichromate and chromium (VI/III) oxide aerosols inhaled by male Wistar rats, *Toxicology*, 42, 219–232, 1986.

Grogan, C.H. and Oppenheimer, H., Experimental studies in metal carcinogenesis. III. Behavior of chromium compounds in the physiological pH range, *J. Am. Chem. Soc*., 77, 152–157, 1955.

Hayes, R.B., Review of occupational epidemiology of chromium chemicals and respiratory cancer, *Sci. Total Environ*., 71, 331–339, 1988.

Hayes, R.B., Lilienfeld, A.M., and Snell, L.M., Mortality in chromium chemical production workers: A prospective study, *Int. J. Epidemiol*., 8, 365–374, 1979.

Hilaski, R., Katz, S.A., and Salem, H., Inhalation toxicity of chromium from Whetlerite dust in rats, *Toxicol. Lett.*, 62, 25–31, 1992.

Hill, W.J. and Ferguson, W.S., Statistical analysis of epidemiological data from chromium chemical manufacturing plant, *J. Occup. Med*., 21, 103–106, 1979.

Hueper, W.C., Experimental studies in metal carcinogenesis. X. Carcinogenic effects of chromite ore roast deposited in muscle tissue and pleural cavity of rats, *Arch. Ind. Health*, 18, 284–291, 1958.

Hueper, W.C., Environmental carcinogenesis and cancers, *Cancer Res*., 21, 842–857, 1961.

IARC (International Agency for Research on Cancer), *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Chromium, Nickel and Welding,* Vol. 49, International Agency for Research on Cancer, Lyon, 1990, pp. 90–91.

Iqbal, M.Z., Qadir, M., and Zaidi, J.H., Health evaluation of an aerosol formed in the fumes of mild steel welding electrodes, *J. Trace Elem. Electrolytes Health Dis*., 6, 63–66, 1993.

Ishikawa, Y., Nakagawa, K., Satoh, Y., Kitagawa, T., Sugano, H., Hirano, T., and Tsuchiya, E., "Hot Spots" of chromium accumulation at bifurcations of chromate workers' bronchi, *Cancer Res*., 54, 2342–2346, 1994a.

Ishikawa, Y., Nakagawa, K., Satoh, Y., Kitagawa, T., Sugano, H., Hirano, T., and Tsuchiya, E., Characteristics of chromate workers' cancers, chromium lung deposition and precancerous bronchial lesions: an autopsy study, *Br. J. Cancer*, 70, 160–166, 1994b.

Izzotti, A., Cartiglia, C., Balansky, R., D'Agostini, F.D., Longobardi, M., and De Floria, S., Selective induction of gene expression in rat lung by hexavalent chromium, *Mol. Carcinog*., 35, 75–84. 2002.

Källiomäki, P.L., Harknoen, E., Vaaranen, V., Källiomäki, K., and Sittioniene, A., Lung-retained contaminants: Urinary chromium and nickel among stainless steel welders, *Int. Arch. Occup. Environ. Health*, 49, 67–75, 1981.

Koponen, M., Gustafsson, T., Kalliomäki, P.L., and Pyy, L., Chromium and nickel aerosols in stainless steel manufacturing, grinding, and welding, *Am. Ind. Hyg. J*., 42, 596–601, 1981.

Kortenkamp, A., O'Brien, P., and Beyersmann, D., The reduction of chromate is a prerequisite of chromium binding to cell nuclei, *Carcinogenesis*, 12, 1143–1144, 1991.

Koyama, T., Evaluation of the exposure to the occupational and environmental contaminants in the context of anthracotic lesions and metal contents in surgically resected tissue from 255 lung cancer patients, *J. Saitama Med. School*, 22, 129–145, 1995.

Langard, S., A survey of respiratory symptoms and lung function in ferrochromium and ferrosilicon workers, *Int. Arch. Occup. Environ. Health*, 46, 1–9, 1980.

Langard, S. and Norseth, T.A., cohort study of bronchial carcinomas in workers producing chromate pigments, *Br. J. Ind. Me*d., 32, 62–65, 1975.

Laskin, S., Kuschner, M., and Drew, R.T., Studies in pulmonary carcinogenicity, in *Inhalation Carcinogens,* Hanna, M.G., Nettel-shein, P., and Gilbert, J.R., Eds., U.S. Atomic Energy Commission, Oak Ridge, TN, 1970, pp.321–351.

Lee, K.P., Ulrich, C.E., Geil, R.G., and Trochimowicz, H.J., Effects of inhaled chromium dioxide dust in rats exposed for two years, *Fundam. Appl. Toxicol*., 10, 125–145, 1988.

Lerchen, M.L., Wiggins, C.L., and Samet, J.L., Lung cancer and occupation in New Mexico, *J. Natl. Cancer Inst*., 79, 39–44, 1987.

Levy, L.S., Martin, P.A., and Bidstrup, P.L., Investigation of the potential carcinogenicity of a range of chromium containing materials in rat lung, *Br. J. Ind. Med*., 43, 243–256, 1986.

Levy, L.S. and Venitt, S., Carcinogeniciry and mutagenicity of chromium compounds: the association between bronchial metaplasia and neoplasia, *Carcinogenesis*, 7, 831–836, 1986.

Lewalter, J. and Korallus, U., The significance of ascorbic acid and glutathione for chromate metabolism in man, *Toxicol. Environ. Chem*., 24, 25–33, 1989.

Lindberg, E. and Vesterberg, O., Monitoring exposure to chromic acid in chrome-plating by measuring chromium in urine, *Scand. J. Work Environ. Health*, 9, 333–337, 1983.

Lu, F.C., *Basic Toxicology. Fundamentals, Target Organs, and Risk Assessment,* Hampshire Publishing, Washington, 1985, pp. 13–14.

Lurie, P. and Wolfe, A.W., Continuing exposure to hexavalent chromium, a known lung carcinogen: an analysis of OSHA compliance inspections, 1990–2000, *Am. J. Ind. Med*., 42, 378–383, 2002.

Machle, W. and Gregorius, F., Cancer of the respiratory system in the United States chromate-producing industry, *Public Health Rep*., 63, 114–127, 1948.

Maltoni, C., Morisi, L., and Chieco, P., Experimental approach to the assessment of the carcinogenic risk of industrial inorganic pigments, *Adv. Mod. Environ. Toxicol.*, 2, 77–92, 1982.

Mancuso, T.F., Chromium as an industrial carcinogen, Part I, *Am. J. Ind. Med.*, 31, 129–139, 1997a.

Mancuso, T.F., Chromium as an industrial carcinogen, Part II. Chromium in human tissues, *Am. J. Ind. Med*., 31, 140–147, 1997b.

Mancuso, T.F. and Hueper, W.C., Occupational cancer and other health hazards in a chromate plant: A medical appraisal. I. Lung cancer in chromate workers, *Ind. Med. Surg.*, 20, 358–363, 1951.

Matos, E.L., Vilensky, M., Mirabelli, D., and Boffetta, P., Occupational exposure and lung cancer in Buenos Aires, Argentina, *J. Occup. Exp. Med.*, 42, 653–659, 2000.

Moulin, J.J., Clavel, T., Roy, D., Dananché, B., Marquis, N., Févotte, J., and Fontana, J.M., Risk of lung cancer in workers producing stainless steel and metallic alloys, *Int. Arch. Occup. Environ. Health*, 73, 171–180, 2000.

Moulin, J.J., Portexaix, P., Wild, P., Mur, J.M., Smagghe, G., and Mantout, B., Mortality study among workers producing ferro-alloys and stainless steel in France, *Br. J. Ind. Med*., 47, 537–543, 1990.

Mutti, A., Pedroni, G., Arfini, G., Borghi, A., Giaroli, C., and Franchini, I., Biological monitoring of occupational exposure to different chromium compounds at various valence states, in *Carcinogenic and Mutagenic Metal Compounds. Environmental and Analytical Chemistry and Biological Effects,* Merian, E., Frei, R.W., Hardi, W., and Schlatter, C., Eds., Gordon and Breach, London, 1985, pp. 119–125.

Nettesheim, P., Hanna, M.G., Doherty, D.G., Newell, R.F., and Hellman, A., Effects of calcium chromate dust, influenza virus, and 100 R whole body X radiation on lung tumor incidence in mice, *J. Natl. Cancer Inst.,* 47, 1129–1144, 1971.

Newman, D., A case of adenocarcinoma of the left inferior turbinate body and perforation of the nasal septum in the person of a worker in chrome pigments, *Glasgow Med. J.*, 33, 469–470, 1980.

O'Flaherty, E.J., A pharmacokinetic model for chromium, *Toxicol. Lett*., 68, 145–158, 1993.

O'Flaherty, E.J., A physiologically based model of chromium kinetics in the rat, *Toxiol. Appl. Physiol*., 138, 54–64, 1996.

Oláh, B. and Tölgyessy, J., Determination of heavy metals in welder's working environment using nuclear analytical methods, *J. Radioanal. Nucl. Chem*., 93, 43–54, 1985.

Park, R.M., Bena, J.F., Stayner, and L.T., Hexavalent chromium and lung cancer, presented at The Chromate Industry: A Quantitative Risk Assessment, 23rd Annual Meeting of the Society for Risk Analysis, Baltimore, 7–10 December 2003.

Paustenbach, D.J., Panko, J.M., Frederick, M.M., Finley, B.L., and Proctor, D.M., Urinary chromium as a biological marker of environmental exposure: what are the limitations?, *Regul. Toxicol. Pharmacol*., 26, S23–S34, 1997.

Payne, W.W., The role of roasted chromite ore in the production of cancer, *Arch. Environ. Heal*th, 1, 20–26, 1960a.

Payne, W.W., Production of cancers in mice and rats by chromium compounds, *Arch. Ind. Health*, 21, 530–535, 1960b.

Pfeil, E., Lungentumoren als Berufskrankung in Chromatbetrieben, *Dtsch. Med. Wochenschr.*, 61, 1197–1202, 1935.

Popper, H.H., Grygar, E., Ingolic, E., and Wawschinek, O., Cytotoxicity of chromium-iii and -vi compounds. i. *in vitro* studies using different cell culture systems, *Inhalation Toxicol.,* 5, 345–369, 1993.

Popper, H.H. and Woldrich, A., Oxygen radical formation a probable mechanism for chromate toxicity, *Prog. Histochem. Cytochem*., 23, 220–225, 1991.

Proctor, D.M., Otani, J.M., Finley, B.L., Paustenbach, D.J., Bland, J.A., Speizer, N., and Sargent, E.V., Is hexavalent chromium carcinogenic via ingestion? A weight-of-evidence review, *J. Toxicol. Environ. Health A*, 65, 701–746, 2002.

Proctor, D.M., Panko, J.P., Liebig, E.W., Scott, P.K., Mundt, K.A., Buczynski, M.A., Barnhart, R.J., Harris, M.A., Morgan, R.J., and Paustenbach, D.J., Workplace airborne hexavalent chromium concentrations for the Painsville, Ohio, chromate production plant (1943–1971), *Appl. Occup. Environ. Hea*lth, 18, 430–449, 2003.

Roberts, W.C., The ferroalloy industry, hazards of the alloys and semimetallics, Part I, *J. Occup. Med*., 7, 30–36, 1965a.

Roberts, W.C., The ferroalloy industry, hazards of the alloys and semimetallics, Part II, *J. Occup. Med.*, 7, 71–77, 1965b.

Royle, H., Toxicity of chromic acid in the chromium plating industry (1), *Environ. Res*., 10, 39–53, 1975.

Salem, H. and Katz, S.A., Speciation, bioavailability and systemic distribution of chromium from Whetlerite dust, *Sci. Total Environ*., 86, 59–64, 1989.

Samitz, M.H. and Katz, S.A., Protection against inhalation of chromic acid mist, *Arch. Environ. Health*, 11, 770–772, 1965.

Samitz, M.H., Scheiner, D.M., and Katz, S.A., Ascorbic acid in the prevention of chrome dermatitis, *Arch. Environ. Health*, 17, 44–45, 1968.

Sferlazza, S.J. and Beckett, W.S., The respiratory health of welders, *Am. Rev. Respir. Dis*., 143, 1134–1148, 1991.

Shainkin-Kestenbaum, R., Caruso, C., and Berlyne, G.M., Effect of chromium on oxygen free radical metabolism, inhibition of super- oxide dismutase and enhancement of 6-hydroxydopamine oxidation, *J. Trace Elem. Electrolytes Health Dis*., 5, 197–201, 1991.

Shi, X. and Dalal, N.S., On the hydroxyl radical formation in the reaction between hydrogen peroxide and biologically generated chromium (V) species, *Arch. Biochem. Biophys*., 277, 342–350, 1990a.

Shi, X. and Dalal, N.S., Evidence for a Fenton-type mechanism for the generation of ·OH radicals in the reduction of Cr(VI) in cellular media, *Arch. Biochem. Biophys*., 281, 90–95, 1990b.

Silverstein, M., Mirer, F., Kotelchuck, D., Silverstein, B., and Bennett, M., Mortality among workers in a die-casting and electroplating plant, *Scand. J. Environ. Health*, 7, 156–165, 1981.

Simonato, L., Fletcher, A.C., Andersen, A., Andersen, K., Becker, N., Cheng-Claude, J., Ferro, G., Gérin, M., Gray, C.N., Hansen, K.S., Kalliomäki, P-L, Kuppa, K., Långard, S., Merló, F., Moulin, J.J., Newhouse, M.L., Peto, J., Pukkala, B., Sjögren, B., Wild, P., Winklemann, R., and Saracci, R., A historical prospective study of European stainless steel, mild steel, and shipyard welders, *Br. J. Ind. Med*., 48, 145–154, 1991.

Singleton, J. A., and Beaumont, J. J., COMS II. California Occupational Mortality, 1979–1981, Adjusted for Smoking, Alcohol, and Socioeconomic Status, California Department of Health Services, Data and Statistics Branch, Demographics Section, Sacramento, 1989.

Sjogren, B., A retrospective chort study of mortality among stainless steel welders, *Scand. J. Work Environ. Health*, 6, 197–200, 1980.

Sjogren, B., A cohort study of welders exposed to stainless steel welding fumes, *Arbete Och. Halsa*, 9, 1–8, 1985.

Sjogren, B., Hedstrom, L., and Ulfvarson, U., Urine chromium as an estimator of air exposure to stainless steel welding fumes, *Int. Arch. Occup. Environ. Health*, 51, 347–354, 1983.

Sorahan, T., Burges, D.C.L., and Waterhouse, J.A.H., A mortality study of nickel/chromium platers, *Br. J. Ind. Med*., 44, 250–258, 1987.

Sorahan, T. and Harrington, J.M., Lung cancer in yorkshire chrome platers, 1972–1997, *Occup. Environ. Med*., 57, 385–389, 2000.

Standeven, A.M. and Wetterhahn, K.E., Possible role of glutathione in chromium (VI) metabolism and toxicity in rats, *Pharmacol. Toxicol*., 69, 469–476, 1991.

Steffee, C.H. and Baetjer, A.M., Histopathologic effects of chromate chemicals. Report of studies in rabbits, guinea pigs, rats, and mice, *Arch. Environ. Health*, 11, 66–75, 1965.

Steinhoff, D., Gad, S., Hatfield, K., and Mohr, U., Carcino-genicity study with sodium dichromate in rats, *Exp. Pathol*., 30,129–141, 1986.

Stern, R.M., Chromium compounds: production and occupational exposure, in *Biological and Environmental Aspects of Chromium,* Langård, S., Ed., Elsevier, Amsterdam, 1982, pp. 17–33.

Stern, R.M., Assessment of risk of lung cancer for welders, *Arch. Environ. Health*, 38, 148–155, 1983.
Sugiyama, M., Role of physiological antioxidants in chromium (VI) induced cell injury, *Free Radical Biol. Med*., 12, 379–407, 1992.
Suzuki, Y., Synergism of ascorbic acid and glutathione in the reduction of hexavalent chromium in vitro, *Ind. Health*, 28, 9–19, 1990.
Suzuki, Y. and Fukuda, K., Reduction of hexavalent chromium by ascorbic acid and glutathione with special reference to the rat lung, *Arch. Toxicol*., 64, 169–176, 1990.
Suzuki, T., Miyata, N., Horitsu, H., Kawai, K., Takamizawa, K., Tai, Y., and Okazaki, M., NAD(P)H-dependent chromium (VI) reductase of *Pseudomonas ambigua* G-1: a Cr(V) intermediate is formed during the reduction of Cr(VI) to Cr(III), *J. Bacteriol.,* 174, 5340–5345, 1992.
Tadao, T., Kazuo, F., Norihiko, K., Hiroko, K., and Heihachiro, A., Hexavalent chromium responsible for lung lesions induced by intratracheal instillation of chromium fumes in rats, *Ind. Health*, 37, 36–46, 1999.
Takemoto, K., Kawai, H., Kuwahara, T., Nishina, M., and Adachi, S., Metal concentrations in human lung tissue, with special reference to age, sex, cause of death, emphysema, and contamination of lung tissue, *Int. Arch. Occup.. Environ. Health*, 62, 579–586, 1991.
Tsuchiyama, F., Hisanaga, N., Shibata, E., Aoki, T., Takagi, H., Ando, T., and Takeuchi, Y., Pulmoinary metal distributions in urban dwellers, *Int. Arch. Occup. Environ. Health*, 70, 77–84, 1997.
U.S. EPA (U.S. Environmental Protection Agency), Health Assessment Document for Chromium, , EPA-600/8-83-014F, U.S. EPA, Research Triangle Park, NC, August 1984, p. 5–1.
Valentine, W.N., Chromate induced inhibition of glutathione reductase (GSSG-R) in the human erythrocyte, *Trans. Assoc. Am. Phys*., 27, 88–98, 1964.
Vanoeteren, C., Cornelis, R., Versieck, J., Hoste, J., and DeRoose, J., Trace element patterns in human lung tissue, *J. Radioanal. Chem*., 70, 219–238, 1982.
Welinder, H., Littorin, M., Gullberg, B., and Skerfving, S., Elimination of chromium in urine after stainless steel welding, *Scand. J. Work Environ. Health*, 9, 397–403, 1983.
Wetterhahn, K.E. and Hamilton, J.W., Molecular basis of hexavalent chromium carcinogenicity: effect and expression, *Sci. Total Environ*., 86, 113–129, (1989),
Wiegand, H.J., Ottenwalder, H., and Bolt, H.M., Bioavailability and metabolism of hexavalent chromium compounds, *Toxicol. Environ. Chem.*, 14, 263–275, 1987.
World Health Organization *Chromium in Air Quality Guid*elines, 2nd ed., Chap. 6.4, Copenhagen, 2000, 166.

24 Safety Assessment of Therapeutic Agents Administered by the Respiratory Route

Shayne C. Gad

CONTENTS

24.1 INTRODUCTION

Drugs and medicinal agents administered by the inhalation route include gaseous and vaporous anesthetics, coronary vasodilators, aerosols of bronchodilators, corticosteroids, mucolytics, expectorants, antibiotics, and an increasing number of peptides and proteins where there is significant nasal absorption (Cox et al., 1970; Williams, 1974; Paterson et al., 1977, 1979; Hodson et al., 1981; Lourenco and Cotromanes, 1982; Tamulinas and Leach, 2000). Concerns with the environmental affects of chlorofluorocarbons have also led to renewed interest in dry-powder inhalers (DPIs), which, in addition, have shown promise for better tolerance and absorption for some new drugs.

TABLE 24.1 Nasal Delivery Systems

*** Liquid Nasal Formulations**

-Instillation and rhinyle catheter	-Drops
-Unit Dose Containers	-Squeezed Bottle
-Metered Doe Pump Sprays	-Airless and Preservative free sprays
-Compressed Air Nebulizers	

*** Powder Dosage Forms**

-Insufflators	-Mono-dose powder inhaler
-Multi-dose dry powder System	

*** Pressurized MDIs (dose inhalers)**

*** Nasal Gels**

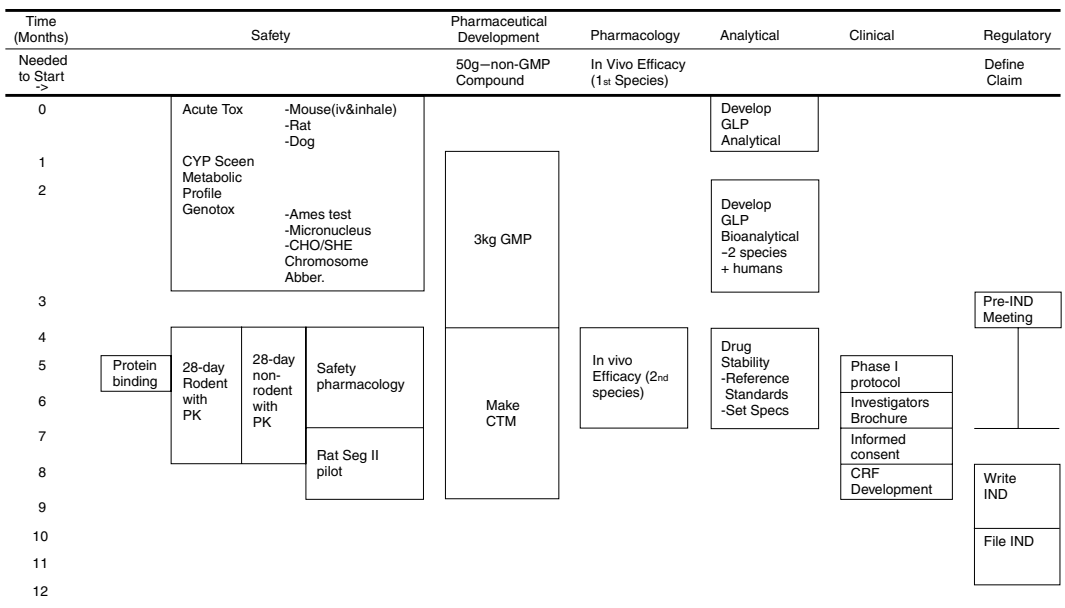


FIGURE 24.1 Typical pharmaceutical development activities up to the first tests in human (phase I) studies for respiratory-route drugs (Gad, 2002).

Recent advances have also led to new nasal delivery systems, such as in Table 24.1. Excessive inhalation of a drug into the pulmonary system during therapy or manufacturing may result in adverse local and/or systemic effects. Consequently, safety assessment of medicinal preparations delivered via respiratory routes with respect to local tissue toxicity, systemic toxicity, and the therapeutic-to-toxicity ratio are essential. The data generated are essential for charting the course of evaluation and development of a potential therapeutic agent, the general course of which is summarized in Figure 24.1.

Different portions of the respiratory system are subjected to specific potential patterns of damage, as summarized in Table 24.2 (adapted from Davis, 1961; Weibel, 1963; Horsfield and Cunning, 1968; Weibel, 1983).

TABLE 24.2 Responses of the Lungs to Toxic Injury

Aveoli		
Acute	Epithelial injury	Examples:
		Simple repair O2
		Exudation and NO2, SO2, Cd2+ fibrosis
	Endothelial injury	Thrombosis, O2, O2
	Interstitial injury	Fibrosis, proteolytic enzymes
Chronic	Granulomatus injury	Tuberculosis, beryllium, etc.
	Immunologic injury	Farmer's lung
Bronchi		
Acute	Mucosal ulceration	
	Metaplasia	
Chronic	Atypia	
	Dysplasia	
	Neoplasia	
Systemic Damage		
	Central nervous system	
	Target organ toxicity	

Regions			Generations	Total Cross section (cm^2)	Particle size penetration limits (μm)	Distribution			
						Cell types	Mucous Glands	Smooth Muscle	p-sym. Innerv.
Extrathoracic	Head	Nasopharynx			60				
Intrathoracic	Tracheo-bronchial	Trachea	0	2.54		Cilliated			
		Primary bronchi	1	2.33	20	Goblet			
		Secondary bronchi	2	2.13	10	Brush Border Squamus			
			3	2.00					
			4	2.48		Columnar			
			10	13.4					
		Bronchioles	11	19.6	6				
			15	113					
		Respiratory bronchioles	16	180	4	Cuboidal Less cilliated			
	Alveolar	Terminal bronchioles	17	300	3				
			18	534		Less goblet			
			19	944		Non cilicated clara			
		Alveoli ducts	10	1.60K	<3				
			21	3.22K		Type I			
			22	5.88K					
		Alveoli	23	11.8K	<3	Type II			

FIGURE 24.2 The distribution of cell types in the respiratory tract and lungs.

24.2 PENETRATION AND ABSORPTION OF INHALED GASES AND VAPORS

Pulmonary dynamics, the dimension and geometry of the respiratory tract and the structure of the lungs, together with the solubility and chemical reactivity of the inhalants greatly influence the magnitude of penetration, retention, and absorption of inhaled gases, vapors (Dahl, 1990), and aerosols (Phalen, 1984). The quantity of an inhalant effectively retained in the pulmonary system constitutes the inhaled "dose" that causes pharmacotoxic responses.

Highly reactive and soluble gaseous or vaporous drugs react and dissolve readily in the mucosal membrane of the nasopharynx and the upper respiratory tract (URT), thereby exerting pharmacological effects or causing local irritation and/or adverse effects on the ciliated, goblet, brush border columnar, and squamous cells of the epithelium. The dissolved drug is also absorbed into the bloodstream and transported to the target organ where it exerts systemic effects. Less reactive and less soluble gaseous or vaporous drugs are likely to penetrate beyond the URT and reach the bronchial and alveolar regions causing local and systemic effects. The unabsorbed gases or vapors are then exhaled. For example, ammonia gas generated from a 10% ammonia water may be inhaled for reflex respiratory stimulation purposes (Budavari, 1989). Ammonia is extremely soluble in water at a concentration of 715 ml of ammonia per ml of water (Phalen, 1984), and is readily solubilized in the mucous lining, causing URT irritation. By contrast, oxygen is only sparingly soluble in water (0.031 cc of oxygen per ml of water) and capable of penetrating deeply into the alveoli where gas exchange takes place. Oxygen that binds reversibly with the hemoglobin of erythrocytes is unloaded at the target tissues, whereas the unbound oxygen is exhaled. Inhalation of properly humidified oxygen is life supporting, but inhalation of unhumidified oxygen may cause a reduction in the mucociliary clearance of secretions in the trachea of animals (Pavia, 1984) and humans (Lichtiger et al., 1975; Gamsu et al., 1976). Gases or vapors of low lipid solubility are also poorly absorbed in the lungs, with much of the inhaled vapor exhaled. Other pharmacological gases and vapors, such as the anesthetics (nitrous oxide, halothane, enflurane, isoflurane, etc.) and the coronary vasodilators (amyl nitrite), likewise affect the epithelium of the respiratory tract and the lungs. The absorbed drugs exert local effects on various types of epithelial cells of the respiratory tract, and on type I and II cells and the alveolar macrophages (AMs) in the alveoli. Repeated inhalation of some halogenated hydrocarbon anesthetics will result in accumulation of the vapors and systemic toxicity (Chenoweth et al., 1972). By contrast, vapors such as the fluorocarbons (FC 11 and FC 12), which are used extensively as propellants for bronchodilator and corticosteroid aerosols, are absorbed rapidly but are not accumulated in the body even upon repeated inhalation (Aviado and Micozzi, 1981).

In general, dissolved gases or vapors at a nontoxic concentration are absorbed and metabolized locally by the lungs and systemically by the liver. The unchanged parent drug and its metabolites may be excreted to some extent via exhalation but mainly via the renal system. A dissolved gas or vapor at a toxic concentration, however, is likely to exert local effects such as altering the surface tension of the alveoli linings or disrupting the normal functions of the epithelial cells, the pneumocytes, and the AMs. The disrupted AMs in turn release their intracellular enzymes, causing destruction of the alveolar septa and contributing to histopathological changes of the respiratory tract and the lungs. Again, the magnitude of the adverse effects depends on pulmonary dynamics and the solubilities of the inhalants in the mucous membrane of the URT and in the plasma or lipids of the erythrocytes.

24.3 DEPOSITION OF INHALED AEROSOLS

For inhaled aerosols, particle size is the major factor affecting the penetration, deposition, and hence the "dose" and site of pharmacological action (Dautreband, 1962a, 1962b; Agnew, 1984). Particle size is expressed in terms of *aerodynamic diameter* (AD), defined as the diameter of a spherical particle of unit density (lg/cm^3) that has the same terminal settling velocity as the particle in question, regardless of its shape and density (Marple and Rubow, 1980). The unit for AD is micrometers (μm). A sample of aerosol particles having ADs within a narrow size range is considered to be a

monodisperse aerosol, whereas a sample of aerosols with a wide range of ADs is a heterodisperse, or polydisperse, aerosol. The pattern of particle-size distribution is usually bell shaped, with smaller and larger particles on both sides of the mean AD. An aerosol sample with a high proportion of particles of similar size has a narrow particle-size distribution, or small geometric standard deviation (GSD). An aerosol sample with a GSD of less than 2 is considered to be a monodisperse aerosol. Therefore, both the AD and GSD of 2 or less are considered to be optimal for pulmonary penetration and distribution in the respiratory tract and the lungs. An example follows.

In nose breathing, aerosol particles with ADs $>$ 15 μm are likely to be trapped in the nasopharynx (extrathoracic, or head, region) by filtration and impaction. Particles deposited in the nasopharynx are considered to be "noninhalable" (Lippmann, 1970; Miller et al., 1979).

In mouth breathing, only 10–15% of 15-μm particles penetrate through the larynx to the intrathoracic tracheobronchial (TB) region. Particles reaching the TB region are considered to be "inhalable" (Lippmann, 1970; Miller et al., 1979).

In natural nose and mouth breathing, only a negligible proportion of aerosol particles of AD $>$ 10 μm reach the lungs (Swift and Proctor, 1982). Aerosol particles of 3–4 μm in AD are considered to be optimal sizes for TB deposition. The mechanisms of deposition are by impaction along the trachea and at bronchial branchings where the direction of airflow changes and by gravity settlement in the fine airways in amounts proportional to the particle-settling velocity and the time available for settlement (Hatch and Gross, 1964; Heyder et al., 1980). Aerosol particles of 1–2 μm in AD, however, decrease in TB deposition because the particles are too small for effective impaction and sedimentation (Lippmann, 1977; Chan and Lippmann, 1980; Lippman et al., 1980; Stahlhofen et al., 1980). Consequently, the majority of the very fine particles are exhaled. However, the deposition of the ultrafine particles of approximately 0.5 μm in AD on the walls of the finest bronchioles and the alveoli increases again because of molecular diffusion processes. Even so, some 90% of the inhaled 0.5-μm particles will still be exhaled during quiet tidal breathing and much more under forced exhalation (Davis et al., 1972; Taulbee et al., 1978). Those fine particles reaching the finest bronchioles and alveoli are considered to be "respirable" (Lippmann, 1970).

In general, particles of AD $>$ 10 μm deposit mainly in the URT, whereas particles of 1–5 μm AD, with a GSD of less than 2, are likely to reach the lower respiratory tract, which includes the TB region and the alveoli, with small oropharyngeal loss.

The proportion of an aerosol sample suitable for inhalation can also be determined on the basis of mass median aerodynamic diameter (MMAD), which is defined as the AD which 50% (by weight) of an aerosol sample is less than or equal to the stated median AD. For example, a sample with an MMAD of 5 μm means that 50% by weight of that sample has ADs of 5 μm and smaller. The MMAD is, therefore, a good index for determining the proportion of an aerosol sample that is "noninhalable," "inhalable," or "respirable." An aerosol sample with an MMAD of 5 μm and a GSD of less than 2 is considered to be optimal for pulmonary deposition and retention (Task Group on Lung Dynamics, 1966).

In addition to AD and GSD, the pulmonary dynamics of a subject also greatly influence the distribution of aerosol particles in various regions of the respiratory tract (Agnew, 1984). For example, the velocity of airflow in the respiratory tract significantly influences the pattern of TB deposition. An increase in airflow velocity in the airways increases the effectiveness of particle impaction at the bifurcations of the large airways (Dennis, 1961; Hatch and Gross, 1964; Parent, 1991). As a result, spots impacted with a high concentration of particles (hot spots) are frequently present at the carina and the bifurcations of the airways (Lee and Wang, 1977; Bell, 1978; Stahlhofen et al., 1981). Furthermore, the depth of each breath (tidal volume) also influences the distribution of aerosols. A small tidal volume permits greater impaction in the proximal conducting airways and less sedimentation in the distal airways.

In general, slow, deep inhalation followed by a period of breath holding increases the deposition of aerosols in the peripheral parts of the lungs, whereas rapid inhalation increases the deposition in the oropharynx and in the large central airways. Thus, the frequency of respiration (the flow velocity) and the depth of breath (tidal volume) influence the pattern of pulmonary penetration and

deposition of inhaled aerosols. Therefore, an aerosol of ideal size will penetrate deeply into the respiratory tract and the lungs only when the aerosols are inhaled in the correct manner.

24.4 ABSORPTION AND CLEARANCE OF INHALED AEROSOLS

Soluble aerosols deposited on the epithelial linings of the respiratory tract are absorbed and metabolized in the same way as soluble gases and vapors.

Insoluble medicinal aerosols are few in number. Sodium cromoglycate (SCG) is probably the only insoluble powder to be administered as a prophylactic antiasthmatic (Wanner, 1979). Insoluble particles deposited on the ciliated linings of the URT are removed by a mucociliary clearance mechanism. Particles deposited on a terminal airway devoid of ciliated cells may be endocytosed into the epithelial cells. At a toxic concentration, the cells die and the debris is then phagocytosed and transported into the interstitial space for removal via the lymph or vascular drainages, or reenters the ciliated zone of the airway. Particles deposited in the alveolar walls are phagocytosed by the AMs and transported from the low surface tension surfactant in the alveolar lining to the high surface tension bronchial fluid of the ciliate airways for elimination by the mucociliary clearance mechanism (Laurweryns and Baert, 1977). The particle sizes optimal for phagocytosis are 2–3 μm, whereas particles smaller than 0.26 μm are less effective in activating the macrophages (Holma, 1967). In any case, AMs can phagocytose only a small fraction of a large number of deposited particles. The nonphagocytosed particles are translocated to the lymphatic system for elimination (Ferin, 1977).

Like the inhaled gases or vapors, soluble and insoluble aerosol particles can directly exert desirable and undesirable local effects at the site of deposition and/or systemic effects after solubilization, absorption, and metabolization.

24.5 PHARMACOTOXICITY OF INHALED AEROSOLS

The inhalation route for administering drugs into the pulmonary system for treatment of respiratory diseases eliminates many bioavailability problems such as plasma binding and "first-pass" metabolism, which are encountered in parenteral or oral administration. Consequently, a small inhalation dose is adequate for achieving the desirable therapeutic response without inducing many undesirable side effects. Furthermore, the direct contact of the drug with the target site ensures rapid action. Nevertheless, the effects from inhaled drug aerosols also depend on the pharmacological properties of the aerosols and the location of their deposition in the respiratory system. For example, the classic experiments on bronchodilation drugs (Dautrebande, 1962a, 1962b) showed that fine aerosol particles of isoproterenol penetrate deeply in to the lower respiratory airways (LRAs). In this way, a high concentration of the drug aerosol can reach the beta-adrenergic receptors of the bronchial smooth muscles. Stimulation of the receptors causes relaxation of the smooth muscle fibers and results in bronchodilation (Weiner, 1984; McFadden, 1986; Cherniak, 1988). Such rapid bronchial responses can be produced in healthy and asthmatic subjects without inducing any cardiac effects. By contrast, the same dose of isoproterenol of large particle sizes deposits mainly along the URT, with a minimal amount reaching the smooth muscles of the LRA. The drug is quickly absorbed into the tracheal and bronchial veins and delivered immediately to the left ventricle of the heart. A high plasma concentration of the drug in the heart causes prominent cardiovascular effects such as tachycardia and hypertension. Other aerosols of beta-adrenergic drugs, such as epinephrine, isoprenaline, terbutaline, and salbutamol, induce bronchodilation effects in animals and humans (Pavia, 1984) via inhalation and stimulate ciliary beat frequency and mucus production at the site of deposition in the trachea (Wanner, 1981). Thus, the tracheobronchial mucociliary clearance mechanism is also stimulated. By contrast, anticholinergic bronchodilators, such as atropine and ipratropium bromide, cause mucus retention in the lungs (Pavia et al., 1983a, 1983b). Therefore, in pharmacological or safety assessments of inhalant beta-adrenergic bronchial dilatation drugs, aerosols should be of small particle sizes suitable for deposition in the peripheral airways to minimize side effects.

However, anticholinergic agents should be of larger particle sizes suitable for deposition in the large airways (Ingram et al., 1977; Hensley et al., 1978).

Other therapeutic aerosols, such as beclomethasone dipropionate, betamethasone valerate, and budesonide corticosteroid (Williams, 1974); the carbenicillin and gentamicin antibiotics (Hodson et al., 1981); the 2-mercaptoethane-sulfonate (Pavia et al., 1983b) and *n*-acetylcysteine mucolytics; and even vaccines for the prevention of influenza and tuberculosis (Lourenco and Cotromanes, 1982) are active by inhalation and/or oral administration. When these drugs are administered as aerosols, certain particle sizes may be targeted to a specific region or to multiple regions of the pulmonary system depending on the therapeutic target site(s). In any case, when aerosols are delivered as fine particles, the rate of absorption is increased because of an increase in the distribution area per unit mass of the drug. Thus, an effective aerosol dose of corticosteroid for treatment of asthma and bronchitis is merely a fraction of an oral dose (Williams, 1974). An aerosol of disodium cromoglycate (DSG) dry powder, a prophylactic for preventing the onset of bronchoconstriction in asthmatic attacks (Cox, 1970), is effective mainly by local inhibition of the release of chemical mediators from mast cells in bronchial smooth muscle. Therefore, DCG particle sizes should be approximately 2 μm in AD for the most effective penetration into the bronchial regions (Godfrey et al., 1974; Curry et al., 1975). Likewise, therapeutic aerosols of local anesthetics and surfactants may require appropriate particle sizes to be targeted to a specific region of the pulmonary system.

Other than undesirable pharmacological effects, toxic concentrations of soluble or insoluble aerosol particles may lead to adverse physiological and/or histopathological responses (Menzel and Amdur, 1986; Leong et al., 1987). For example, irritating aerosols cause dose-related reflex depression of the respiratory rate (Alarie 1966, 1981a), whereas phagocytosed particles cause chemotaxis of AMs and neutrophils to the site of deposition (Brain, 1971). The maximum response usually occurs at 24 h and returns to normal in approximately 3 d postexposure (Kavet et al., 1978). Furthermore, a toxic quantity of phagocytosed particles may interact with the lysosomal membrane within a macrophage, releasing cytotoxic lysosomal enzymes, proteases, and free radicals that in turn damage the adjacent lung tissue (Hocking and Golde, 1979).

In general, a specific category of drug delivered to a specific site of the pulmonary system will exert a specific pharmacological or toxicological action locally or systemically. Therefore, in safety assessments of inhalants, a drug should be delivered to the target sites of the pulmonary system according to the toxicological information required.

Finally, many drugs in the categories of amphetamines, anorectics, antihistamines, antipsychotics, tricyclic antidepressants, analgesics and narcotics, and beta-adrenergic blocking agents are known to accumulate in the lung (Wilson, 1982), even though these drugs are not administered via the inhalation route. Therefore, in safety assessments of these drugs, their pulmonary toxicity should also be evaluated. There are also *in vitro* techniques that are proposed for use in evaluating inhalation effects on respiratory tissues (Agu, 2002).

24.6 NASAL DELIVERY OF THERAPEUTICS

The biotechnology revolution in therapeutics has led to renewed interest in the nasal route as a means of safely delivering an effective dose of many therapeutics. This, in turn, has lead to investigations of new approaches to drug administration by this route which have proved very rewarding (Smaldone, 1997; Logemaan and Rankin, 2000; Sharma et al., 2001; Aldridge, 2003; Lawrence, 2002). Table 24.3 presents a comparison of this route versus others which point out the advantages. These advantages have lead to the development (and successful marketing) of an impressive number of new drugs with both topical (Table 24.4) and systemic (Table 24.5) therapeutic uses. At the same time, this has also lead to new guidelines on the subject being promulgated by the U.S. Food and Drug Administration (FDA) (CDER, 2002) and concerns about end-use safety (Kannisto et al., 2002). These guidelines and concerns will have a direct impact on the development of new drugs, such as those in Table 24.6.

TABLE 24.3 Factors Influencing Selection of the Nasal Route

Major Considerations	Routes of Administration					
	Oral	**IV**	**IM/SC**	**Transdermal**	**Nasal**	**Pulmonary**
Delivery interface to blood	Indirect; absorbed through GI system	Direct bolus administration into vein	Indirect; absorbed from muscular/ subcutaneous tissue	Indirect; absorbed through relatively impermeable skin	Indirect; absorbed through the highly permeable nasal mucosa	Indirect; but drug delivered to a large highly permeable epithelia
Delivery issues and concerns	Subject to digestive process, first-pass metabolism	Requires administration by healthcare professional	Painful injection, may require administration by healthcare professional	Highly variable, slow delivery; potential for skin reactions	Self administration. Requirement of high solubility	Requires deep, slow inhalation of small aerosol particles
Patient convenience	High	Low	Low	Moderate	Moderate to high	Moderate to high
Onset of action	Slow	Rapid	Moderate	Slow	Rapid	Moderate to rapid
Delivery of macromolecules	No	Yes	Yes	No	Yes	Yes
Bioavailability	Low to high	Reference standard	Moderate to high	Low	High	Moderate to high
Dose control	Moderate	Good	Moderate	Poor	Moderate	Moderate to good

Sources: Moren (1993); Durham (2002); Wills and Greenstone (2001).

TABLE 24.4 Marketed Nasal Products (for Topical Activity)

Product	Drug	Indication	Manufacturer
Astelin® Nasal Spray	Azelastine hydrochloride	Treatment of seasonal allergic rhinitis	Wallace Laboratories
Beconse® AQ Nasal Spray	Beclomethasone dipropionate monohydrate	Symptomatic treatment of seasonal and perennial allergic rhinitis	Allen and Hanbury's/ Glaxo Wellcome Inc
Vancenase® AQ Nasal Spray	Beclomethasone dipropionate monohydrate	Symptomatic treatment of seasonal and perennial allergic rhinitis	Schering Plough Corp
Rhinocort® Nasal Inhaler	Budesonide	Management of symptoms of seasonal and perennial allergic rhinitis and non-allergic perennial rhinitis	Astra USA, Inc
Nasalcrom® Nasal Solution	Cromolyn sodium	Symptomatic prevention and treatment of seasonal and perennial allergic rhinitis	Sandoz Pharmaceutical Coop
Adrenalin® Chloride	Epinephrine hydrochloride	Nasal decongestant	Parke Davis
Nasalide® Nasal Solution	Flunisolide	Treatment of seasonal and perennial allergic rhinitis	Dura trading Co LTD
Flonase® Nasal Spray	Fluticasone propronate	Symptomatic treatment of seasonal and perennial allergic rhinitis	Glaxo SmithKline, Inc
Atrovent® Nasal Spray	Ipratropium bromide	Symptomic relief of rhinitis	Boehringer Ingelhein Pharmaceuticals, Inc
Livostin® Nasal Spray	Levocabastine	Treatment of allergic rhinitis	Janssen Research FDN Div/ Johnson and Johnson
Privine® Nasal Spray, Nasal Solution and Nasal Drops	Naphazoline hydrochloride	Prompt and prolonged relief of nasal congestion due to common colds, sinusitis	Ciba Consumer Pharmaceuticals
Flunisolide Nasal Solution	Flunisolide	Nasal Decongestant	Bausch & Lomb
Afrin® Nasal Spray	Oxymetazoline hydrochloride	Temporary relief of nasal congestion associated with colds, hay fever and sinusitis	Schering Plough Healthcare Products
Vicks® Sinex® Regular Decongestant Nasal Spray and Ultra Fine Mist	Phenylephrine hydrochloride	Temporary relief of nasal congestion due to colds, hay fever, upper respiratory allergies or sinitis	Proctor and Gamble
Vick® Vapor Inhaler *OTC	1-Desoxyephedrine	Nasal Decongestant	Proctor and Gamble
Nasonex®	Mometasone	Treatment of seasonal and perennial nasal allergy symptoms	Schering Corp
Nasacort®	Triamcinole acetonide	Treatment of seasonal and perennial allergic rhinitis	Aventis

TABLE 24.5 Marketed Nasal Products (for Systemic Activity)

Product	Drug	Indication	Manufacturer
Stadol NS® Nasal Spray	Butorphanol tartrate	Management of pain including migraine headache pain	Bristol Myers Squibb
Miacalcin® Nasal Spray	Calcitonin-salmon	Treatment of hypercalcemia and osteoporosis	Novartis
DDAVP® Nasal Spray	Demopressin acetate	Diabetes insipidus	Aventis Pharmaceuticals
Migranal® Nasal Spray	Dihydroergotamine mesylate	Treatment of migraine	Novartis
Medihaler-ISO® Spray	Isoproterenol sulfate	Treatment of bronchospasm	3M Pharmaceuticals INC
Nitrolingual® Spray	Nitroglycerin	Prevention of angina pectoris due to coronary artery disease	G Pohl Boskamp GMBH and Co.
Synarel® Nasal Solution	Nafarelin acetate	Central precocious puberty, endometriosis	Roche Laboratories
Nicotrol® Inhalation	Nicotine	Smoking cessation	Pharmacia
Syntocinon® Nasal Spray	Oxytocin	Promote milk ejection in breast feeding mothers	Novartis
Imitrex® Nasal Spray	Sumatriptan	Migrane	Glaxo SmithKline
Relenza® Powder for Inhalation	Zanamivir	Treatment of uncomplicated acute illness due to influenza A and B	Glaxo SmithKline

The target for nasal administration is the nasal cavity, with a volume (in adults) of only 20 ml but a total surface area (in humans) of ~180 cm^2 (National Academy of Sciences, 1958). The cavity surface is covered with a 2- to 4-mm-thick nasal mucosa, composed of both respiratory and olfactory components.

There are three separate mechanisms for transmucosal transport of potential drugs (and, of course, toxicants):

a) Simple diffusion—a nonsaturable mechanism with no carrier or energy involvement
b) Facilitated transport—saturable, with carrier involvement but no energy directly expended, and
c) Active transport—a saturable mechanism that involves both a carrier and energy expenditure

"Nose to Brain" Delivery

- Transport of substances from nasal cavity to the central nervous system (CNS) was observed in the mid-1900s when it was observed that viruses can move from nose to brain via the olfactory pathways.
- A number of studies have also reported the transport of heavy metals from nose to brain via olfactory pathways.
- Studies with tracer materials like potassium ferricyanide, horseradish peroxidase, colloid gold, and albumin have shown the transport of these substances from nose to brain.
- Various low- molecular-weight drugs like estradiol, cephalexin, cocaine, and certain peptides have been shown to reach the cerebral spinal fluid (CSF), the olfactory bulb, and some parts of the brain after nasal administration.

What has increased the utility of the nasal route is the development of strategies for enhancing nasal delivery of drugs. The two major categories of such approaches are manipulation of formulation (by either coadministration with an enzyme inhibitor or an absorption enhancer or use of a

TABLE 24.6 Drugs in Development

Product	Indication	Manufacturer	Development
Nasal Nicotine Spray (NNS)	Smoking Cessation	Pharmacia Corporation	Phase III trials
formoterol, Oxis Tturbuhaler®	Asthma	Astra Zeneca PLC	Phase III trials
Zomig (zolmitriptan)®	Migraine Therapy	Astra Zeneca PLC	NDA Filed
ciches onide	Bronchial-Respiratory	Aventis Pharmaceuticals	Phase III trials
FluMist	Vaccine	Aiviron	Approval Recommended
Inhaled Insulin	Diabetes therapy	Eli Lilly & Co.	Phase II trials
GW	Bronchial-Respiratory	Glaxo Smith Kline	Phase II trials
INS 37217 Intranasal	Bronchial-Respiratory	Inspire Pharmaceuticals	Phase II trials
Beclomethasone	Bronchial-Respiratory	Mediera Pharmaceuticals	Clinical
Salbutanol	Bronchial-Respiratory	Mediera Pharmaceuticals	Clinical
VLA-4 antagonist	Bronchial-Respiratory	Merck & Co., Inc.	Phase II trials
PT-141	Reproductive System Therapy	Palatin Technologies, Inc.	Phase II trials
PA-1806	Bronchial-Respiratory	Patho Genisis Corporation	Clinical
Exubera-inhaled insulin	Diabetes Therapy	Pfizer	Phase III trials
NNS	Smoking Cessation Aid	Pharmacia Corporation	Phase III trials
Iloprost	Cardiovascular Agent	Schering-Plough Corporation	Phase III trials
Salloutanol	Bronchial-Respiratory	Sheffield Pharmaceutical	Phase II trials
Pulmicort	Bronchial-Respiratory	Astra Zeneca PLC	New Labeling Approval
Serevent Dis Kus	Bronchial-Respiratory	Glaxo Smith Kline	New Indication Approval
Nicotrol	Smoking Cessation Aide	Pharmacia	New Formation Approval
Flunisolide	Severe Asthma and Lung Disease	Bausch & Lomb Pharma	Approved
Cromolyn Sodium	Persistent Asthma	Novex Pharma	Approved

bioadhesive system) or by structural modification of the drug molecule (that is, a prodrug approach) (Ugwoke et al., 2001).

Advantage of Formulations

- Increase the permeability of the nasal mucosa by interaction of the formulation components with the nasal membrane in a safe, effective, and reversible manner
- Increase in drug solubility and protection against enzymatic degradation
- Increase in the residence time of the drug in the nasal cavity

Commonly used formulations include:

- Liquid formulations
  - Aqueous solutions
    - Synthetic surfactants
    - Bile salts
    - Phospholipids
    - Cyclodextrins

- Micelles
- Liposomes
- Emulsions
- Polymeric microspheres

24.6.1 Excipients Used in Formulation

Surfactants:

Effect of surfactant on drug absorption across nasal mucosa has been studied since the 1970s. Hirai et al. compared nonionic, anionic, amphoteric synthetic surfactants, and natural anionic surfactants for *in vivo* nasal absorption of insulin:

- A majority of surfactants enhances insulin absorption relative to the extraction of membrane components.
- Enhancing effects correlate with the extraction efficiency of the membrane components by the surfactants.
- Alkyl glycosides constitute a novel class of sugar-derived surfactants used in cosmetics.
- Maltoside derivatives with an alkyl chain length between 12 and 14 enhance insulin absorption at low sufactant concentrations.
- Mechanism involves the loosening of tight junctions and increasing the paracellular transport.
- Enhancement effect produced by surfactant monomers is related to the ability of the surfactant molecules to penetrate and fluidize the lipid bilayers.
- Above the critical micelle concentration (CMC), monomers aggregate into micelles that can soulubilize components, particularly cholesterol and phospholipids.

Mixed micelles:

- Nasal absorption of insulin in the presence of sodium glycocholate (NaGC) and linoleic acid increases relative to the increase by NaGC and linoleic acid alone.
- Mixed micelles of bile salts and fatty acid appear to have a synergistic effect on the absorption of peptides.
- Maximal nasal absorption enhancement of [D-Arg] kyotorphin has been observed with mixed micelles of sodium glycocholate and linoleic acid, an effect greater than that with glycocholate alone.

Bile salts:

- Insulin absorption is enhanced but the effects on the biomembrane are milder.
- Absorption-promoting effects appear to arise from an inhibitory effect on the enzyme degradation of insulin
- Bile salts appeared to be most promising and effective absorption enhancers of peptides and proteins and still remain widely used as permeation enhancers.

Cyclodextrins:

- Cyclodextrins are cyclic oligosaccharides containing a minimum of 6-D-glycopyranose units attached by an alpha-1, 4 linkage.
- They are produced by enzymatic conversion of prehydrolized starch.
- Natural cyclodextrins are designated by a Greek letter—α, β, γ.
- β form is the most soluble of the three.
- The ring structure resembles a truncated cone with characteristic cavity volume.

- The internal surface of cavity has slight hydrophobic properties whereas the outer surface is hydrophilic.
- Cyclodextrins form inclusion complexes with lipophillic molecules.
- Generally appear to be less irritating to the nasal mucosa than bile salts and surfactants.
- Large increase in solubility through complex formation and protection from enzymatic degradation improve nasal absorption of lipophillic drugs.
- Very potent absorption enhancers for hydrophilic peptides that are not complexed.
- Shao et al. (1992) evaluated several cyclodextrins for their absorption-promoting effect on insulin.
- Best results for absorption-promoting effect on insulin were obtained with dimethyl-β-cyclodextrin
- Promoting order correlated well with the extent of nasal mucosal perturbation.

Formulation and mucosal damage:

- Improved absorption involves interactions with the mucosal membrane.
- Proposed enhancement mechanisms are:
  - Extraction of membrane components
  - Penetration and fluidization of membrane
  - Loosening of tight junctions
  - Perturbation of nasal mucociliary clearance system
  - Simultaneous transport of environmental toxins
- Adverse effects have to be of short duration, mild and rapidly reversible.
- Kinetics of lipid and protein extraction from the membrane is measures of the extent of damage evaluated by measuring activity of membrane marker enzymes:
  - Lactate dehydrogenase: cytosolic enzyme related to intracellular damage
  - 5′-Nucleotidase: membrane-bound enzyme, indicator of membrane perturbations
  - Alkaline phosphatase: membrane-bound enzyme, related to membrane damage
- Ideal enhancers include:
  - Pharmacological inertness
  - Nonirritant, nontoxic, and nonallergenic
  - Effect on nasal mucosa should be transient and completely reversible
  - Potent in low concentrations
  - Compatible with other adjuvants
  - Possess no offensive odor or taste
  - Inexpensive and readily available
- The factors influencing mucosal damage include:
  - Drug administration
  - Dose
  - Frequency
  - Interspecies difference
  - Sensitivity toward absorption enhancers
- Clinical signs of nasal irritation studies in rats include:
  - In studies for less than 90 days:
    - Struggling, sneezing, salivation, head shaking, and nose rubbing
  - In studies for more than 90 day:
    - Histological signs of nasal irritation including inflammation of septal and turbinate mucosal surfaces, epithelial and submucosal infiltration of inflammatory cells, purulent exudates, and mucosal hyperplasia.

Zhang and Jiang (2001) recently characterized specific mechanisms for reduction of nasal toxicity of drugs.

Methods to Assess Irritancy and Damage:

- Erythrocytes
  - Used to study the membrane activity of absorption enhancers
- Histology
  - Histological studies of nasal membranes
- Intracellular protein release
  - Index of cellular damage due to exposure to absorption enhancers
- Tolerability
  - These are subjective studies in which individuals report any effects due to the use of enhancers in the formulation. These are blinded studies.
- Cilia A Function
  - Cilia beat frequency is obtained from tissue samples at sacrifice by using video capture systems.
  - Tissues used for ciliary function studies include chicken embryo trachea, cryo-preserved human mucosa taken from sphenoidal sinus, rat nasal mucosa, and, recently, human nasal epithelial cells.

Reported Nasal Irritancies Include:

- Local irritation, burning, and stinging upon both acute and/or chronic administration were reported for Laureth-9, bile salts, and sodium taurodihydrofusidate.
- Slight nasal itch was reported when dimethyl-cyclodextrin was used for nasal insulin delivery.
- Nasal burning and sinusitis were reported during studies involving nasal insulin delivery with glycocholate and methylcellulose.

24.6.2 Delivery Forms

Liquid Nasal Formulation:

- Most widely used dosage forms
- Mainly based on aqueous formulations
- Humidifying effect is convenient because of drying of mucous membranes owing to allergic and chronic diseases.
- Major drawback is the microbiological stability.
- Reduced chemical stability of the drug and short residence time in the nasal cavity are other disadvantages.
- Deposition site and deposition pattern are dependent on delivery device, the mode of administration and the physicochemical properties of formulation.
- Preparations depend on whether administered for local or systemic application.
- Patient compliance, cost effectiveness, and risk assessment

Instillation and Rhinyle Catheter:

- Catheters used for delivery to a defined region
- Combination of an instillation catheter with a Hamilton threaded plunger syringe was used to compare the deposition of drops, nebulizers, and sprays in rhesus monkeys.
- Used only for experimental studies

Drops:

- One of the oldest delivery systems
- Low-cost devices
- Easy to manufacture

- Disadvantages related to microbiological and chemical stability
- Delivered volume cannot be controlled.
- Formulation can be easily contaminated by pipette.

Powder Dosage Forms:

- Dry powders are less frequent in nasal drug delivery
- Major advantages include lack of preservatives and drug stability.
- Prolonged retention times when compared with solutions
- Addition of bioadhesive excipients results in a decreased clearance rate.
- Nasal powders may increase patient compliance, especially for children, if the smell and taste of the drug are unacceptable.

Insufflators and Mono-Dose Powder Inhaler:

- Many insufflators work with predosed powder doses in capsules.
- The use of gelatin capsules enables the filling and application of different amounts of powder.
- In a monodose powder inhaler, pushing a piston results in a precompression of air in chamber.
- The piston pierces a membrane and the expanding air expels air into the nostrils.

Pressurized Metered Dose Inhalers (MDIs):

- They are manufactured by suspending the drug in liquid propellants with the aid of surfactants.
- Physicochemical compatibility between the drug and propellants should be evaluated.
- Phase separation, precipitation, crystal growth, polymorphism, dispersibility, adsorption of drug influence drug-particle size, dose distribution, and deposition pattern.
- Their advantages include:
  - Portability, small size, availability over a wide dose range, dose consistency and accuracy, protection of contents
- Their disadvantages include:
  - Nasal irritation by propellants, depletion of ozone layer by chlorofluorocarbons (CFCs)

Nasal Gels:

- Nasal administration of gels can be carried out by precompression pumps.
- The deposition of gel in the nasal cavity depends on the mode of administration because of its viscosity and poor spreading properties.
- Nasal gel containing vitamin B12 for systemic administration is available in the market.

Patented Nasal Formulations:

West Pharma developed nasal technology (ChiSys) based on the use of chitosan as an absorption enhancer. Chitosan is a natural polysaccharide with bioadhesive properties. It prolongs the retention time of the formulation in the nasal cavity, and it may facilitate absorption through promoting paracellular transport.

24.7 METHODS FOR SAFETY ASSESSMENT OF INHALED THERAPEUTICS

Methods for evaluation of inhalation toxicity should be selected according to the pharmacological and/or the toxicological questions asked, and the design of experiments should specify the delivery

route of a drug to the target sites in the pulmonary system. For example, if an immunological response of the lungs to a drug is in question, then the lymphoid tissues of the lungs should be the major target of evaluation. The following are some of the physiological, biochemical, and pharmacological tests that are applicable for the safety assessment of inhaled medicinal gases, vapors, or aerosols.

Upper respiratory tract (URT) irritation can occur from inhalation of a medicinal gas, vapor, or aerosol. For assessing the potential of an inhalant to cause URT irritation, the mouse body plethysmographic technique (Alarie, 1966, 1981a, 1981b) has proven to be extremely useful. This technique operates on the principle that respiratory irritants stimulate the sensory nerve endings located at the surface of the respiratory tract from the nose to the alveolar region. The nerve endings in turn stimulate a variety of reflex responses (Alarie, 1973; Widdicombe, 1974) that result in characteristic changes in inspiratory and expiratory patterns and, most prominently, depression of respiratory rate. Both the potency of irritation and the concentration of the irritant are positively related to the magnitude of respiratory rate depression. The concentration response can be quantitatively expressed in terms of "RD_{50}," defined as the concentration (in logarithmic scale) of the drug in the air that causes a 50% decrease in respiratory rate. The criteria for positive URT irritation in intact mice exposed to the drug atmosphere are depression in breathing frequency and a qualitative alteration of the expiratory patterns. Numerous experimental results have shown that the responses of mice correlated almost perfectly with those of humans (Alarie et al., 1980; Alarie and Luo, 1984). Thus, this technique is useful for predicting the irritancy of airborne medicinal compounds in humans. From the drug-formulating point of view, an inhalant drug with URT-irritating properties indicates the need for an alternate route of administration. From the industrial hygiene point of view, the recognition of the irritant properties is very important. If a chemical gas, vapor, or aerosol irritates, it has a "warning property." With an adequate warning property a worker will avoid inhaling damaging amounts of the airborne toxicant; without a warning property a worker may unknowingly inhale an harmful amount of the toxicant.

Inhalation of a cardiovascular drug, such as an aerosol of propranolol (a beta-adrenergic receptor agonist), may affect the respiratory cycle of a subject. For evaluating the cardiopulmonary effects of an inhalant, the plethysmograph technique using a mouse or a guinea pig model is useful. The criteria for a positive response in intact mice or guinea pigs are changes in the duration of inspiration and expiration and the interval between breaths (Schaper et al., 1989).

Pulmonary sensitization may occur from inhalation of drug vapors such as enflurane (Schwettmann and Casterline, 1976) and antibiotics such as spiramycin (Davies and Pepys, 1975) and tetracycline (Menon and Das, 1977). To detect pulmonary sensitization from inhalation of drug and chemical aerosols, the body plethysmographic technique using a guinea pig model has been useful (Patterson and Kelly, 1974; Karol, 1988; Karol et al., 1989; Thorne and Karol, 1989). The criteria for positive pulmonary sensitization in intact guinea pigs are changes in breathing frequency and their extent and the time of onset of an airway-constrictive response after induction and after a challenge dose of the test drug (Karol et al., 1989).

The mucociliary transport system of the airways can be impaired by respiratory irritants, local analgenesics and anesthetics, and parasympathetic stimultants (Pavia, 1984). Any one of the agents above will retard the beating frequency of the cilia and the secretion of the serous fluid of the mucous membranes. As a result, the propulsion of the inhaled particles, bacteria, or endogenous debris toward the oral pharynx for expectoration or swallowing will be retarded. Conversely, inhalation of adrenergic agonists increases the activity of the mucociliary transport system and facilitates the elimination of noxious material from the pulmonary system. Laboratory evaluation of the adverse drug effects on mucociliary transport in animal models can be achieved by measuring the velocity off the linear flow of mucus in the trachea of surgically prepared animals (Rylander, 1966). Clinically, the transportation of markers placed on the tracheal epithelium of healthy human subjects can also be observed by using a fiber-optic bronchoscopic technique (Pavia et al., 1980; Mussatto et al., 1988). The criteria of a positive response are changes in the transport time over a given distance of markers placed on the mucus or changes in the rate of mucus secretion (Davis et al., 1976; Johnson et al., 1983, 1987;

Webber and Widdicombe, 1987). More comprehensive discussion on mucociliary clearance can be found in several reviews (Last, 1982; Pavia, 1984).

Cytological studies on the bronchial alveolar lavage fluid (BALF) permit the evaluation of the effects of an inhaled drug on the epithelial lining of the respiratory tract. This fluid can be obtained form intact animals or from excised lungs (Henderson, 1984, 1988, 1989). Quantitative analyses of fluid constituents such as neutrophils, antibody-forming lymphocytes, and antigen-specific immunoglobulin G (IgG) provide information on the cellular and biochemical responses of the lungs to the inhaled agent (Henderson, 1984; Henderson et al., 1985, 1987). For example, BALF parameters were found to be unperturbed by the inhalation of halothane (Henderson and Loery, 1983). The criteria of a positive response are increase in protein content, increase in number of neutrophils and macrophages for inflammation, increase in number of lymphocytes and alteration of lymphocyte profiles for immune response, increase in cytoplasmic enzymes (lactate dehydrogenease) for cell lysis (Henderson, 1989), and the presence of antigen-specific antibodies for specific immune responses (Bice, 1985).

Morphological examination of the cellular structure of the pulmonary system is the foundation of most inhalation toxicity studies. Inhalation of airborne drug vapors or aerosols at harmful concentrations results mainly in local histopathological changes in the epithelial cells of the airways, of which there are two types: nonciliated and ciliated cells. The nonciliated cells are the Clara cells, which contain secretory granules with smooth endoplasmic reticulum (SER); the secretory granules, which lack SER; and the brush cells, which have stubby microvilli and numerous cytoplasmic fibers on their free surfaces. If the concentration gradient of the drug in the lung is high enough to reach the alveoli, the type I alveoli cells will also be affected (Evans, 1982). Drugs that affect the lungs via the bloodstream, such as bleomycin (Aso et al., 1976), cause changes to the endothelial cells of the vascular system that result in diffuse damage to the alveoli. The criteria of cellular damage are loss of cilia, swelling, and necrosis and sloughing of cell debris into the airway lumina. Tissues recovering from injuries are characterized by increases in the number of dividing progenitor cells followed by increases in intermediate cells that eventually differentiate into normal surface epithelium.

Pulmonary drug disposition studies are essential in research and development of new inhalant drugs. Inhaled drugs are usually absorbed and metabolized to some extent in the lungs because the lungs, like the liver, contain active enzyme systems. A drug may be metabolized to an inactive compound for excretion or to a highly reactive toxic metabolite(s) that causes pulmonary damage. In most pulmonary disposition studies, a gas or vapor is delivered via whole-body exposure (Paustenbach et al., 1983) or head-only exposure (Hafner et al., 1975). For aerosols, over 90% of a dose administered by mouth breathing is deposited in the oropharynx and swallowed. Consequently, the disposition pattern reflects that of ingestion in combination with a small contribution from pulmonary metabolism. For determining the disposition of inhaled drugs by the pulmonary system alone, a dosimetric endotracheal nebulization technique (Leong et al., 1988) is useful. In this technique, microliter quantities of a radiolabeled drug solution can be nebulized within the trachea using a miniature air-liquid-nebulizing nozzle. Alternatively, a small volume of liquid can be dispersed endotrachially by using a microsyringe. In either technique, an accurate dose of a labeled drug solution is delivered entirely into the respiratory tract and lungs. Subsequent radioassay of the excreta thus reflects only the pulmonary disposition of the drug without complication from aerosols deposited in the oropharyngeal regions as would be the case if the drug had been delivered by mouth inhalation. For example, in a study of the antiasthmatic drug lodoximide tromethamine, the urinary metabolites produced by beagle dogs after receiving a dose of the radiolabeled drug via endotracheal nebulization showed a high percentage of the intact drug. However, metabolites produced after oral administration were mainly nonactive conjugates. The differences were due to the drug's escape from first-pass metabolism in the liver when it was administered through the pulmonary system. The results thus indicated that the drug had to be administered by inhalation to be effective. This crucial information was extremely important in the selection of the most effective route of administration and formulation of this antiasthmatic drug (Leong et al., 1988).

Cardiotoxicity of inhalant drugs should also be evaluated. For example, adverse cardiac effects may be induced by inhaling vapors of fluorocarbons, which are used extensively as propellants in drug aerosols. Inhalation of vapors of anesthetics have also been shown to cause depression of the heart rate and alteration of the rhythm and blood pressure (Merin, 1981; Leong and Rop, 1989). More importantly, inhalation of antiasthmatic aerosols of beta-receptor agonists delivered in a fluorocarbon propellant have been shown to cause marked tachycardia, electrocardiogram (ECG) changes, and sensitization of the heart to arrhythmia (Aviado, 1981; Balazs, 1981). Chronic inhalation of drug aerosols can also result in cardiomyopathy (Balazs, 1981). For detection of cardiotoxicity, standard methods of monitoring arterial pressures, heart rate, and ECGs of animals during inhalation of a drug, or at frequent intervals during a prolonged treatment period, should be useful in safety assessments of inhalant drugs.

Since the inhalation route is just a method for administering drugs, other nonpulmonary effects, such as behavioral effects (Ts'o et al., 1975), and renal and liver toxicity should also be evaluated. In addition, attention should also be given to drugs that are not administered via the inhalation route but that accumulate in the lungs where they cause pulmonary damage (Wilson, 1982).

24.8 PARAMETERS OF TOXICITY EVALUATION

Paracelsus stated over 400 years ago that "All substances are poison. The right dose differentiates a poison and a remedy." Thus, in safety assessments of inhaled drugs, the "dose," or magnitude of inhalation exposure, in relation to the physiological, biochemical, cytological, or morphological response(s) must be determined. Toxicity information is essential to establishing guidelines to prevent the health hazards of acute or chronic overdosage during therapy, or of unintentional exposure to the bulk drugs and their formulated products during manufacturing and industrial handling.

24.8.1 The Inhaled "Dose"

Most drugs are designed for oral or parenteral administration in which the dose is calculated in terms of drug weight in milligrams (mg) divided by the body weight in kilograms (kg):

$$\text{Dose} = \frac{\text{drug weight (mg)}}{\text{body weight (kg)}} = \text{mg / kg}$$

For inhalant drugs, the inhaled "dose" has been expressed in many mathematical models (Dahl, 1990). However, the practical approach is based on exposure concentration and duration rather than on theoretic concepts. Thus, an inhaled "dose" is expressed in terms of the exposure concentration (C) in milligrams per liter (mg/l) or milligrams per cubic meter (mg/m^3) or, less commonly, parts per million (ppm) parts of air, the duration of exposure (t) in minutes, the ventilatory parameters including the respiratory rate (R) in number of breaths per minute and the tidal volume (Tv) in liters per breath, and a dimensionless retention factor α (alpha), which is related to the reactivity and the solubility of the drug. The product of these parameters divided by the body weight in kilograms gives the dose:

$$\text{Dose} = \frac{C \cdot t \cdot R \cdot Tv \cdot \alpha}{\text{body weight}} = \text{mg / kg}$$

The dosimetric method has been recommended in critical evaluation of the effect of a gas, vapor, or aerosol inhaled in to the respiratory tract of an animal (Oberst, 1961). However, because of the complexity of measuring the various parameters simultaneously, only a few studies on gaseous drugs or chemicals have employed the dosimetric method (Weston and Karel, 1946; Leong and

MacFarland, 1965; Landy et al., 1983; Stott and McKenna, 1984; Dallas et al., 1986, 1989). For studies on liquid or powdery aerosols, modified techniques such as intratracheal instillation (Brain et al., 1976) or endotracheal nebulization (Leong et al., 1988) were used to deliver an exact dose of the test material into the lower respiratory tract (LRT) while bypassing the URT and ignoring the ventilatory parameters.

In routine inhalation studies, it is generally accepted that the respiratory parameters are relatively constant when the animals are similar in age, sex, and body weight. This leaves only C and t to be the major variables for dose consideration.

$$\text{“Dose”} = C \cdot t = \text{mg·min/l}$$

The product Ct is not a true dose because its unit is mg·min/l rather than mg/kg. Nevertheless, Ct can be manipulated as though it were a dose, an approximated dose (MacFarland, 1976).

The respiratory parameters of an animal will dictate the volume of air inhaled and hence the quantity of test material entering the respiratory system. Commonly used parameters for a number of experimental species and humans are given in Table 24.7 to illustrate this point and include the alveolar surface area because this represents the target tissue for most inhaled materials. It can be seen that by taking the ratios of these parameters and comparing the two extremes, i.e., the mouse and humans, that a mouse inhales approximately 30 times its lung volume in one minute whereas a man at rest inhales approximately the same volume as that of his lung (McNeill, 1964). This can increase with heavy work up to the same ratio as the mouse but is not sustained for long periods. This means that the dose per unit lung volume is up to 30 times higher in the mouse than in humans at the same inhaled atmospheric concentration (Touvay and Le Mosquet, 2000). The minute volume of the mouse is in contact with five times less alveolar surface area than humans; hence, the dose per unit area is up to five times greater in the mouse (Akoun et al., 1989). The lung volume in comparison with the alveolar surface area in experimental animals is less than in humans, meaning that the extent of contact of inhaled gases with the alveolar surface is greater in experimental animals.

Although it is possible, and common, to refer to standard respiratory parameters for different species to calculate inhaled dose and deposited dose with time, it is usually the case that inhaled materials influence the breathing patterns of test animals. The most common examples of this are irritant vapors, which can reduce the respiratory rate by up to 80%. This phenomenon results from a reflexive pause during the breathing cycle due to stimulation by the inhaled material of the trigeminal nerve endings situated in the nasal passages. The duration of the pause and hence the reduction in the respiratory rate are concentration related, permitting concentration–response relationships to be plotted. This has been investigated extensively by Alarie (1981a) and forms the basis of a test screen for comparing quantitatively the irritancy of different materials; it has found application in assessing appropriate exposure limits for human exposure when respiratory irritancy is the predominant cause for concern.

While irritancy resulting from the reflex reaction above is one cause of altered respiratory parameters during exposure, there are many others. These include other types of reflex response, such as bronchoconstriction, the narcotic effects of many solvents, the development of toxic signs as exposure progresses, or simply a voluntary reduction in respiratory rate by the test animal due to the unpleasant nature of the inhaled atmosphere. The extent to which these affect breathing patterns and hence inhaled dose can only be assessed by actual measurement.

By simultaneous monitoring of tidal volume and respiratory rate, or minute volume, and the concentration of an inhaled vapor in the bloodstream and the vapor in the exposure atmosphere, pharmacokinetic studies on the $C{\cdot}t$ relationship have shown that the effective dose was nearly proportional to the exposure concentration for vapors such as 1,1,1-trichloroethane (Dallas et al., 1983), which has a saturable metabolism, found that the steady-state plasma concentrations were disproportionally greater at higher exposure concentrations.

TABLE 24.7 Respiratory Parameters for Common Experimental Species and Man

Species	Body weight (kg)	Lung volume (ml)	Minute volume (ml min^{-1})	Alveolar surface area (m^2)	Lung volume % surface area	Minute volume % lung volume	Minute volume % surface area
Mouse	0.023	0.74	24	0.068	10.9	32.4	353
Rat	0.14	6.3	84	0.39	16.2	13.3	215
Monkey	3.7	184	694	13	14.2	3.77	53
Dog	22.8	1501	2923	90	16.7	1.95	33
Human	75	7000	6000	82	85.4	0.86	73

Source: From Altman and Ditmar (1974).

Acknowledging the possible existence of deviations, this simplified approach of using C and t for dose determination provides that basis for dose—response assessments in practically all inhalation toxicological studies.

24.8.2 The Dose–Response Relationship

Often overlooked is that dose–response in toxicology has multiple dimensions. The reader should start by recognizing that at least three dimensions are present, and all must be considered to understand a biological system response. As dose increases:

- Incidence of responders in an exposed population increases (population incidence)
- Severity of response in affected individuals increases (severity)
- Time to occurrence of response or of progressive stage of response decreases (lag time)

The oldest principle of dose–response determination in inhalation toxicology is based on Haber's rule, which states that responses to an inhaled toxicant will be the same under conditions where C varies in complementary manner to t (Haber, 1924). For example, if $C{\cdot}t$ elicits a specific magnitude of the same response; that is, $Ct = K$, where K is a constant for the stated magnitude of response.

This rule holds reasonably well when C or t varies within a narrow range for acute exposure to a gaseous compound (Rinehart and Hatch, 1964) and for chronic exposure to an inert particle (Henderson et al., 1991). Excursion of C or t beyond these limits will cause the assumption $Ct = K$ to be incorrect (Adams et al., 1950, 1952; Sidorenko and Pinigin, 1976; Andersen et al., 1979; Uemitsu et al., 1985). For example, an animal may be exposed to 1000 ppm of diethyl ether for 420 min or 1400 ppm for 300 min at a constant rate without incurring any anesthesia. However, exposure to 420,000 ppm for 1 min will surely cause anesthesia or even death of the animal. Furthermore, toxicokinetic study of liver emzymes affected by inhalation of carbon tetrachloride (Uemitsu et al., 1985), which has a saturable metabolism in rats, showed that $Ct = K$ does not correctly reflect the "toxicity value" of this compound. Therefore, the limitations of Haber's rule must be recognized when it is used in interpolation or extrapolation of inhalation toxicity data.

24.8.3 Exposure Concentration versus Response

In certain medical situations (e.g., a patient's variable exposure duration to a surgical concentration of an inhalant anesthetic, or the repeated exposures of surgeons and nurses to subanesthetic concentrations of an anesthetic in the operating theater) it is necessary to know the duration of safe exposure to a drug. Duration safety can be assessed by determining a drug's median effective time (Et_{50}) or median lethal time (Lt_{50}). These statistically derived quantities represent the duration of exposure required to affect or kill 50% of a group of animals exposed to a specified concentration of an airborne drug or chemical in the atmosphere. This is illustrated in Figure 24.3.

The graph in Figure 24.4 is the probit plot of cumulative percentage response to logarithm of exposure duration. It shows the 1000 mg/m^3 for 10 h or to 10 mg/m^3 for 1000 h, each with a Ct (an approximated dose) of ~10,000 h mg/m^3. Similar to concentration–response graphs, the slopes indicate the differences in the mechanism of action and the margins of safe exposure of the three drugs. The ratio of the ET_{50} or LT_{50} of two drugs indicates their relative toxicity, and the ratio of ET_{50} over LT_{50} of the same drug is the therapeutic ratio.

24.8.4 Product of Concentration and Duration (Ct) versus Responses

To evaluate inhalation toxicity in situations where workers are exposed to various concentrations and durations of a drug vapor, aerosol, or powder in the work environment during manufacturing or packaging, a more comprehensive determination of $E(Ct)_{50}$ or $L(Ct)_{50}$ values are used. The $E(Ct)_{50}$ or $L(Ct)_{50}$ values are statistically derived values that represent the magnitude of exposure, expressed

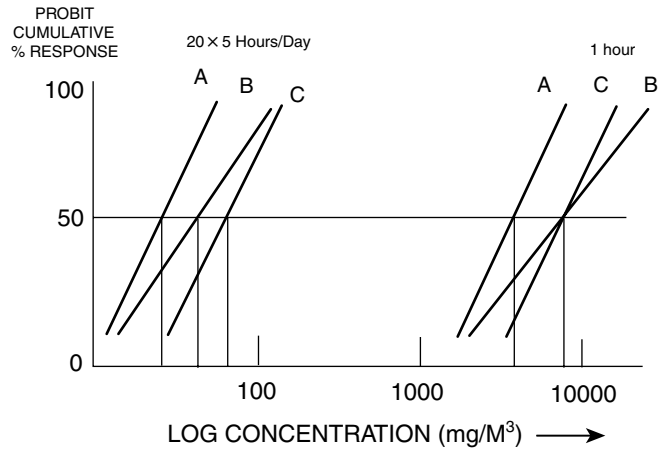


FIGURE 24.3 Dose–response is plotted in terms of the probit of cumulative percentage response to logarithm of the exposure concentrations, where A, B, and C are agents acting by different mechanisms or kinetics.

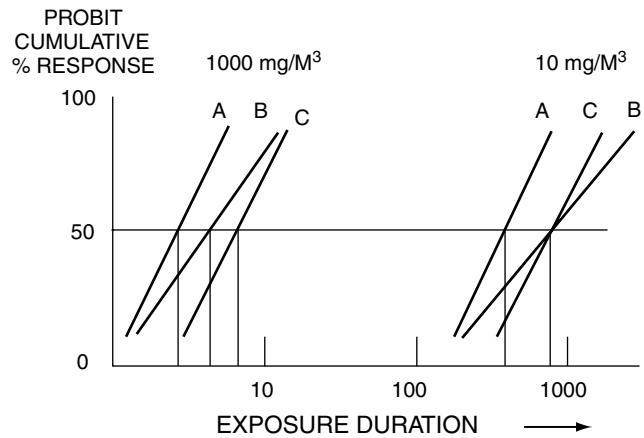


FIGURE 24.4 Dose–response plotted in terms of the probit plot of cumulative percentage response to logarithm of exposure duration.

as a function of the product of C and t, that is expected to affect or kill approximately 50% of the animals exposed. The other curve represents exposures that kill 50% or >50% of each group of animals (Irish and Adams, 1940).

The graph in Figure 24.5 illustrates inhalation exposures to a drug using various combinations of C and t that kill 50% of the animals. For example, a 50% mortality occurs when a group of animals is exposed to drug A at a concentration of 1000 mg/m^3 for a duration of approximately 2 h or at a concentration of 100 mg/m^3 for a duration of approximately 20 h. Furthermore, the graph also illustrates that the inhalation toxicity of drug A is more than one order of magnitude higher than that of drug B. For example, an exposure to drug A at the concentration of 100 mg/m^3 for 100 h kills 100% of the animals, whereas an exposure to drug B at the concentration of 1000 mg/m^3 for 100 h does not kill any animals.

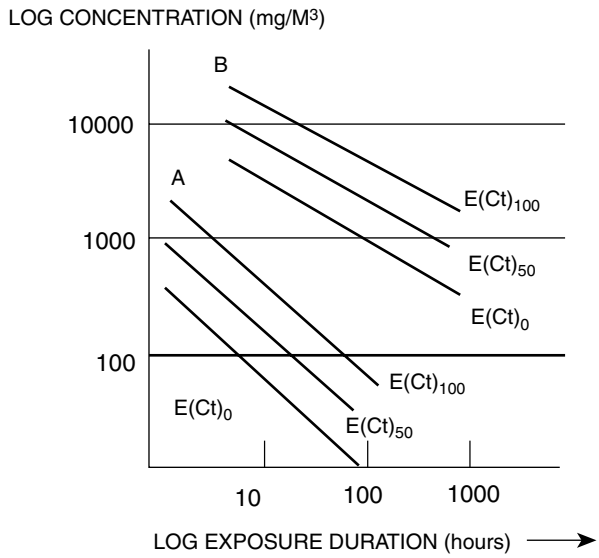


FIGURE 24.5 Dose–response plotted in terms of logarithms of exposure concentration and durations.

24.8.5 Units for Exposure Concentration

For gases and vapors, exposure concentrations are traditionally expressed in parts per million (ppm). The calculation for the ppm of a gas or vapor in an air sample is based on Avogadro's law, which states that "Equal volumes contain equal numbers of molecules under the same temperature and pressure." In other words, under standard temperature and pressure (STP), one gram-molecular weight (mole) of any gas under a pressure of one atmosphere (equivalent to the height of 760 mm mercury) and a temperature of 273 K has the same number of molecules and occupies the same volume of 22.4 l. However, under ambient conditions, the volume of 22.4 l has to be corrected to a larger volume based on Charles' law, which states that at constant pressure the volume of gas varies directly with the absolute temperature. Thus, at a room temperature of 25°C, one mole of a gas occupies a volume of 24.5 l.

$$22.4\,\text{l} \times \frac{298\ \text{K}}{273\ \text{K}} = 24.5\,\text{l}$$

Further correction of volume for an atmospheric pressure deviation from one atmosphere may be done by applying Boyle's Law, which states that the volume of a gas without change of temperature varies inversely with the pressure applied to it.

$$24.5\,\text{l} \times \frac{758\ \text{mmHg}}{760\ \text{mmHg}} = 24.4\,\text{l}$$

In practice, atmospheric pressure in most animal experimental environments usually varies only a few mm Hg, so little or no correction is required.

Using the aforementioned principles, the volume of a vapor generated from a given weight of a liquid can be calculated. For example, 1 mole of water weighs 18 g, whereas 1 mole of ethanol

weighs 46 g. When 1 mole of each liquid is totally vaporized, each will occupy the same volume of 24.5 l at room temperature (25°C) and pressure (760 mmHg). In an inhalation experiment, if the volume of test liquid and the rate of airflow being mixed in the animal exposure chamber are known, the vapor concentration in the chamber atmosphere can be calculated in parts per million or milligrams per liter. A conversion table published by the U.S. Bureau of Mines enables quick conversion between parts per million and milligrams per liter for compounds with molecular weights up to 300 g (Fieldner et al., 1921; Patty, 1958).

For aerosols of nonvolatile liquid and powder compounds, the concentration of the mist or dust atmosphere must be expressed in terms of milligrams per liter or milligrams per cubic meter (mg/m^3) of air. With advances in biotechnology, many pharmacological testing techniques are based on specific receptor bindings, in which the ratio of the number of molecules to those of the receptors are considered, in which case the exposure concentration may be more appropriately expressed in micromoles per unit volume of air ($\mu mol/m^3$).

24.9 RESPIRATORY SAFETY PHARMACOLOGY

Pharmaceuticals differ from industrial and environmental chemicals in that the scope of concern for their adverse safety effects on the respiratory system extend both to reversible functional degradations and to effects on the respiratory systems functionality due to systemically distributed agents administered by routes other than direct respiratory. This is the realm of the relatively new field of safety pharmacology.

As early as 1964, it became apparent that beta-adrenergic blocking agents could lead to bronconstriction (and possible death) in individuals with asthma (McNeill, 1964). Since then, many similar adverse effects have been identified. These known effects of drugs from a variety of pharmacological/therapeutic classes on the respiratory system are summarized in Tables 24.8, 24.9, and 24.10. Resulting worldwide regulatory requirements (Tables 24.11 and 24.12) require the conduct of prescribed respiratory evaluations prior to drug in humans. The objective of such studies is to evaluate the potential for drugs to cause nonintended pharmacological or toxicological effects that influence respiratory function. Changes in respiratory function can result either from alterations in the pumping apparatus that controls the pattern of pulmonary ventilation or from changes in the mechanical properties of the lung that determine the transpulmonary pressures (work) required for lung inflation and deflation.

An example set of data for some standard positive controls is shown in Table 24.13. Note that each of the different agents has a separate pattern of effects.

The respiratory system is responsible for generating and regulating the transpulmonary pressures needed to inflate and deflate the lung. Normal gas exchange between the lung and blood requires breathing patterns that ensure appropriate alveolar ventilation. Ventilatory disorders that alter alveolar ventilation are defined as hypoventilation or hyperventilation syndromes. Hyperventilation results in an increase in the partial pressure of arterial CO_2 above normal limits and can lead to acidosis, pulmonary hypertension, congestive heart failure, headache, and disturbed sleep. Hyperventilation results in a decrease in the partial pressure of arterial CO_2 below normal limits and can lead to alkalosis, syncope, epileptic attacks, reduced cardiac output, and muscle weakness.

Normal ventilation requires that the pumping apparatus provide both adequate total pulmonary ventilation (minute volume) and the appropriate depth (tidal volume) and frequency of breathing. The depth and frequency of breathing required for alveolar ventilation are determined primarily by the anatomic deadspace of the lung. In general, a rapid shallow breathing pattern (tachypnea) is less efficient than a slower deeper breathing pattern that achieves the same minute volume. Thus, any change in minute volume, tidal volume, or the rate of breathing can influence the efficiency of ventilation (Milic-Emili, 1982). The inspiratory and expiratory phases of individual breath rates of airflow and durations are distinct and independently controlled (Boggs, 1992). Thus, by characterizing changes in the airflow rate and duration of each of these phases, mechanisms responsible for changes in tidal volume or respiratory rate can be identified (Milic-Emili, 1982; Indans, 2002).

TABLE 24.8 Agents Known to Cause Pulmonary Disease

Chemotherapeutic

Cytotoxic
- Azathioprine
- Bleomycin*
- Busulfan
- Chlorambucil
- Cyclophosphamide
- Etoposide
- Melphalan
- Mitomycin*
- Nitrosoureas
- Procarbazine
- Vinblastine
- Ifosfamide

Noncytotoxic
- Methotrexate*
- Cytosine arabinoside*
- Bleomycin*
- Procarbazine*

Antibiotic
- Amphotericin B*
- Nitrofurantoin
  - Acute*
  - Chronic
- Sulfasalazine
- Sulfonamides
- Pentamidine

Anti-inflammatory
- Acetylsalicylic acid*
- Gold
- Methtrexate
- Nonsteroidal anit-inflammatory
- Penicillamine* agents

Immunosuppressive
- Cyclosporin
- Interleukin-2*

Analgesics
- Heroin*
- Methadone*
- Noloxone*
- Ethchlorvynol*
- Propoxyphene*
- Salicylates*

Cardiovascular
- Amiodarone*
- Angiotensin-converting enzyme inhibitors

Anticoagulants
- Beta-blockers*
- Dipyridamole
- Fibrinolytic agents*
- Protamine*
- Tocainide

Inhalants
- Aspirated oil
- Oxygen*

Intravenouse
- Blood*
- Ethanolamine oleate (sodium morrhuate)*
- Ethiodized oil (lymphangiogram)
- Talc
- Fat emulsion

Miscellaneous
- Bromocripitine
- Hydrochlorothiazide*
- Methysergide
- Oral contraceptives
- Tocolytic agents*
- Tricyclics*
- L-Tryptophan
- Radiation
- Systemic lupus erythematosus (drug-induced)*
- Complement-mediated leukostasis*

* Typically cause acute or subacute respiratory insufficiency.

Sources: Toubay and Mosquet (2000); Akoun et al. (1989); Dorato (1994); Lalej-Bennis et al. (2001); Mauderly (1989); Rosnow et al. (1992).

TABLE 24.9 Drugs That Adversely Affect Respiratory Function

Drugs Known to cause or Aggravate Bronchospasm	Agents Associated with Pleural Effusion
Vinblastine	Chemotherapeutic agents
Nitrofurantoin (acute)	Nitrofurantoin (acute)
Acetylsalicylic acid	Bromocriptine
Nonsteroidal anti-inflammatory agents	Dantrolene
Interleukin-2	Methysergide
Beta-Blockers	L-Tryptophan
Dipyridamole	Drug inducing systemic lupus erythematosus
Protamine	Tocolytics
Nebulized pentamidine, beclomethasone, and propellants	Amiodarone
Hydrocortisone	Esophageal variceal sclerotherapy agents
Cocaine	Interleukin-2
Propafenone	

Agents Associated with Acute-Onset Pulmonary Insufficiency*	Agents that Cause Subacute Respiratory Failure
Bleomycin plus O_2	Chemotherapeutic agents
Mitocycin	Nitrofurantoin (chronic)
Bleomycin†	Amiodarone
Procarbazine†	L-Trytophan
Methotrexate†	Drug inducing systemic lupus erythematosus
Amphotericin B	
Nitrofurantoin (acute)‡	
Acetylsalicylic acid‡	
Interleukin-2‡	
Heroin and other narcotics‡	
Epinephrine‡	
Ethchlorvynol‡	
Fibrinolytic agents	
Protamine	
Blood products‡	
Fat emulsion	
Hydrochlorothiazide	
Complement-mediated leukostasis	
Hyskon (dextran-70)‡	
Tumor necrosis factor‡	
Intrathecal methotrexate	
Tricyclic antidepressants‡	
Amiodarone plus O_2	
Naloxone	

* Onset at less than 48 h.
† Associated with hypersensitivity with eosinophilia.
‡ Usually reversible within 48–72 h, implying noncardiac pulmonary edema rather than inflammatory interstitial pneumonitis.
Sources: McNeil (1964); Borison (1977); Tattersfield (1986); Illum (1992); Shao et al. (1992); Shao and Mitra (1992); Fariba (2000).

TABLE 24.10 Drugs Known to Influence Ventilatory Control

Depressants	Stimulants
Inhaled anesthetics	Alkaloids
Barbiruartes	Nicotine
Benzodiazepines	Lobeline
Diazepam	Piperdine
Temazapan	Xanthine analogs
Chlordiazepoxide	Theophyline
Serotonin analogs	Caffeine
Methoxy-(dimethyl)-tryptamine	Theobromine
Dopamine analogs	Analeptics
Apomorphine	Doxapram
Adenosine analogs	Salicylates
2-Chloroadenosine	Progestrone analogs
R-Phenylisopropyl-adenosine	Almitrine
(R-PIA)	Glycine analogs
N-Ethylcarboxamide (NECA)	Strychnine
B-Adrenergic antagonists	GABA antagonists
Timolol maleate	Picrotoxin
GABA analogs	Bicuculline
Muscimol	Serotonin synthesis inhibitors
Baclofen	*p*-Chlorophenylalanine
Opiates	Reserpine
Morphine	
Codeine	
Methadone	
Meperidine	
Phenazocine	
Tranquilizers/analgesics	
Chlorpromazine	
Hydroxyzine	
Rompum (xylazine)	
Nalorphine	

For example, a decease in airflow during inspiration (the active phase) is generally indicative of a decrease in respiratory drive, while a decrease in airflow during expiration (the passive phase) is generally indicative of an obstructive disorder.

Mechanisms of ventilatory disorders can also be characterized as either central or peripheral. Central mechanisms involve the neurological components of the pumping apparatus that are located in the central nervous system and include the medullary central pattern generator (CPG) and integration centers located in the medulla, pons, hypothalamus, and cortex of the brain that regulate the output of the CPG (Boggs, 1992). The major neurological inputs from the peripheral

TABLE 24.11 Required Respiratory System Safety Pharmacology Evaluation

Respiratory functions	
Measurement of rate and relative tidal volume in conscious animals	
Pulmonary Function	
Measurement of rate, tidal volume and lung resistance and compliance in anaesthetized animals	

TABLE 24.12 Regulatory Documents Recommending Respiratory Function Testing in Safety Pharmacology Studies

U.S.A.	FDA Guideline for the Format and Content of the Nonclinical Pharmacology/Toxicology Section of an Application (Section IID, p. 12, Feb. 1987)
Japan	Ministry of Health and Welfare Guidelines for Safety Pharmacology Studies Required for the Application for Approval to Manufacture (Import) Drugs. Notification YAKUSHIN-YAKU No. 4, Jan 1991.
Australia	Guidelines for preparation and presentation of Applications for Investigational Drugs and Drug Products Under the Clinical Trials Exemption Scheme (STET 12, 15).
Canada	RA5 Exhibit 2, Guidelines for Preparing and Filing Drug Submissions (p. 21).
U.K.	Medicines Act 1968, Guidance Notes on Applications for Product Licenses (MAL 2, p. A3F-1).

TABLE 24.13 Functional Respiratory Responses to Standard Pharmacologic Agents[a]

Parameters	Theophyline 10 mg/kg PO	Pentobarbital 35 mg/kg IP	Diazapam 35 mg/kg IP	Codeine 100 mg/kg IP
F(breaths/min)	+ + +	− − −	− − −	No Change
TV (ml)	No Change	No Change	No Change	−
Ti (s)	− −	+ +	+ +	+
Te (s)	− −	+ + +	+ +	−
PIF (ml/s)	+ +	−	−	−
PEF (ml/s)	+ +	No Change	+	−
Penh	−	+	+	No Change

[a] F, frequency; TV, tidal volume; Ti, inhalation duration; Te, exhalation duration; PIF, pulmonary inhalation flow rate; PEF, pulmonary exhalation flow rate; Penh, pulmonary efficiency; +, is an increase; −, decrease; s, seconds.

Source: Touvay and Le Mosquet (2000).

nervous system that influence the CPG are the arterial chemoreceptors (Boggs, 1992). Many drugs stimulate or depress ventilation by selective interaction with the CNS (Eldridge and Millhorn, 1981; Keats, 1985; Mueller et al., 1982) or arterial chemoreceptors (Heymans, 1955; Heymans and Niel, 1958).

Defects in the pumping apparatus are classified as hypo- or hyperventilation syndromes and are best evaluated by examining ventilatory parameters in a conscious animal model. The ventilatory parameters include respiratory rate, tidal volume, minute volume, peak (or mean) inspiratory flow, peak (or mean) expiratory flow, and fractional inspiratory time. Defects in mechanical properties of

the lung are classified as obstructive or restrictive disorders and can be evaluated in animal models by performing flow-volume and pressure-volume maneuvers, respectively. The parameters used to detect airway obstruction include peak expiratory flow, forced expiratory flow at 25 and 75% of forced vital capacity, and a timed forced expiratory volume, while the parameters used to detect lung restriction include total lung capacity, inspiratory capacity, functional residual capacity, and compliance. Measurement of dynamic lung resistance and compliance, obtained continuously during tidal breathing, is an alternative method for evaluating obstructive and restrictive disorders, respectively, and is used when the response to drug treatment is expected to be immediate (within minutes post-dose). The species used in the safety pharmacology studies are the same as those generally used in toxicology studies (rats and dogs) because pharmacokinetic and toxicological/pathological data are available in these species. These data can be used to help select test measurement intervals and doses and to aid in the interpretation of functional change. The techniques and procedures for measuring respiratory function parameters are well established in guinea pigs, rats, and dogs (Murphy, 1994; Amdur and Mead, 1958; Diamond and O'Donnell, 1977; King, 1966; Mauderly, 1974).

The key questions in safety pharmacology of the respiratory system are:

- Does the substance affect the mechanisms of respiratory control (central or peripheral) leading to hypoventilation (respiratory depression) or hyperventilation (respiratory stimulation)?
- Does the substance act on a component of the respiratory system to induce, for example, bronchospasm, obstruction, or fibrosis?
- Does the substance induce acute effects or can we expect chronic effects?
- Are the effects observed dose dependent or independent?

24.9.1 Plethysomography

The classic approach to measuring respiratory function in laboratory animals is plethysomography. It has two basic governing principles (Boggs, 1992; O'Neil Raub, 1984; Palecek, 1969; Brown and Miller, 1987).

- The animal (mice, rat, or dog), anesthetized or not, restrained or not, is placed in a chamber (single or double) with pneumotachographs.
- The variations of pressure in chamber(s) at the time of the inspiration and the expiration make it possible to obtain the respiratory flow of the animal.

There are three main types of body plethysmographs: constant volume, constant pressure, and pressure volume. The constant-volume body plethysmograph is a sealed box that detects volume change by measurement of pressure changes inside the box. While inside the plethysmograph, inhalation of room air (from outside the plethysmograph) by the test animal induces an increase in lung volume (chest expansion) and thus an increase in plethysmograph pressure. On the other hand, exhalation to the atmosphere (outside the plethysmograph) induces a decrease in the plethysmograph pressure. The magnitude of lung volume change can be obtained via measurement of the change in plethysmographic pressure and the appropriate calibration factor. The plethysmograph is calibrated by injecting or withdrawing a predetermined change in box pressure. To avoid an adiabatic artifact, the rate of air injection or withdrawal is kept the same as that of chest expansion, indicated by the same dP/dt.

The constant-pressure body plethysmograph is a box with a pneumotachograph port built into its wall. This plethysmograph detects volume change via integration of the flow rate, $\int \Delta \text{Flow}$, which is monitored by the pneumotachograph port. There is an outward flow (air moving from the plethysmograph to the atmosphere) during inspiration and inward flow during expiration. Alternatively, in place of a pneumotachograph, a spirometer can be attached to the constant pressure plethysmograph

to detect volume changes. For detection of plethysmographic pressure and flow rate, sensitive pressure transducers are usually employed. It is important that the transducer be capable of responding to volume changes in a linear fashion within the volume range studied. The plethysmograph should have negligible leaks and temperature should not change during all respiratory maneuvers. The plethysmograph should also have linear characteristics with no hysteresis. Dynamic accuracy requires an adequate frequency response. A fast integrated flow plethysmograph, with a flat amplitude response for sinusoidal inputs up to 240 Hz, has been developed for rats, mice, and guinea pigs (Sinnet et al., 1981). Similar plethysmographs can also be provided for use with large mammals.

A third type of pressure-volume plethysmograph has the mixed characteristics of the two types of body box mentioned above. For a constant-pressure plethysmograph, the change in volume at first is associated with gas compression or expansion. This fraction of the volume change can be corrected by electronically adding the plethysmographic pressure change to the volume signal. Therefore, the combined pressure-volume plethysmograph has excellent frequency–response characteristics and a wide range of sensitivities (Leigh and Mead, 1974).

If volume, flow rate, and pressure changes are detected at the same time, several respiratory variables can be derived simultaneously from the raw signals. The whole-body plethysmograph method can then be used to measure most respiratory variables, such as tidal volume, breathing frequency, minute variables, such as tidal volume, breathing frequency, minute ventilation, compliance, pulmonary resistance, functional residual capacity, pressure-volume characteristics, and maximal expiratory flow-volume curves. Table 24.5 defines the parameters that are typically determined by these methods, whereas Figure 24.1 shows how they actually appear in tracings.

Selection of the proper reference values for interpretation of findings is essential (American Thoracic Society, 1991; Drazen, 1984).

24.9.2 Design of Respiratory Function Safety Studies

The objective of a safety pharmacology evaluation of the respiratory system is to determine whether a drug has the potential to produce a change in respiratory function. Because a complete evaluation of respiratory function must include both the pumping apparatus and the lung, respiratory function safety studies are best designed to evaluate both of these functional components. The total respiratory system is evaluated first by testing for drug-induced changes in ventilatory patterns of intact conscious animals. This is followed by an evaluation of drug-induced effects on the mechanical properties of the lung in anesthetized/paralyzed animals. Together, these evaluations are used to determine whether drug-induced changes in the total respiratory system have occurred (McNeill, 1964) and whether these changes are related to pulmonary or extrapulmonary factors (Touvay and Le Mosquet, 2000).

The time intervals selected for measuring ventilatory patterns following oral administration of a drug should be based on pharmacokinetic data. The times selected generally include the time to reach peak plasma concentration of drug (T_{max}), at least one time before and one after T_{max}, and one time that is approximately 24 h after dosing to evaluate possible delayed effects. If the drug is given as a bolus i.v. injection, ventilatory parameters are monitored for approximately 5 min predose and continuously for 20–30 min postdose. One, 2-, 4,- and 24-h time intervals are also monitored to evaluate possible delayed effects. If administered by inhalation or intravenous infusion, ventilatory parameter would generally be monitored continuously during the exposure period and at 1-, 2-, 3-, and 24-h intervals after dosing.

The time interval showing the greatest ventilatory change is selected for evaluating lung mechanics. However, if no ventilatory change occurred, the T_{max} would be used. If the mechanical properties of the lung need to be evaluated within 30 min after dosing, then dynamic measurements of compliance and resistance are performed. Measurements include a predose baseline and continuous measurements for up to approximately 1 h postdose. If the mechanical properties of the lung need to be measured at 30 min or longer after dosing, then a single time point is selected and the pressure-volume and flow-volume maneuvers are performed.

Supplemental studies including blood gas analysis, end-tidal CO_2 measurements, or responses to CO_2 gas and NaCN can be conducted to gain after the ventilatory and lung mechanical findings have been evaluated. In general, these would be conducted as separate studies.

24.9.3 Capnography

The measurements of rates, volumes, and capacities provided by plethysmograph measurements have a limited ability to detect and evaluate some ventilatory disorders (Murphy, 1994) which markedly affect blood gases.

Detection of hypo- or hyperventilation syndromes requires measurement of the partial pressure of arterial CO_2 (PCO_2). In humans and large animal models this can be accomplished by collecting arterial blood with a catheter or needle and analyzing for $PaCO_2$ by using a blood gas analyzer. In conscious rodents, however, obtaining arterial blood samples by needle puncture or catheterization during ventilatory measurements is generally not practical. An alternative and noninvasive method for monitoring $PaCO_2$ is the measurement of peak expired (end-tidal) CO_2 concentrations. This technique has been successfully used in humans (Nuzzo and Anton, 1986) and recently has been adapted for use in conscious rats (Murphy et al., 1994). Measuring end-tidal CO_2 in rats requires the use of a nasal mask and a microcapnometer (Columbus Instruments, Columbus, OH) for sampling air from the mask and calculating end-tidal CO_2 concentrations. End-tidal CO_2 values in rats are responsive to ventilatory changes and accurately reflect changes in $PaCO_2$ (Murphy et al., 1994).

A noninvasive procedure in conscious rats has been developed for use in helping distinguish between the central and peripheral nervous system effects of drugs on ventilation. Exposure to CO_2 gas stimulates ventilation primarily through a central mechanism (Borison, 1977). In contrast, a bolus injection of NaCN produces a transient stimulation of ventilation through a mechanism that involves selective stimulation of peripheral chemoreceptors (Heymans and Niel, 1958). Thus, to distinguish central from peripheral nervous system effects, our procedure measures the change in ventilatory response (pretreatment vs. posttreatment) to both a 5-min exposure to 8% CO_2 gas and a bolus intravenous (i.v.) injection of 300 μg/kg of NaCN. In this paradigm, a central depressant (e.g., morphine sulfate) inhibits the CO_2 response and has little effect on the NaCN response.

The species selected for use in safety pharmacology studies should be the same as those used in toxicology studies. The advantages of using these species (rat, dog, or monkey) is that the pharmacokinetic data generated in these species can be used to define the test measurement intervals (McNeill, 1964) and acute toxicity data can be used to select the appropriate high dose (Touvay and Le Mosquet, 2000). Further, the toxicological/pathological findings in these species can be used to help define the mechanism of functional change. The rat is the primary choice because rats are readily available, and techniques for measuring pulmonary function are well established in this species.

24.10 INHALATION EXPOSURE TECHNIQUES

Many inhalation exposure techniques, such as the whole-body, nose-only, mouth-only, or head-only technique (Drew and Laskin, 1973; MacFarland, 1976; Leong et al., 1981; Phalen, 1984), the intranasal exposure technique (Elliott and DeYoung, 1970), the endotracheal nebulization technique (Leong et al., 1985, 1988; Schreck et al., 1986), and the body plethysmographic techniques (Alarie, 1966; Thorne and Karol, 1989) have been developed for inhalation toxicity studies. Table 24.14 provides a summary of the advantages and disadvantages of each of the major inhalation exposure methodologies.

The main criteria for the design and operation of any dynamic (as opposed to static) inhalation exposure system are:

- The concentration of the test atmosphere must be reasonably uniform throughout the chamber and should increase and decrease at a rate close to theoretical at the start or end

TABLE 24.14 Advantages, Disadvantages, and Considerations Associated with Modes of Respiratory Exposure

Mode of exposure	Advantages	Disadvantages	Design considerations
Head only	Good for repeated exposure Limited routes of entry into animal More efficient dose delivery	Stress to animal Losses can be large Seal around neck Labor in loading/unloading	Even distribution Pressure fluctuations Sampling and losses Air temperature, humidity Animal comfort Animal restraint
Nose/mouth only	Exposure limited to mouth and respiratory tract Uses less material (efficient) Containment of material Can pulse the exposure	Stress to animal Seal about face Effort to expose large number of animals	Pressure fluctuations Body temperature Sampling Airlocking Animals' comfort Losses in plumbing/masks
Lung only (tracheal administration)	Precision of dose One route of exposure Uses less material (efficient) Can pulse the exposure	Technically difficult Anesthesia or tracheostomy Limited to small numbers Bypasses nose Artifacts in deposition and response Technically more difficult	Air humidity/temperature Stress to the animal Physiologic support
Partial lung	Precision of total dose Localization of dose Can achieve very high local doses Unexposed control tissue from same animal	Anesthesia Placement of dose Difficulty in interpretation of results Technically difficult Possible redistribution of material within lung	Stress to animal Physiologic support

Source: From Gad and Chengelis, 1998.

of the exposure. Silver (1946) showed that the time taken for a chamber to reach a point of equilibrium was proportional to the flow rate of atmosphere passing through the chamber and the chamber volume. From this, the concentration–time relationship during the "run-up" and "run-down" phase could be expressed by the equation

$$t_x = k \frac{V}{F}$$

Where t_x = time required to reach x% of the equilibrium concentration, k = a constant of value determined by the value of x, V = chamber volume, and F = chamber flow rate. The t_{99} value is frequently quoted for exposure chambers, representing the time required to reach 99% of the equilibrium concentration and providing an estimate of chamber efficiency. Thus, at maximum efficiency, the theoretical value of k at t_{99} is 4.605, and the closer to this that the results of evaluation of actual chamber performance fall, the greater is the efficiency and the better the design of the chamber.

- Flow rates must be controlled in such a way that they are not excessive, which might cause streaming effects within the chamber, but must be adequate to maintain normal oxygen levels, temperature, and humidity in relation to the number of animals being

exposed. A minimum of 10 air changes per hour is frequently advocated and is appropriate in most cases. However, the chamber design and housing density also need to be taken into account and some designs, such as that of Doe and Tinston (1981), function effectively at lower air-change rates.

- The chamber or exposure manifold materials should not affect the chemical or physical nature of the test atmosphere.

For critical laboratory studies on inhaled drugs, a monodisperse aerosol of a specified range of MMAD should be used to increase the probability that the aerosol reaches the specified target area of the lungs. The Dautrebande aerosol generators (Dautrebande, 1962c) and the DeVilbiss nebulizer (Drew and Lippmann, 1978) are the classic single-reservoir generators for short-duration inhalation studies. For long-duration inhalation studies, the multiple-reservoir nebulizer (Miller et al., 1981) or the continuous syringe metering and elutriating atomizer (Leong et al., 1981) are frequently used. The nebulizers generate a polydisperse droplet aerosol either by the shearing force of a jet of air over a fine stream of liquid or by ultrasonic disintegration of the surface liquid in a reservoir (Drew and Lippmann, 1978). The aerosols emerging from a jet nebulizer generally have MMADs ranging between 1.2 and 6.9 μm with GSDs of 1.7 to 2.2, and aerosols from an ultrasonic nebulizer have MMADs ranging between 3.7 and 10.5 μm with GSDs of 1.4 to 2.0 (Mercer, 1981).

For testing therapeutic formulations, the liquid aerosols are usually generated by the pressurized MDI (Newman, 1984; Gad and Chengelis, 1998; Newton, 2000). The pressurized MDI generates a bolus of aerosols by atomizing a well-defined quantity of a drug that is solubilized in a propellant. Of concern in such formulations are the propellants (though these are generally inert gases) and excipients such as stabilizers (see Table 24.15). The aerosols, thus, consist of the drug particles with a coating of the propellant. As the aerosols emerge from the orifice, the mean particle size may be as large as 30 μm (Moren, 1981). After traveling through a tubular or cone-shaped spacer, the propellant may evaporate, reducing the MMADs to a range of 2.8 to 5.5 μm with GSDs of 1.5 to 2.2 (Hiller et al., 1978; Sackner et al., 1981; Newman, 1984) and making the aerosols more stable for inhalation studies. In a prolonged animal exposure study, multiple MDIs have to be actuated sequentially

TABLE 24.15 Some Examples of Excipients Used for Dry-Powder Aerosols

Active Ingredient	Excipient Carrier
Salbutamol sulfate	Lactose (63-90 μm): regular, spray-dried, and recrystallized
Budesonide	Lactose (α-monohydrate (<32 μm, 63-90 μm, 125–180 μm))
rhDNase	Lactose (50 wt% < 42 and 115 mm)
	Mannitol (50 wt% < 43 mm)
	Sodium Chloride (50 wt% < 87 mm)
Bovine serum	Lactose (a-monohydrate (63–90 mm))
albumin-	Fine particle lactose (76 wt% < 10 mm)
maltodextrin (50-50)	Micronized polyethylene glycol 6000 (97.5 wt% < 10mm)
Recombinant human	Polyethylene glycol 8000 (38–75 mm, 90–125 mm)
granulocyte-colony	
stimulating factor-mannitol	

with an electromechanical gadget (Ulrich et al., 1984) to maintain a slightly pulsatile but relatively consistent chamber concentration.

For generating an aerosol from dry powders, various dust generators, such as the Wright dust feed, air elutriator or fluidized-bed dust generator, and air impact pulverizer, have been developed for acute and chronic animal inhalation studies and have been described in many articles (Hinds, 1980; Leong et al., 1981; Phalen, 1984; Gad and Chengelis, 1998; Hext, 2000; Valentine and Kennedy, 2001). For generating powdery therapeutic agents, a metered-dose dry-powder inhaler, spinhaler, or a rotahaler is used (Newman, 1984). The particle size of the drug powder is micronized to a specific size range during manufacture and the spinhaler or the rotahaler only disperse the powders.

More recently, another approach for administering dry powders to both humans and test animals has arisen. Dry powders, though less frequently used in nasal drug delivery, are becoming more popular. Powders can be administered from several devices, the most common being the insufflator. Many insufflators work with predosed powder in gelatin capsules. To improve patient compliance a multidose powder inhaler has been developed that has been used to deliver budesonide. These devices can also be used for administration to test animals delivery, both in terms of amounts and aerodynamic size of the particles. Although early dry-powder inhalers such as the Rotohaler® used individual capsules of micronized drug, which were difficult to handle, modern devices use blister packs (e.g., Diskus®) or reservoirs (e.g., Turbuhaler®). The dry-powder inhalers rely on inspiration to withdraw drug from the inhaler to the lung and hence the effect of inhalation flow rate through various devices has been extensively studied. The major problem to be overcome with these devices is to ensure that the finely micronized drug is thoroughly dispersed in the airstream. It has been recommended that patients inhale as rapidly as possible from these devices to provide the maximum force to disperse the powder. The quantity of drug and deposition pattern varies enormously depending on the device, for example, the Turbuhaler® produces significantly greater lung delivery of salbutamol than the Diskus®. Vidgren and coworkers (1987) demonstrated by gamma scintigraphy that a typical dry-powder formulation of sodium cromoglycate suffers losses of 44% in the mouth and 40% in the actuator nozzle itself.

It must also be emphasized that the major mass of a heterodispersed aerosol may be contained in a few relatively large particles, because the mass of a particle is proportional to the cube of its diameter. Therefore, the particle-size distribution and the concentration of the drug particles in the exposure atmosphere should be sampled using a cascade impactor or membrane filter-sampling technique, monitored by using an optical or laser particle-size analyzer, and analyzed by using optical or electron microscopy techniques.

In summary, many techniques have been developed for generating gas, vapor, and aerosol atmospheres for inhalation toxicology studies. By proper regulation of the operating conditions of the nebulizers and the formulation of MDIs, together with the use of spacer or reservoir attachments to MDIs, more particles within the respirable range can be generated for inhalation. An accurately controlled exposure concentration is essential to an accurate determination of the dose–response relationship in a safety assessment of an inhalant drug.

Finally, comparisons of various techniques for animal exposures indicate that the whole-body exposure technique is the most suitable for safety assessment of gases and vapors and permits simultaneous exposure of a large number of animals to the same concentration of a drug; however, this technique is not suitable for aerosol and powder exposures because the exposure condition represents the resultant effects from inhalation, ingestion, and dermal absorption of the drug (Phalen, 1984; Gad and Chengelis, 1998).

24.11 THE UTILITY OF TOXICITY DATA

Regardless of the type of test and the parameters to be monitored, the ultimate goal is to interpolate or extrapolate from the dose–response data to find a no-observable-adverse-effect level (NOAEL) or a no-observable-effect level (NOEL). By applying a safety factor of 1 to 10 to the NOAEL, a safe

single-exposure dose for a phase I clinical trial may be obtained. By applying a more stringent safety factor, a multiple-exposure dose for a clinical trial may also be obtained. After the drug candidate has successfully passed all the drug safety evaluations and entered in the production stage, more toxicity tests may be needed for the establishment of a threshold limit value-time-weighted average (TLV-TWA). A TLV-TWA is defined as "the time-weighted average concentration for a normal 8-hour workday and a 40-hour workweek, to which nearly all workers may be repeatedly exposed, day after day, without adverse effect" (American Conference of Governmental Industrial Hygienists [ACGIH], 1991). Using TLVs as guides, long-term safe occupational exposures during production and industrial handling of a drug may be achieved. Appropriate safety assessments of pharmaceutical chemicals and drugs will ensure the creation and production of a safe drug for the benefit of humans and animals. Furthermore, inhalation toxicity data are needed for compliance with many regulatory requirements of the Food and Drug Administration, the Occupational Health and Safety Administration, and the Environmental Protection Agency (Gad and Chengelis, 1998).

More comprehensive descriptions and discussions on inhalation toxicology and technology may be found in several monographs, reviews, and textbooks (Willeke, 1980; Leong et al., 1981; Witschi and Nettesheim, 1982; Clarke and Pavia, 1984; Phalen, 1984; Witschi and Brain, 1985; Barrow, 1986; McFadden, 1986; Witschi and Last, 2001; Salem, 1986; Gardner et al., 1988; Gad and Chengelis, 1998; Valentine and Kennedy, 2001; McClellan and Henderson, 1989; Hext, 2000; Pauluhn, 2002).

GLOSSARY OF TERMS

Acceptance Criteria Numerical limits, ranges, or other criteria for the test described.

Batch A specific quantity of a drug or other material that is intended to have uniform character and quality, within specified limits, and is produced according to a single manufacturing order during the same cycle of manufacture (21 CFR 210.3(b)(2)).

Container Closure System The sum of packaging components that together contain, protect, and deliver the dosage form. This includes primary packaging components and secondary packaging components if the latter are intended to provide additional protection to the drug product (e.g., foil overwrap). The container closure system also includes the pump for nasal and inhalation sprays. For nasal spray and inhalation solution, suspension, and spray drug products, the critical components of the container closure system are those that contact either the patient or the formulation, components that affect the mechanics of the overall performance of the device, or any protective packaging.

Drug Product The finished dosage form and the container closure system.

Drug Substance An active ingredient that is intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease or to affect the structure or any function of the human body (21 CFR 314.3(b)).

Excipients Any intended formulation component other than the drug substance.

Extractables Compounds that can be extracted from elastomeric or plastic components of the container closure system when in the presence of a solvent.

Inhalation Solutions, Suspensions, and Sprays Drug products that contain active ingredients dissolved or suspended in a formulation, typically aqueous based, which can contain other excipients and are intended for use by oral inhalation. Aqueous-based drug products for oral inhalation must be sterile (21 CFR 200.51). Inhalation solutions and suspensions are intended to be used with a specified nebulizer. Inhalation sprays are combination products in which the components responsible for metering, atomization, and delivery of the formulation to the patient are a part of the container closure system.

Insufflator Dry-powder nasal inhaler used with Rynacrom cartridges. Each cartridge contains one dose; the inhaler opens the cartridge, allowing the powder to be blown into the nose by squeezing the bulb.

Leachables Compounds that leach into the formulation from elastomeric or plastic components of the drug product container closure system.

MDI Metered-dose inhaler, consisting of an aerosol unit and plastic mouthpiece. This is currently the most common type of inhaler and is widely available.

Nasal Sprays Drug products that contain active ingredients dissolved or suspended in a formulation, typically aqueous based, which can contain other excipients and are intended for use by nasal inhalation. Container closure systems for nasal sprays include the container and all components that are responsible for metering, atomization, and delivery of the formulation to the patient.

Nociception Perception of pain in the nose.

Placebo A dosage form that is identical with the drug product except that the drug substance is absent or replaced by an inert ingredient.

Pump All components of the container closure system that are responsible for metering, atomization, and delivery of the formulation to the patient.

Specification The quality standard (i.e., test, analytical procedures, and acceptance criteria) provided in the approved application to confirm the quality of drug substances, drug products, intermediates, raw material reagents, components, in-process materials, container closure systems, and other materials used in the production of drug substances or drug products.

Specified Impurity An identified or unidentified impurity that is selected for inclusion in the drug substance or drug product specification and is individually listed and limited to ensure that reproducibility of the quality of the drug substance and/or drug product.

Spinhaler A dry-powder inhaler used with Intal capsules specifically designed for the spinhaler. Each capsule contains one dose; the inhaler opens the capsule such that the powder may be inhaled through the mouthpiece.

Syncroner MDI with elongated mouthpiece, used as training device to see if medication is being inhaled properly.

Turbuhaler A dry-powder inhaler. The drug is in form of a pellet; when body of inhaler is rotated, prescribed amount of drug is ground off this pellet. The powder is then inhaled through a fluted aperture on top.

REFERENCES

ACGIH (American Conference of Governmental Industrial Hygienists), *Documentation of the Threshold Limit Values and Biological Exposure Indices*, 6th ed., American Conference of Governmental Industrial Hygienists, Cincinnati, OH, 1991.

Adams, E.M., Spencer, H.C., Rowe, V.K., and Irish, D.D., Vapor toxicity of 1,1,1,-trichloroethane (methylchloroform) determined by experiments on laboratory animals, *Arch. Ind. Hyg. Occup. Med.*, 1, 225–236, 1950.

Adams, E.M., Spencer, H.C., Rowe, V.K., McCollister, D.D., and Irish, D.D., Vapor toxicity of carbon tetrachloride determined by experiments on laboratory animals, *Arch. Ind. Hyg. Occup. Med.*, 6, 50–66, 1952.

Agnew, J.E., Physical properties and mechanisms of deposition of aerosols, in *Aerosols and the Lung, Clinical Aspects*, Clarke, S.W. and Pavia, D., Eds., Butterworth, London, 1984, pp. 49–68.

Agu, R.U., Jorissen, M., Kinget, R., Verbeke, N., and Augustigns, P., Alternatives to in vivo nasal toxicological screening for nasally administered drugs, *STP Pharm. Sci.*, 12, 13–22, 2002.

Akoun, G.M., et al., Natural history of drug-induced pneumonitis, in *Drug Induced Disorders*, Vol. 3. *Treatment Induced Respiratroy Disorders*, Akoun, G.M. and White, J.P., Eds., Elsevier Scientific Pulbishers B.V., New York, 1989, pp. 3–9.

Alarie, Y., Irritating properties of airborne material to the upper respiratory tract, *Arch. Environ. Health*, 13, 433–449, 1966.

Alarie, Y., Sensory irritation by airborne chemicals, *CRC Crit. Rev. Toxicol.*, 2, 299–363, 1973.

Alarie, Y., Toxicological evaluation of airborne chemical irritants and allergens using respiratory reflex reactions, in *Inhalation Toxicology and Technology*, Leong, B.K.J., Ed., Ann Arbor Science, Ann Arbor, MI, 1981a, pp. 207–231.

Alarie, Y., Bioassay for evaluating the potency of airborne sensory irritants and predicting acceptable levels of exposure in man, *Food Cosmet. Toxicol.*, 19, 623–626, 1981b.

Alarie, Y., Kane, L., and Barrow, C., Sensory irritation: the use of an animal model to establish acceptable exposure to airborne chemical irritants, in *Toxicology: Principles and Practice 1*, Reeves, A.L., Ed., John Wiley & Son, New York, 1980.

Alarie, Y. and Luo, J.E., Sensory irritation by airborne chemicals: A basis to establish acceptable levels of exposure, in *Toxicology of the Nasal Passages*, Barrow, C.S., Ed., Hemisphere, New York, 1984, pp. 91–100.

Aldridge, S., Inhaled antibodies work better for chronic sinusitis, *My Health and Age*, March 4, 2003.

Altman, P.L. and Dittmer, D.S., *Biological Data Book*, Vol. III, Federation of American Societies for Experimental Biology, Bethesda, MD, 1974.

Amdur, M.O. and Mead, J., Mechanics of respiration in unanesthetized guinea pigs, *Am J. Physiol.*, 192, 364–368, 1958.

American Thoracic Society, Lung function testing: selection of reference values and interpretative strategies, *Am. Rev. Respir. Dis.*, 144, 1202–1218, 1991.

Andersen, M.E., French, J.E., Gargas, M.L., Jones, R.A., and Jenkins, L.J., Jr., Saturable metabolism and the acute toxicity of 1,1-dichloroethylene, *Toxicol. Appl. Pharmacol.*, 47, 385–393, 1979.

Aso, Y., Yoneda, K., and Kikkawa, Y., Morphologic and biochemical study of pulmonary changes induced by bleomycin in mice, *Lab. Investig.*, 35, 558–568, 1976.

Aviado, D.M., Comparative cardiotoxicity of fluorocarbons, in *Cardiac Toxicology*, Vol. II, Balazs, T., Ed., CRC Press, Boca Raton, FL, 1981. pp. 213–222.

Aviado, D.M. and Micozzi, M.S., Fluorine-containing organic compounds, in *Patty's Industrial Hygiene and Toxicology*, Vol. 2B, Clayton, G.D. and Clayton, F.E., Eds., Wiley, New York, 1981, pp. 3071–3115.

Balazs, T., Cardiotoxicity of adrenergic bronchodililator and vasodilating antihypertensive drugs, in *Cardiac Toxicology*, Vol. II, Balazs, T., Ed., CRC Press, Boca Raton, FL, 1981, pp. 61–73.

Barrow, C.S., *Toxicology of the Nasal Passages*, Hemisphere, New York, 1986.

Bell, K.A., Local particle deposition in respiratory airway models, in *Recent Developments in Aerosol Science*, Shaw, D.T., Ed., Wiley, New York, 1978, pp. 97–134.

Bice, D.E., Methods and approaches to assessing Immunotoxicology of the lower respiratory tract, in *Immunotoxicology and Immunopharmacology*, Dean, J.H., Luster, M.I., Munson, A.E., and Amos, H.A., Eds., Raven Press, New York, 1985, pp.145–157.

Boggs, D.F., Comparative control of respiration, in *Comparative Biology of the Normal Lung*, Vol. I, Parent, R.A., Ed., CRC Press, Boca Raton, FL, 1992, pp. 309–350.

Borison, H.L., Central nervous system depressants: control-systems approach to respiratory depression, *Pharmacol Ther B.*, 3, 211–226, 1977.

Brain, J.D., The effects of increased particles on the number of aveolar macrophages, in *Inhaled Particles III, Proceedings of ISOHS Symposium*, Union Brothers Ltd., London, 1971, pp. 220–233.

Brain, J.D., Knudson, D.E., Sorokin, S.P., and Davis, M.A., Pulmonary distribution of particles given by intratracheal instillation or be aerosol inhalation. *Environ. Res.*, 11, 13–33, 1976.

Brown, L.K. and Miller, A., Full lung volumes: functional residual capacity, residual volume and total lung capacity, in *Pulmonary Function Tests: A Guide for the Student and House Officer*, Miller, A., Ed., Grune & Stratton, Inc., New York, 1987, pp. 53–58.

Budavari, S., Ed., *Merck Index*, 11th ed., Merck & Co., Rahway, NJ., 1989, p. 82.

Burham, S.R., The ideal nasal carticosteroid: balancing efficacy, safety and patient preference, *Clin. Exp. Allergy Rev*., 2, 32–37, 2002.

CDER (Center for Drug Evaluation and Research), *Guidance for Industry: Nasal Spray and Inhalation Solution, Suspension, and Spray Drug Products-Chemistry, Manufacturing and Control Documentation*, Food and Drug Administration, Washington, DC, 2002.

Chan, T.L. and Lippmann, M., Experimental measurements and empirical modelling of the regional deposition of inhaled particles in humans, *Am. Ind. Hyg. Assoc. J.*, 41, 399–409, 1980.

Chenoweth, M.B., Leong, B.K.J., Sparschu, G.L., and Torkelson, T.R., Toxicities of methoxyflurane, halothane and diethyl ether in laboratory animals on repeated inhalation at subanesthetic concentrations, in *Cellular Biology and Toxicity of Anesthetics*, Fink, B.R., Ed., Williams & Wilkins, Baltimore, 1972, pp. 275–284.

Cherniack, N.S., Disorders in the control of breathing: hyperventialtion syndromes, in *Textbook of Respiratory Medicine*, Murray, J.F. and Nadal, J.A., Eds., W.B. Saunders Co., Philadelphia, 1988, pp. 1861–1866.

Clarke, S.W. and Pavia, D., Eds., *Aerosol and the Lung: Clinical and Experimental Aspects*, Butterworth, London, 1984.

Cox, J.S.G., Beach, J.E., Blair, A.M.J.N., Clarke, A.J., King, J., Lee, T.B., Loveday, D.E.E., Moss, G.F., Orr, T.S.C., Ritchie, J.T., and Sheard, P., Disodium cromoglycate, *Adv. Drug. Res.*, 5, 115–196, 1970.

Curry, S.H., Taylor, A.J., Evans, S., Godfrey, S., and Zeidifard, E., Disposition of disodium cromoglycate administered in three particle sizes, *Br. J. Clin. Pharmacol.*, 2, 267–270, 1975.

Dahl, A.R., Dose concepts for inhaled vapors and gases, *Toxicol. Appl. Pharmacol.*, 103, 185–197, 1990.

Dallas, C.E., Bruckner, J.V., Maedgen, J.L., and Weir, F.W., A method for direct measurement of systemic uptake and elimination of volatile organics in small animals, *J. Pharmacol. Methods*, 16, 239–250, 1986.

Dallas, C.E., Ramanathan, R., Muralidhara, S., Gallo, J.M., and Bruckner, J.V., The uptake and elimination of 1,1,1-trichloroethane during and following inhalation exposure in rats, *Toxicol. Appl. Pharmacol.*, 98, 385–397, 1989.

Dautrebande, L., Importance of particle size for therapeutic aerosol efficiency, in *Microaerosols*, Academic Press, New York, 1962a, pp. 37–57.

Dautrebande, L., Practical recommendation for administering pharmacological aerosols, in *Microaerosols*, Academic Press, New York, 1962b, pp. 86–92.

Dautrebande, L., Production of liquid and solid micromicellar aerosols, in *Microaerosols*, Academic Press, New York, 1962c, pp. 1–22.

Davies, R.J. and Pepys, J., Asthma due to inhaled chemical agents—the macrolide antibiotic spiramycin, *Clin. Allergy*, 5, 99–107, 1975.

Davis, B., Marin, M.G., Fischer, S., Graf, P., Widdicombe, J.G., and Nadel, J.A., New method for study of canine mucus gland secretion *in vivo:* cholinergic regulation (abstr.), *Am. Rev. Respir. Dis.*, 113, 257. 1976.

Davis, C.N., A formalized anatomy of the human respiratory tract, in *Inhaled Particles and Vapours*, Davis, C.N., Ed., Pergamon Press, London, 1961, pp. 82–91.

Davis, C.N., Heyder, J., and Subba Ramv, M.C., Breathing of half micron aerosols. I. Experimental, *J. Appl. Physicol.*, 32, 591–600, 1972.

Dennis, W.L., The discussion of a paper by C.N. Davis: a formalized anatomy of the human respiratory tract, in *Inhaled Particles and Vapours*, Davis, C.N., Ed., Pergamon Press, London, 1961, p.88.

Diamond, L. and O'Donnell, M., Pulmonary mechanics in normal rats, *J. Appl. Physiol. Respir. Environ. Exerc. Physiol.*, 43, 942–948, 1977.

Doe, J.E. and Tinston, D.J., Novel chamber for long-term inhalation studies, in *Inhalation Toxicology and Technology*, Leong, K.J., Ed., Ann Arbor Science, Ann Arbor, MI, 1981.

Dorato, M.A., Toxicological evaluation of intranasal peptide and protein drugs, *Drugs Pharm. Sci.*, 62, 345–381, 1994.

Drazen, J.M., Physiological basis and interpretation of indices of pulmonary mechanics, *Environ. Health Perspect.*, 56, 3–9, 1984.

Drew, R.T. and Laskin, S., Environmental inhalation chambers, in *Methods of Animal Experimentation*, Vol. IV, Academic Press, New York, 1973, pp. 1–41.

Drew, R.T. and Lippmann, M., Calibration of air sampling instruments, in *Air Sampling Instruments for Evaluation of Atmospheric Contaminants*, 5th ed., Sect. I, American Conference of Governmental Industrial Hygienists, Cincinnati, OH, 1978, pp. 1–32.

Durham, S.R., he ideal nasal corticosteroid: Balancing efficacy, safety and patient preference, *Clin. Exp. Allergy Rev.*, 2, 32–37, 2002.

Eldridge, F.L. and Millhorn, D.E., Central regulation of respiration by endogenous neurotransmitters and neuromodulators, *Annu. Rev. Physiol.*, 3, 121–135, 1981.

Elliot, G.A. and DeYoung E.N., Intranasal toxicity testing of antiviral agents, *Am. N. Y. Acad. Sci.*, 173, 169–175, 1970.

Evans, M.J., Cell death and cell renewal in small airways and alveoli, in *Mechanisms in Respiratory Toxicology*, Vol. 1, Witschi, H. and Nettesheim, P., Eds., CRC Press, Boca Raton, FL, 1982, pp. 189–218.

Fariba, A., Kellie, M., Stephen, M., Oune, O., Kafi, A., and Mary, H., Repeated doses of antenatal coricosteroids in animals: a systematic review, *Am. J. Obstet. Gynecol.*, 186,843–849, 2002.

Ferin, J., Effect of particle content of lung on clearance pathways, in *Pulmonary Macrophage and Epithelial Cells*, Sanders, C.L., Schneider, R.P., Dagle, G.E., and Ragan, H.A., Eds., Technical Information Center, Energy Research & Development Administration, Springfield, VA, 1977, pp. 414–423.

Fieldner, A.C., Kazt, S.H., and Kinney, S.P., Gas Masks for Gases Met in Fighting Fires, U.S. Bureau of Mines, Technical paper 248, 1921.

Gad, S., *Drug Safety Evaluation*, Wiley, New York, 2000.
Gad, S. and Chengelis, C.P., *Acute Toxicology Testing: Perspectives and Horizons*, 2nd ed., Academic Press, San Diego, CA, 1998, pp. 404–466.
Gamsu, G., Singer, M.M., Vincent, H.H., Berry, S., and Nadel, J.A., Postoperative impairment of mucous transport in the lung, *Am. Rev. Respir. Dis.*, 114, 673–679, 1976.
Gardner, D.E., Crapo, J.D., and Massaro, E.J., *Toxicology of the Lung*, Raven Press, New York, 1988.
Godfrey, S., Zeidifard, E., Brown, K., and Bell, J.H., The possible site of action of sodium cromoglycate assessed by exercise challenge, *Clin. Sci. Mol. Med.*, 46, 265–272, 1974.
Haber, F.R., Funf Vortage aus den jahren 1920–23, No. 3, *Die Chemie im Kriege*, Julius Springer, Berlin, 1924.
Hafner, R.E., Jr., Watanabe, P.G., and Gehring, P.J., Preliminary studies on the fate of inhaled vinyl chloride monomer in rats, *Am. N. Y. Acad. Sci.*, 246, 135–148, 1975.
Hatch, T.F. and Gross, P., *Pulmonary Deposition and Retention of Inhaled Aerosols*, Academic Press, New York, 1964, pp. 16–17, 51–52, 147–168.
Henderson, R., Use of bronchoalveolar lavage to detect lung damage, in *Toxicology of the Lung*, Gardner, D.E., Crapo, J.D., and Massaro, E.J., Eds., Raven Press, New York, 1988, ss pp. 239–268.
Henderson, R., Bronchoalveolar lavage: a tool for assessing the health status of the lung, in *Concepts in Inhalation Toxicology*, McClellan, R.O. and Henderson, R.F., Eds., Hemisphere, Washington DC, 1989, pp. 414–442.
Henderson, R.F., Use of bronchoalveolar lavage to detect lung damage, *Environ. Health Perspect.*, 56, 115–129, 1984.
Henderson, R.F., Barr, E.B., and Hotchkiss, J.A., Effect of exposure rate on response of the lung to inhaled particles (abstr.), *The Toxicologists*, 11, 234, 1991.
Henderson, R.F., Benson, J.M., Hahn, F.F., Hobbs, C.H., Jones, R.K., Mauderly, J.L., McClellan, R.O., and Pickrell, J.A., New approaches for the evaluation of pulmonary toxicity: Bronchoalveolar lavage fluid analysis, *Fundam. Appl. Toxicol.*, 5, 451–458, 1985.
Henderson, R.F. and Loery, J.S., Effect of anesthetic agents on lavage fluid parameters used as indicators of pulmonary injury, *Lab. Anim. Sci.*, 33, 60–62, 1983.
Henderson, R.F., Mauderly, J.L., Pickrell, J.A., Hahn, F.F., Muhle, H., and Rebar, A.H., Comparative study of bronchoalveolar lavage fluid: effect of species, age, method of lavage, *Exp. Lung Res.*, 1, 329–342, 1987.
Henry, C.M., New wrinkles in drug delivery, *C & E News*, March 1, 2004, pp. 37–42.
Hensley, M.J., O'Cain, C.F., McFadden, E.R., Jr., and Ingram, R.H., Jr., Distribution of bronchodilatation in normal subjects: Beta agonist versus atropine, *J. Appl. Physiol.*, 45, 778–782, 1978.
Hext, P.M., Inhalation toxicology, in *General and Applied Toxicology*, Ballantyne, B., Marrs, T., and Syversen, T., Eds., Macmillan, London, 2000, pp. 587–601.
Heyder, J., Gebhart, J., and Stahlhofen, W., Inhalation of aerosols: particle deposition and retention, in *Generation of Aerosols and Facilities for Exposure Experiments*, Willeke, K., Ed., Ann Arbor Science, Ann Arbor, MI, 1980, pp. 80–99.
Heymans, C., Action of drugs on carotid body and sinus, *Pharmacol. Rev.*, 7, 119–142, 1955.
Heymans, C. and Niel, E., The effects of drugs on chemoreceptors, in *Reflexogenic Areas of the Cardiovascular Systems*, Heymans, C., and Neil, E., Eds., London, Churchill, Ltd., (1958). pp 192–199.
Hiller, F.C., Mazunder, M.K., Wilson, J.D., and Bone, R.C., Aerodynamic size distribution of metered dose bronchodilator aerosols, *Am. Rev. Respir. Dis.*, 118, 311–317, 1978.
Hinds, W.C., Dry–dispersion aerosol generators, in *Generation of Aerosols and Facilities for Exposure Experiments*, Willeke, K., Ed., Ann Arbor Science, Ann Arbor, MI, 1980, pp. 171–187.
Hocking, W.G. and Golde, D.W., The pulmonary alveolar macrophage, *N. Engl. J. Med.*, 310, 580–587, 639–645, 1979.
Hodson, M.E., Penketh, A.R., and Batten, J.C., Aerosol carbenicillin and gentamicin treatment of *Pseudomonas aeruginosa* infection I patients with cystic fibrosis, *Lancet*, 2, 1137–1139, 1981.
Holma, B., Lung clearance of mono- and di-disperse aerosols determined by profile scanning and whole body counting: a study on normal and SO_2 exposed rabbits, *Acta Med. Scand.* 473 (Suppl.), 1–102, 1967.
Horsfield, K. and Cunning, G., Morphology of the bronchial tree in man, *J. Appl. Physiol.*, 24, 373–383, 1968.
Illum, L. and Davis, S.S., Intranasal insulin, *Clin. Pharmacokinet.*, 23, 30–41, 1992.
Indans, I., Non-lethal end-points in inhalation toxicology, *Toxicol. Lett.*, 135, 53, 2002.
Ingram, R.H., Wellman, J.J., McFadden, E.R., Jr., and Mead, J., Relative contributions of large and small airways to flow limitation in normal subjects before and after atropine and isoproterenol, *J. Clin. Investig.*, 59, 696–703, 1977.

Irish, D.D. and Adams, E.M., Apparatus and methods for testing the toxicity of vapors, *Ind. Med. Surg.*, 1, :1–4, 1940.

Jonhnson, H.G., McNee, M.L., and Braughler, J.M., Inhibitors of metal catalyzed lipid peroxidation reactions inhibit mucus secretion and 15 HETE levels in canine trachea, *Prostaglandins Leukot. Med.*, 30, 123–132, 1987.

Johnson, H.G., McNee, M.L., Johnson, M.A., and Miller, M.D., Leukotriene C_4 and dimethylphenylpiperazinium-induced responses in canine airway tracheal muscle contraction and fluid secretion, *Int. Arch. Allergy Appl. Immunol.*, 71, 214–218, 1983.

Kannisto, S., Voutilainen, R., Remes, K., and Korppi, M., Efficacy and safety of inhaled steroid and cromane treatment in school-age children: a randomized pragmatic pilot study, *Pediatr. Asthma Allergy Immunol.*, 13, 24–34, 2002.

Karol, M.H., Immunologic responses of the lung to inhaled toxicants, in *Concepts in Inhalation Toxicology*, McClellan, R.O. and Henderson, R., Eds., Hemisphere Publishing, Washington, DC, 1988, pp. 403–413.

Karol, M.H., Hillebrand, J.A., and Thorne, P.S., Characteristics of weekly pulmonary hypersensitivity responses elicited in the guinea pig by inhalation of ovalbumin aerosols, in *Toxicology of the Lung*, Gardner, D.E., Crapo, J.D., and Massaro, E.J., Eds., Raven Press, New York, 1989, pp. 427–448.

Kavet, R.I., Brain, J.D., and Levens, D.J., Characteristics of weekly pulmonary macrophages lavaged from hamsters exposed to iron oxide aerosols, *Lab. Investig.*, 38, 312–319, 1978.

Keats, A.S., The effects of drugs on respiration in man, *Annu. Rev. Pharmacol. Toxicol.*, 25, 41–65, 1985.

King, T.K.C., Measurement of functional residual capacity in the rat, *J. Appl. Physiol.*, 21. 233–236, 1966.

Koushik, K. and Kompella, U.B., Particle and device engineering for inhalation drug delivery, *Drug Delivery Technol.*, 4, 39–50, 2004.

Lalej-Bennis, D., et al., Six month administration of gelified intranasal insulin in 16 type 1 diabetic pateints under multiple injections: efficacy vs subcutaneous injections and local tolerance, *Diabetes Metab.*, 27, 372–377, 2001.

Landy, T.D., Ramsey, J.C., and McKenna, M.J., Pulmonary physiology and inhalation dosimetry in rats: Development of a method and two examples, *Toxicol. Appl. Pharmacol.*, 71, 72–83, 1983.

Last, J.A., Mucus production and ciliary escalator, in *Mechanisms in Respiratory Toxicology*, Vol. 1, Witschi, H., and Nettesheim, P., Eds., CRC Press, Boca Raton, FL, 1982, pp. 247–268.

Lauweryns, J.M. and Baert, J.H., Alveolar clearance and the role of the pulmonary lymphatics, *Am. Rev. Respir. Dis.*, 115, 625–683, 1977.

Lawrence, S., Intranasal delivery could be used to administer drugs directly to the brain, *Lancet*, 359, 1674, 2002.

Lee, W.C. and Wang, C.S., Particle deposition in systems of repeated bifurcating tubes, in *Inhaled Particles IV*, Walton, W.H., Ed., Pergamon, Oxford, 1977, pp.49–60.

Leigh. D.E. and Mead, J., Principals of Body Plethysmography, *National Heart and Lung Institute*, NIH, Bethesda, MD, 1974.

Leong, B.K.J., Coombs, J.K., Petzold, E.N., and Hanchar, A.J., A dosimetric endotracheal nebulization technique for pulmonary metabolic disposition studies in laboratory animals, *InhalAtion Toxicol.*, Premier issue, 37–51, 1988.

Leong, B.K.J., Coombs, J.K., Petzold, E.N., Hanchar, A.H., and McNee, M.L., Endotracheal nebulization of drugs into the lungs of anesthetized animals (abstr.), *Toxicologist*, 5, 31, 1985.

Leong, B.K.J., Lund, J.E., Groehn, J.A., Coombs, J.K., Sabaitis, C.P., Weaver, R.J., and Griffin, L., Retinopathy from inhaling 4,4'-methylenedianiline aerosols, *Fundam. Appl. Toxicol.*, 9, 645–658, 1987.

Leong, B.K.J. and MacFarland, H.N., Pulmonary dynamics and retention of toxic gases, *Arch. Environ. Health*, 11, 555–563, 1965.

Leong, B.K.J., Powell, D.J., and Pochyla, G.L., A new dust generator for inhalation toxicological studies, in *Inhalation Toxicology and Technology*, Leong, B.K.J., Ed., Ann Arbor Science, Ann Arbor, MI, 1981, pp. 157–168.

Leong, B.K.J. and Rop, D.A., The combined effects of an inhalation anesthetic and an analgesic on the electrocardiograms of beagle dogs, in *International Congress of Toxicology*, Taylor & Francis, London, 1989, Abstr. 475, p. 159.

Lichtiger, M., Landa, J.F., and Hirsch, J.A., Velocity of tracheal mucus in anesthetized women undergoing gynaecologic surgery, *Anesthesiology*, 42,753–756, 1975.

Lippmann, M., "Respirable" dust sampling, *Am. Ind. Hyg. Assoc. J.*, 31, 138–159, 1970.

Lippmann, M., Regional deposition of particles in the human respiratory tract, in *Handbook of Physiology*, section 9: *Reactions of Environmental Agents*, Lee, D.H.K., Falk, L.K., and Murphy, S.D., Eds., American Physiology Society, Bethesda, MD, 1977, pp. 213–232.

Lippmann, M., Yeates, D.B., and Albert, R.E., Deposition, retention and clearance of inhaled particles, *Br. J. Ind. Med.*, 37, 337–362, 1980.

Logemaan, C.D. and Rankin, L.M., Newer intranasal migraine medications, *Am. Fam. Physican*, 61, 180–6, 2000.

Lourenco, R.V. and Cotromanes, E., Clinical aerosols. II. Therapeutic aerosols, *Arch. Intern. Med.*, 142, 2299–2308, 1982.

MacFarland, H.N., Respiratory toxicology, in *Essays in Toxicology*, Vol. 7, Hayes, W.J., Ed., Academic Press, New York, 1976, pp. 121–154.

Marple, V.A. and Rubow, K.L., Aerosol generation concepts and parameters, in *Generation of Aerosols and Facilities for Exposure Experiments*, Willeke, K., Ed., Ann Arbor Science, Ann Arbor, MI, 1980, p. 6.

Mauderly, J.L., The influence of sex and age on the pulmonary function of the beagle dog, *J. Gerontol.*, 29, 282–289, 1974.

Mauderly, J.L., Effects of inhaled toxicants on pulmonary function, in *Concepts in Inhalation Toxicology*, McClellan, R.O. and Henderson, R.F., Eds., Hemisphere Publishing Corp, New York, 1989, pp. 347–401.

McClellan, R.O. and Henderson, R.F., *Concepts in Inhalation Toxicology*, Hemisphere, Washington, DC, 1989.

McFadden, E.R., Jr., *Inhaled Aerosol Bronchodilators*, Williams & Wilkins, Baltimore, 1986, pp. 40–41.

McNeill, R.S., Effect of a β-adrenergic blocking agent, propranol, on asthmatics, *Lancet*, Nov 21, 1101–1102, 1964.

Menon, M.P.S. and Das, A.K., Tetracycline asthma—a case report, *Clin. Allergy*, 7, 285–290,1977.

Menzel, D.B. and Amdur, M.O., Toxic responses of the respiratory system, in: *Casarett and Doull's Toxicology*, 3rd ed., Klaassen, C.D., Amdur, M.O., and Doull, J., Eds., Macmillan, New York, 1986, pp. 330–358.

Mercer, T.T., Production of therapeutic aerosols; principles and techniques, *Chest*, 80 (Suppl. 6), 813–818, 1981.

Merin, R.G., Cardiac toxicity of inhalation anesthetics, In *Cardiac Toxicology*, Vol. II, Balazs, T., Ed., CRC Press, Boca Raton, FL, 1981, pp. 4–10.

Milic-Emili, J., Recent advances in clinical assessment of control of breathing, *Lung*, 160, 1–17, 1982.

Miller, F.J., Gardner, D.E., Graham, J.A., Lee, R.E., Jr., Wilson, W.E., and Bachmann, J.D., Size considerations for establishing a standard for inhalable particles, *J. Air Poll. Cont. Assoc.*, 29, 610–615, 1979.

Miller, J.L., Stuart, B.O., Deford, H.S., and Moss, O.R., Liquid aerosol generation for inhalation toxicology studies, in *Inhalation Toxicology and Technology*, Leong, B.K.J., Ed., Ann Arbor Science, Ann Arbor, MI, 1981, pp. 121–207.

Moren, F., Pressurized aerosols for oral inhalation, *Int. J. Pharm.*, 8, 1–10, 1981.

Moren, F., Aerosol dosage forms and formulations, in *Aerosols in Medicine*, 2nd ed., Moren, S., Dolovich, M.B., Newhouse, M.T., Newman, S.P.. Eds., Elsevier, Amsterdam, 1993, pp. 329–336 .

Mueller, R.A., et al., The neuropharmacology of respiratory control, *Pharmacol. Rev.*, 34, 255–285, 1982.

Murphy, D.J., Safety pharmacology of the respiratory system: techniques and study design, *Drug Dev. Res.*, 32, 237–246, 1994.

Murphy, D.J., Joran, M.E., and Grando, J.C., Microcapnometry: a non-invasive method for monitoring arterial CO_2 tension during ventilatory measurements in conscious rats, *Toxicol. Methods*, 4, 177–187, 1994.

Mussatto, D.J., Garrad, C.S., and Lourenco, R.V., The effect of inhaled histamine on human tracheal mucus velocity and bronchial mucociliary clearance. *Am. Rev. Respir. Dis.* 138:775–779. (1988).

NAS (National Academy of Sciences), *Handbook of Respiration*, NAS National Research Council, W.B. Saunders, Philadelphia, 1958, p. 41.

Newman, S.P., Therapeutic aerosols, in *Aerosols and the Lung, Clinical and Experimental Aspects*, Clarke, S.W. and Pavia, D., Eds., Butterworth & Company, London, 1984, pp. 197–224.

Newton, P.E., Techniques for Evaluating Hazards of Inhaled Products, in *Product Safety Evaluation Handbook*, 2nd ed., Marcel Dekker, New York, 2000, pp. 243–298.

Nuzzo, P.F. and Anton, W.R., Practical applications of capnography, *Respir. Ther.*, 16, 12–17, 1986.

Oberst, F.W., Factors affecting inhalation and retention of toxic vapours, in *Inhaled Particles and Vapours*, Davis, C.N. Ed., Pergamon Press, New York, 1961, pp. 249–266.

O'Neil, J.J. and Raub, J.A., Pulmonary function testing in small laboratory mammals, *Environ. Health Perspect.*, 53, 11–22, 1984.

Palecek, F., Measurement of ventilatory mechanics in the rat, *J. Appl. Physiol.*, 27, 149–156, 1969.

Parent, R.A., *Comparative Biology of the Normal Lung*, CRC Press, Boca Raton, FL, 1991.

Paterson, J.W., Bronchodilators, in *Asthma*, Clark, T.J.H. and Godfrey, S., Eds., Chapman and Hall, London, 1977, pp. 251–271.

Paterson, J.W., Woocock, A.J., and Shenfield, G.M., Bronchodilator drugs, *Am. Rev. Respir. Dis.*, 120, 1149–1188, 1979.

Patterson, R. and Kelly, J.F., Animal models of the asthmatic state, *Annu. Rev. Med.*, 25, 53–68, 1974.

Patty, F.A., *Industrial Hygiene and Toxicology*, 2nd ed., Interscience, New York, 1958.

Pauluhn, J., Overview of testing methods used in inhalation toxicology: from facts to artifacts, in *Eurotox 2002*, 2002, pp. 5–7.

Paustenbach, D.J., Carlson, G.P., Christian, J.E., Born, G.S., and Rausch, J.E., A dynamic closed-loop recirculating inhalation chamber for conducting pharmacokinetic and short-term toxicity studies, *Fundam. Appl. Toxicol.*, 3, 528–532, 1983.

Pavia, D., Lung mucociliary clearance, in *Aerosols and the Lung, Clinical and Experimental Aspects,* Clarke, S.W. and Pavia, D., Eds., Butterworth & Co, London, 1984, pp. 127–155.

Pavia, D., Bateman, J.R.M., Sheahan, N.F., Agnew, J.E., Newman, S.P., and Clarke, S.W., Techniques for measuring lung mucociliary clearance, *Eur. J. Respir. Dis.*, 67 (Suppl. 110),157–177, 1980.

Pavia, D., Sutton, P.P., Agnew, J.E., Lopez-Vidriero, M.T., Newman, S.P., and Clarke, S.W., Measurement of bronchial mucociliary clearance, *Eur. J. Respir. Dis.*, 64 (Suppl. 127), 41–56, 1983a.

Pavia, D., Sutton, P.P., Lopez-Vidriero, M.T., Agnew, J.E., and Clarke, S.W., Drug effects on mucociliary function, *Eur. J. Respir. Dis.*, 64 (Suppl. 128). 304–317. 1983b.

Phalen, R.F., *Inhalation Studies: Foundations and Techniques*, CRC Press, Boca Raton, FL, 1984, pp. 35–46, 51–57.

Rinehart, W.E. and Hatch, T., Concentration –time product (Ct) as an expression of dose in sublethal exposures to phosgene, *Am. Ind. Hyg. Assoc. J.*, 25, 545–553, 1964.

Rosnow, E.C., et al., Drug-induced pulmonary disease: an update, *Chest*,102, 239–250, 1992.

Rylander, R., Current techniques to measure alterations in the ciliary activity of intact respiratory epithelium, *Am. Rev. Respir. Dis.*, 93 (Suppl), 67–85, 1966.

Sackner, M.A., Brown, L.K., and Kim, C.S., Basis of an improved metered aerosol delivery system, *Chest*, 80 (Supp. 6), 915–918, 1981.

Salem, H., Principles of inhalation of inhalation toxicology, in *Inhalation Toxicology: Research Methods, Applications, and Evaluation*, Marcel Dekker, New York, 1986, pp. 1–33.

Schaper, M., Detwiler, K., and Alaire, Y., Alteration of respiratory cycle timing by propanolol, *Toxicol. Appl. Pharmacol*., 1989, pp. 538–547.

Schreck, R.W., Sekuterski, J.J., and Gross, K.B., Synchronized intratracheal aerosol generation for rodent studies, in *Aerosols, Formation and Reactivity*, Second International Aerosol Conference, Berlin, Pergamon Press, New York, 1986, pp. 37–51.

Schwettmann, R.S. and Casterline, C.L., Delayed asthmatic response following occupational exposure to enflurane, *Anesthesiology*, 44, 166–169, 1976.

Shao, Z., Krishnamoorthy, R., and Mitra, A.K., Cyclodextrins as nasal absorption promoters of insulin-mechanistic evaluations, *Pharm. Res.*, 9. 1157–1163. 1992.

Shao, Z. and Mitra, A.K., Nasal membrane and intracellular proteein and enzyme release by bile salts and bile salt fatty-acd mixed micelles-correlation with facilitated drug transport, *Pharm. Res.*, 9,1184–1189, 1992.

Sharma, S., White, G., Imondi, A.R., Plaelse, M.E., Vail, D.M., and Dris, M.G., Development of inhalation agents for oncological use, *J. Clin. Oncol.*, 19, 1839–1847, 2001.

Sidorenko, G.I. and Pinigin, M.A., Concentration-time relationship for various regimens of inhalation of organic compounds, *Environ. Health Perspect.*, 13, 17–21, 1976.

Silver, S.D., Constant flow gassing chambers: principles influencing design and operation, *J. Lab. Clin. Med.*, 31, 1153–1161, 1946.

Sinnet, E.E., et al. Fast integrated flow plethysmograph for small mammals, *J. Appl. Physiol.*, 50, 1104–1110, 1981.

Smaldone, G.C., Determinants of dose and response to inhaled therapeutic agents in asthma, in *Inhaled Glucocorticoids in Asthma: Mechanisms and Clinical Actions*, Schleimer, R.P., Busse, W.W., and O'Byrne, P.M., Eds., Marcel Dekker, New York, 1997, pp. 447–477.

Stahlhofen, W., Gebhart, J., and Heyder, J., Experimental determination of the regional deposition of aerosol particles in the human respiratory tract, *Am. Ind. Hyg. Assoc. J.*, 41,385–398, 1980.

Stahlhofen, W., Gebhart, J., and Heyder, J., Biological variability of regional deposition of aerosol particles in the human respiratory tract, *Am. Ind. Hyg. Assoc. J.*, 42, 348–352, 1981.

Stott, W.T. and McKenna, M.J., The comparative absorption and excretion of chemical vapors by the upper, lower and intact respiratory tract of rats, *Fundam. Appl. Toxicol.* 4, 594–602, 1984.

Swift, D.B. and Proctor, D.F., Human respiratory deposition of particles during oronasal breathing, *Atmos. Environ.*, 16, 2279–2282, 1982.

Tamulinas, C.B. and Leach, C.L., Routes of Exposure: Inhalation and Intranasal, *Excipient Toxicology and Safety*, Weiner, M.L. and Kotkoskie, L.A., Eds., Marcel Dekker, New York, 2000, pp. 185–205.

Task Group on Lung Dynamics, Deposition and retention models for internal dosimetry of the human respiratory tract, *Health Phys.*, 12, 173–207, 1966.

Tattersfield, A.E., Beta adrenoreceptor antagonists and respiratory disease, *J. Cardiovasc. Pharmacol.*, 8 (Suppl. 4), 535–539, 1986.

Taulbee, D.B., Yu, C.P., and Heyder, J., Aerosol transport in the human lung from analysis of single breaths, *J. Appl. Physiol.*, 44, 803–812, 1978.

Thorne, P.S. and Karol, M.H., Association of fever with late-onset pulmonary hypersensitivity responses in the guinea pig, *Toxicol. Appl. Pharmacol.*, 100, 247–258, 1989.

Touvay, C. and Le Mosquet, B., Systeme resiratoire et pharmacology de securite, *Therapie*, 55, 71–83, 2000.

Ts'o, T.O.T., Leong, B.K.J., and Chenoweth, M.B., Utilities and limitations of behavioral techniques in industrial toxicology, in *Behavioral Toxicology*, Weiss, B. and Laties, V.G., Eds., Plenum Press, New York, 1975, pp. 265–291.

Uemitsu, N., Minobe, Y., and Nakayoshi, H., Concentration-time-response relationship under conditions of single inhalation of carbon tetrachloride, *Toxicol. Appl. Pharmacol.*, 77, 260–266, 1985.

Ugwoke, M.I., Verbeke, N., and Kinget, R., The biopharmaceutical aspects of nasal mucoadhesive drug delivery, *J. Pharm. Pharmacol.*, 53, 3–21, 2001.

Ulrich, C.E., Klonne, D.R., and Church, S.V., Automated exposure system for metered-dose aerosol pharmaceuticals, *Toxicologist*, 4, 48, 1984.

Valentine, R. and Kennedy, G.L., Jr., Inhalation toxicology, in *Principles and Methods of Toxicology*, 4th ed., Hayes, W., Ed., Raven Press, New York, 2001, pp. 1085–1143.

Vidgren, M.T., Karkkainen, A., Paronen, T.P., and Karjalainen, P., Respiratory tract deposition of ^{99}Tc-labeled drug particles administered via a dry powder inhaler, *Int. J. Pharm.*, 39, 101–105, 1987.

Wanner, A., The role of mucociliary dysfunction in bronchial asthma, *Am. J. Med.*, 67, 477–485, 1979.

Wanner, A., Alteration of tracheal mucociliary transport in airway disease. Effect of pharmacologic agents, *Chest*, 80 (Suppl. 6), 867–870, 1981.

Webber, S.E. and Widdicombe, J.G., The actions of methacholine, phenylephrine, salbutamol and histamine on mucus secretion from the ferret in-vitro trachea, *Agents Actions*, 22,82–85, 1987.

Weibel, E.R., *Morphometry of the Human Lung*, Springer-Verlag, Heidelberg, 1963.

Weibel, E.R., How does lung structure affect gas exchange? *Chest*, 83, 657–665, 1983.

Weiner, N., Norepinephrine, epinephrine, and the sympathomimetic amines, in *Goodman and Gilman's, The Pharmacological Basis of Therapeutics*, 7th ed., Gilman, A.G., Goodman, L.S., Rall, T.W., and Murad, F., Eds., Macmillan, New York, 1984, pp. 145–180.

Weston, R., and Karel, L., An application of the dosimetric method for biologically assaying inhaled substances, *J. Pharmacol. Exp. Ther.*, 88, 195–207, 1946.

Widdicombe, J.G., Reflex control of breathing, in *Respiratory Physiology*, Vol. 2, Widdicombe, J.G., Ed., University Park Press, Baltimore, MD, 1974.

Willeke, K., *Generation of Aerosols and Facilities for Exposure Experiments*, Ann Arbor Science, Ann Arbor, MI, 1980.

Williams, M.H., Steroids and antibiotic aerosols, *Am Rev. Respir. Dis.*, 110, 122–127, 1974.

Wills, P. and Greenstone, M., Inhaled hyperosmolar agents for bronchiectasis, *Cochrane Database Syst. Rev.*, CD002996, 2001.

Wilson, A.G.E., Toxicokinetics of uptake, accumulation, and metabolism of chemicals by the lung, in *Mechanisms in Respiratory Toxicology*, Vol. 1, Witschi, H. and Nettesheim, P., Eds., CRC Press, Boca Raton, FL, 1982, pp. 162–178.

Witschi, H.P. and Brain, J.D., *Toxicology of Inhaled Materials*, Springer-Verlag, New York, 1985.

Witschi, H.P. and Nettesheim, P., *Mechanisms in Respiratory Toxicology*, Vol. 1, CRC Press, Boca Raton, FL, 1982.

Zhang, Y. and Jiang, X., Detoxification of nasal toxicity of nasal drug delivery system, *Zhongguo Yiyao Gongze Zazhi*, 32, 323–327, 2001.

25 Nonclinical Pharmacology and Safety Studies of Insulin Administered to the Respiratory Tract

Gregory L. Finch

CONTENTS

25.1 INTRODUCTION

The importance of insulin in the treatment of patients with diabetes mellitus has been recognized since the 1920s. Given that diabetes is characterized by an inability to produce or to effectively utilize insulin, exogenous administration of insulin has been central to diabetes therapy since that time. During the approximately 80 years of use, a number of improvements in insulin therapy have occurred. These include the purification and application of animal insulins and the development, approval, and clinical use of both recombinant human insulin and insulin analogs.

Over this time, subcutaneous injections have been almost exclusively the only therapeutically available route of administration of insulin. An exception to this was the development and use of implanted intraperitoneal pumps capable of slow, sustained insulin release. A variety of other routes have been investigated; most notably, these include pulmonary, oral, nasal, and dermal delivery. A review of the nonpulmonary routes is beyond the scope of this chapter; the interested reader is directed to other reviews (for example, see Heinemann et al., 2001). The importance of advancements in insulin therapy in diabetes mellitus has been recognized by many commentators given the prevalence of the disease, projections for future increases in disease incidence, the incomplete glycemic control in many patients, and the societal and economic burden of the disease.

This chapter focuses on selected aspects of the pulmonary delivery of insulin. Gansslen (1925) investigated the delivery of insulin via the lungs soon after the identification of insulin. However, early efforts were hampered in large measure by the inhalation device technology available and an

TABLE 25.1 Selected Nonclinical Studies of Insulin Absorption from the Respiratory Tract[a]

Animal model	Method of exposure	Exposure material and endpoints	Results	Comments	Reference
Rabbits, male New Zealand whites	INH, IT, or IV injection; crossover design. Aerosolization by jet nebulization.	Bovine insulin in saline at pH 4. Pharmacokinetics and gamma scintigraphy.	Gamma scintigraphy showed ratio of peripheral:central deposition was 1.52 for INH and 0.32 for IT, and bioavailability relative to IV was 57% versus 5.6%.	Authors concluded that enhanced deep lung deposition led to enhanced absorption. Commented that the pharmacokinetics were absorption rate limited.	Colthorpe et al., 1992.
Monkey, male and female Rhesus	INH, 20 minutes. Two types of nebulizers used.	Human insulin solution containing ^{99m}Tc. Gamma scintigraphy, pharmacokinetics, and pulmonary function parameters.	Aerosols with MMADs of 0.81 μm or 4.2 μm were produced. The smaller aerosol produced increased deep lung deposition, absorption, and bioefficacy. No effect on pulmonary function.	Authors concluded that increased deep lung deposition produced greater absorption and greater bioefficacy as measured by glucose lowering.	Pillai et al., 1996.
Rats, male Sprague-Dawley	IT. Additional *in vitro* experiments.	Human recombinant insulin, solution, with/without enzyme inhibitors: *N*-ethylmaleimide, *p*-chloromercuribenzoic acid, and 1,10-phenanthroline. Measured plasma insulin and glucose.	The inhibitors given IT but not IV increased insulin absorption, suggesting local enzymatic inhibition. *In vitro* studies confirmed the presence of IDE in cultured Type II alveolar epithelium.	Authors concluded that the results suggested that IDE is present in alveolar epithelium and might be involved in limiting insulin absorption from the lungs.	Hsu and Bai, 1998.

Rats, male Sprague-Dawley	IT. Additional *in vitro* experiments.	Porcine insulin in saline. With/without enzyme inhibitors bacitracin or sodium cholate. Also formulations at pH 3 or 7. Evaluated glucose lowering effect.	Both enzyme inhibitors and decreased pH lead to decreased glucose concentrations versus insulin solution alone. *In vitro* results were consistent in suggesting that enzyme inhibition increased absorption.	Authors concluded that protease activity in the lungs was responsible for insulin degradation. Effect of lower pH to increase absorption was attributed to either enzyme inhibition or tissue damage.	Shen et al., 1999.
Rats, male Sprague-Dawley	IT. Additional *in vitro* experiments.	Porcine insulin: formulations (1) in saline, (2) in saline within liposomes, and (3) in saline and DPPC. Endpoints: glucose lowering, insulin levels.	Through 4 h, order of glucose lowering response was formulation $3 > 2 > 1$.	Authors concluded that encapsulation in liposomes increased absorption, and DPPC without liposomes increased absorption further.	Mitra et al., 2001.
Beagle dogs, male and female	INH via endotracheal tube. The methodology for aerosol generation was not described.	Dry powder human insulin, 1 or 2 mg, compared with SC insulin. Plasma levels in blood, and glucose required to maintain euglycemia.	Quantified insulin C_{max} in arterial, portal, and deep venous blood.	Authors concluded that (1) arterial and venous levels were not significantly different, and (2) at comparable AUCs, more glucose was needed for euglycemia for 2 mg inhaled versus SC insulin.	Cherrington et al., 2003.
Beagle dogs	INH via endotracheal tube. The methodology for aerosol generation was not described.	Dry powder insulin or a derivitized PEG-insulin. Cross-over type design. Serum insulin and glucose.	Both aerosols produced a rapid rise in serum insulin and a reduction in glucose levels. Prolonged duration of action for PEG-insulin.	Authors concluded that the PEG-insulin derivitization produced elevated serum insulin and decreased glucose for as long at 10 hours after exposure.	Leach et al., 2003.

[a] Abbreviations: INH, inhalation; i.t., intratracheal instillation; i.v., intravenous injection; s.c., subcutaneous injection; IDE, insulin-degrading enzyme; DPPC, dipalmitoyl phosphatidylcholine; MMAD, mass median aerodynamic diameter; C_{max}, concentration maximum; PEG, polyethylene glycol.

incomplete appreciation of the relationship between aerosol characteristics and deep-lung deposition. As the available literature shows, renewed experimental interest in inhaled insulin dates largely from the 1980s and has led to several inhaled-insulin development projects sponsored and performed by a variety of researchers and pharmaceutical companies. Many characteristics of the pulmonary delivery route have spurred interest in inhaled insulin therapy. These include the relatively large area of the alveoli, minimal mucociliary clearance in the deep lung, adequate permeability to macromolecules with relatively low (compared with the gastrointestinal tract) enzymatic activity, and extensive vascularization of the lung (Patton, 1996).

This chapter provides a selective review of existing data on the absorption of insulin from the lungs and published nonclinical safety studies in animal models. The focus here is on the application of nonclinical models to investigate aspects of insulin absorption from the lungs. Table 25.1 provides an expanded presentation of study details for selected studies described more briefly in the text. This chapter is not intended to provide a comprehensive review of all aspects of delivery of insulin via the lungs, and in several places, the interested reader is referred to other complementary review papers available in the literature.

25.2 ABSORPTION OF INSULIN FROM THE LUNGS

25.2.1 Mechanisms of Macromolecular Absorption from the Lungs

The alveolar barrier, consisting of the alveolar epithelium, basement membrane, and capillary endothelium, is generally accepted as the predominant site for absorption of peptides such as insulin (Wolff, 1998). This seems logical because of the relatively high alveolar surface area and the thin barrier between the airspaces and blood. However, the dominant contribution of alveolar absorption has not been definitively proven, nor have there been measurements of the relative contribution of absorption (if any) across the thicker epithelial barriers found in the conducting airways.

Several studies suggest that deep-lung absorption is the principal way in which insulin gains systemic distribution. Patton (1996) noted that the predominant mechanism is thought to be paracellular transport, consisting of passive diffusion of insulin across the alveolar epithelium through extracellular tight junctions. Although transcytosis has also been demonstrated for the absorption of proteins from the lung, this mechanism may involve transport within alveoli and is probably more important for proteins of 40 kDa or larger (Patton, 1996; Crandall and Matthay, 2001). It should be recognized, however, that the predominance of alveolar absorption over potential airway absorption is not necessarily reflective of fundamental differences in absorption rates through these epithelia. Apparent transepithelial rates across alveolar versus airway epithelium were found to be comparable for hydrophilic solutes (Mathias et al., 1996), but the low diffusional distance across the alveoli compared with airway cells would appear to favor the alveolar route.

A number of *in vivo* studies suggest the importance of the alveolar spaces in the absorption of inhaled insulin (Colthorpe et al., 1992; Pillai et al., 1996; Laube et al., 1998). These studies, discussed in the next section, support the generally held view that absorption of inhaled insulin from the alveolar region is the predominant manner in which insulin gains access to the systemic circulation.

The alveolar epithelium consists of two morphologically distinct cell types termed type I and type II alveolar epithelial cells. Type I cells are relatively large, thin squamous cells, and type II cells are smaller cuboidal cells. Both tight junctions and gap junctions join type I and type II cells, providing a barrier function (Crandall and Matthay, 2001). Absorption of proteins from the lung is dependent on a variety of factors, including molecular size, charge, and site of deposition, whether diffusion is active or passive (Byron and Patton, 1994; Patton, 1996), and endogenous host factors such as cigarette smoking. Active transport mechanisms for insulin from the airspace lumen to blood have not been described in the literature. Thus, passive diffusion down a concentration gradient is the mechanism presumed to be responsible for driving systemic absorption of insulin following inhalation (Folkesson et al., 1996). Available evidence suggests that inhaled peptides remain in alveolar lining

fluid as they are being absorbed; this has been reported for superoxide dismutase (Welty-Wolf et al., 1997) and human growth hormone (Patton et al., 1989–1990).

25.2.2 Results from Selected Nonclinical Studies

Absorption of insulin has been studied in a variety of *in vitro* and *in vivo* model systems. A review of the *in vitro* experimentation is beyond the scope of this chapter. An example of *in vitro* research is the work of Kim et al. (1993), who measured an apparent permeability coefficient of 4.1×10^{-7} cm sec^{-1} for radiolabeled insulin *in vitro* in rat pneumocytes, and further observed fragmentation of insulin absorbed through this model system.

When considering *in vivo* studies, bioavailability of inhaled insulin following inhalation, as well as other proteins and peptides, is often an important parameter of interest in clinical investigations. With respect to animal studies, several studies describing either the kinetics or extent of insulin absorption can be found in the literature. Farr and Taylor (1997) have described many of the early studies. In general, it has been clearly shown that insulin can be absorbed from the lungs and delivered to the systemic circulation.

When comparing across studies, it is usually difficult to compare studies directly by examining bioavailability alone. This can be because of differences in the exposure methodology and differences in dose metrics. For an inhaled dose, bioavailability can be calculated on the basis of the amount of material in a starting container, the amount of material inhaled, or the amount of material estimated to reach the deep lung. Bioavailability can be described in relation to a subcutaneous dose, or absolutely by comparing with an intravenously injected dose. A further complicating factor seen in many animal studies is the use of an intratracheal instillation route of exposure. There are significant differences in regional and local deposition for the instillation route compared with the inhalation route (Driscoll et al., 2000), and thus estimates of absorption are not directly comparable for the two routes.

As noted in the previous section, several studies have suggested that absorption from the deep lung is important in insulin absorption. Pillai et al. (1996) measured the bioavailability of inhaled insulin produced by two methods of nebulization that produced differing particle sizes. Gamma camera imaging using ^{99m}Tc radiolabel demonstrated that more of a larger-sized aerosol was delivered than a smaller-sized aerosol. However, at comparable doses, the smaller-sized aerosol, which had increased deep-lung deposition compared with the larger-sized aerosol as determined from the scintigraphic images, produced a greater bioavailability and a greater bioefficacy as determined by blood glucose lowering. Using gamma scintigraphy and pharmacokinetic monitoring in rabbits, Colthorpe et al. (1992) measured a 10-fold greater bioavailability for inhaled versus intratracheally instilled insulin, and demonstrated that the greater bioavailability for inhaled insulin was associated with a greater peripheral deposition. Similarly in a clinical setting, Laube et al. (1998) evaluated the influence of the relative ratio of apical to basal deposition (as determined by gamma camera imaging) on blood glucose lowering and determined that relatively greater basal deposition was associated with greater percentage decreases in blood glucose levels.

Cherrrington et al. (2003) conducted a study in Beagle dogs to determine whether inhalation delivery was associated with different distributions of insulin concentrations in arterial, deep venous, and portal circulation, compared with subcutaneously administered insulin. Indwelling catheters were used to facilitate blood sampling, dogs received somatostatin to suppress endogenous insulin production, and euglycemia was maintained by glucose infusion. The maximum plasma concentration (C_{max}) values for the three blood compartments were reported, and it was determined that arterial and venous insulin concentrations were not significantly different following either the inhalation or subcutaneous routes of exposure.

As has been seen, many species have been used to examine absorption of insulin from the lungs. Further, the wide variety of exposure techniques has confounded a comparison of results across studies. Nonetheless, the animal studies have provided useful data on some of the parameters that are important

in delivering inhaled insulin and have set the stage for more comprehensive pharmacokinetic/pharmacodynamic studies of inhaled insulin in humans.

25.2.3 Role of Pulmonary Enzymatic Activity in Insulin Absorption

Numerous studies have demonstrated enzymatic activity in the deep lung or in cultured type II alveolar epithelial cells. In addition, some studies have shown the enzymatic degradation of insulin, whereas others have shown that enzyme inhibition results in enhanced bioavailability of insulin administered to the lungs. Alveolar macrophages are also known to possess a potent array of lytic enzymes.

Several ectopeptidases were described in rat alveolar epithelial cells, A549 cells, and pulmonary macrophages, and aminopeptidase activity in particular was seen as constituting a significant metabolic barrier to systemic delivery of peptides via the lungs (Forbes et al., 1999). As noted above, cell-associated metabolism of insulin during transport across a cell barrier was described in monolayer-cultured rat pneumocytes (Kim et al., 1993); fragmentation of insulin was noted in downstream fluids following apical to basal absorption, demonstrating metabolism of insulin in this model system. The biodegradation of insulin was reported in cytosolic or subcellular pellet fractions of rat lung homogenate; it was further noted that degradation was less in lung tissue obtained from diabetic rats, and that degradation could be inhibited by the enzyme inhibitors bacitracin and sodium cholate (Shen et al., 1999). It has been shown *in vitro* in the presence of lung lavage fluid supernatant that alveolar type II cells take up insulin (Mitra et al., 2001), so the uptake and subsequent cytosolic degradation of insulin is a likely route of removal of insulin delivered to the deep lung.

Okumura et al. (1992) reported relatively weak proteolytic degradation of insulin administered to the lungs of rats. Coadministration of nafamostat, a serine protease inhibitor, but not bacitracin, increased the relative bioavailability of intratracheally administered insulin by approximately 2-fold; the authors concluded that lung proteolytic activity was weaker than in subcutaneous tissues. In a follow-up study, these investigators demonstrated that glycocholate, a surfactant-like agent reported to suppress aminopeptidase activity, increased relative bioavailability by approximately 5-fold in rats receiving intratracheal instillations of aqueous insulin (Komada et al., 1994).

Insulin-degrading enzyme (IDE) is a 110-kDa metalloproteinase widely distributed in all cells, not only insulin-sensitive tissues (Duckworth et al., 1998). This enzyme has multiple cellular functions in addition to insulin degradation, including binding and regulatory functions. Most IDE is localized to the cytosolic compartment of cells, but has also been found on the plasma membrane, in endosomes, and in peroxisomes; the multiple cellular locations where IDE is found support a multifunctional role for this enzyme. Low levels of IDE relative to other tissues have been reported in rat lungs (Kuo et al., 1993). However, *in vitro* studies in type II alveolar epithelial cells demonstrated that IDE accounted for 81% of their insulin-degrading activity (Hsu and Bai, 1998). These investigators further demonstrated that the enzyme inhibitors *N*-ethylmaleimide, *p*-chloromercuribenzoic acid, and 1,10-phenanthroline not only inhibited IDE activity in crude cell homogenate and cytosolic preparations, but also significantly enhanced the bioavailability of intratracheally administered insulin in rats, thus leading them to suggest that IDE might be involved in limiting the pulmonary absorption of insulin. In a review of IDE, Duckworth et al. (1998) concluded that most evidence supports IDE as the primary degradative mechanism for the removal of insulin, but other systems (protein disulfide isomerase, lysosomes, and other enzymes) undoubtedly contribute to insulin metabolism.

Alveolar macrophages are scavenger leukocytes of the alveolar spaces. These cells are motile, able to internalize particles and solutes by phagocytosis and/or pinocytosis, and contain a range of lytic enzymes (Schlesinger, 1995). Materials internalized by macrophages can be cleared from the alveolar lumen by macrophage translocation and mucociliary clearance, translocation to lymphatic drainage, and by *in situ* degradation. Rat alveolar macrophages were shown to contain high levels of aminopeptidase activity (Forbes et al., 1999). On the other hand, in reviewing mechanisms of absorption and clearance of proteins from the lung, Wolff (1998) concluded that macrophage

phagocytosis does not appear to be as important a clearance mechanism as absorption, presumably because phagocytosis is more important in clearing relatively insoluble materials from the lung. Uptake of the inhaled enzyme superoxide dismutase by primate alveolar macrophages has been demonstrated (Welty-Wolf et al., 1997), and it has been concluded that macrophage uptake probably plays an increasingly important role as lung residence time increases (Wolff, 1998).

Taken together, it appears likely that both IDE and other nonspecific proteolytic enzymes are involved in the enzymatic degradation of insulin as it is being absorbed across the air–blood barrier, although this has not been directly reported *in vivo* for inhaled insulin.

25.2.4 Modifiers of Absorption

Animal studies are useful for evaluating various methods of altering insulin absorption from the lungs. Broadly, these include both physicochemical and biological techniques. Physicochemical modifications can include formulation alterations to include molecules such as penetration enhancers or also modifications to the insulin itself. Biologically, experiments have been conducted in which the enzymatic properties of the lung have been modified, as discussed in the previous section.

Many experiments have investigated the role of absorption enhancers. Although such agents can increase absorption, it has also been noted that many of these agents have historically been associated with tissue damage (Patton et al., 1999), and thus long-term safety data would be needed to support product registration. Research in this area is continuing. In a recent example, Hussain et al. (2003) characterized two absorption enhancers and further examined the reversibility of absorption enhancement. Following intratracheal spray administration to the lungs of rats, concentration-dependent increases in plasma insulin concentrations and decreases in plasma glucose concentrations were seen with the absorption enhancers tetradecyl-β-maltoside and dimethyl-β-cyclodextrin. When insulin in saline alone was administered 2 h after administration of one of these agents, absorption was comparable with that seen originally with the insulin controls, indicating the reversibility of absorption enhancement over this period.

Modifications of insulin itself have been investigated. Hinds and Kim (2002) prepared and characterized a variety of insulins conjugated with polyethylene glycol (insulin-PEG) and demonstrated that the conjugates remained in the systemic circulation for longer periods than unmodified insulin following subcutaneous administration to Beagle dogs. Leach et al. (2003) extended this type of approach to the inhalation route and showed that conjugation of human insulin to a 750 molecular weight PEG resulted in prolonged serum levels with a concomitant suppression of glucose concentrations, when compared with unmodified insulin.

25.2.5 Human Data

As noted above, clinical interest in inhaled insulin dates from the 1920s (Gansslen, 1925). A number of studies in humans have shown that insulin is bioavailable following inhalation and that administered doses can be biologically effective, as determined by pharmacodynamic effects on blood glucose concentrations. Current efforts involve both dry-powder and liquid formulations of insulin, and a wide variety of inhalation devices (Selam, 2003). It has been noted that clinical experience with inhaled insulin seems promising, and although several limitations and questions have been recognized, resolution of these questions and subsequent approval of marketing applications would result in a very valuable treatment method for patients with both type 1 and type 2 diabetes mellitus. Furthermore, it has been suggested that the noninvasive nature of inhaled insulin administration could lead to improved patient satisfaction and convenience, with potentially better glycemic control (Cefalu et al., 2002).

A comprehensive review of the clinical pharmacokinetics, safety, and efficacy of inhaled insulin is beyond the scope of this chapter. For additional discussion, the interested reader is referred to recent reviews by Patton et al. (1999), Heinemann et al. (2001), Cefalu et al. (2002), Selam (2003), and Patton et al. (2004). In general, and given the numerous caveats in comparing results across

studies and across species, the nonclinical data appear consistent with the human data. One potential difference between animals and humans regarding the bioavailability of inhaled insulin is related to regional differences in deposition. Given that the alveolar spaces appear to predominate as absorption sites for inhaled insulin (see above), differences across species in alveolar deposition fraction could lead to differing fractions of inhaled insulin being absorbed. It is well known that the alveolar deposition fraction of an inhaled dose is substantially lower for species such as rats and mice than for humans (Schlesinger, 1995).

25.3 SAFETY OF INHALED INSULIN

25.3.1 Safety/Toxicology Studies of Inhaled Insulin

This section contains a review of the literature describing the nonclinical safety/toxicology studies conducted with insulin administered to the respiratory tract. Only limited data are available in the published literature. Longer-term toxicology studies, which would be necessary to support more advanced stages of clinical development, have not been reported.

Jendle et al. (1995) performed a multiple-dose study including toxicological end points in piglets. A saline solution of porcine insulin with the surfactants polysorbate 80 or lipal-10 LA was nebulized to provide six treatments (time between treatments was not specified) at doses of 10, 20, 40, or 80 units. One animal served as a control, two received the insulin solution alone, two received insulin plus polysorbate 80, and two received insulin plus lipal-10 LA. Light microscopic histological evaluation of sections taken from the upper and lower lung lobes and the main bronchi showed no adverse effects of insulin inhalation.

There are several published reports including safety-related end points following single intratracheal or inhalation exposures of insulin in intact animals. Todo et al. (2001) exposed rats by single intratracheal instillation or intratracheal insufflation to a variety of aqueous and dry-powder bovine insulin formulations and then performed bronchioalveolar lavage (BAL) 24 h later for analysis of lactate dehydrogenase (LDH), a marker of cell injury. Rats given phosphate-buffered saline (PBS) instillation with a solution of 0.25% Triton X-100 served as a positive control. This group had LDH levels severalfold greater than the PBS-treated negative control group. Levels of LDH were the same as controls for dry-powder formulations containing insulin and mannitol, or insulin, mannitol, and citrate. Levels were slightly greater than controls for dry powders of insulin, mannitol, Span 85, and bacitracin.

Choi et al. (2001) exposed rats to a single inhalation (10-min exposure; estimated 25 μg deposited dose) of a nebulized suspension of bovine insulin in ethanol. Bronchioalveolar lavage was conducted at 2, 6, or 24 h on groups of rats. There were no cellular or soluble markers of inflammatory or allergic responses at any time point.

Garcia-Contreras et al. (2001) exposed rats by spray-instillation to a porcine insulin solution (1.3 units/kg insulin) with and without the absorption facilitator hydroxymethyl aminopropionic acid and sacrificed groups of animals at 1, 2, or 3 days after exposure for BAL analysis and lung histological analysis. There were no toxic effects of insulin with or without the absorption facilitator as compared with saline spray-instilled controls.

Pillai et al. (1996) monitored several pulmonary function parameters before, during, and after a 20-min inhalation exposure of two rhesus monkeys to nebulized rhu-insulin, administered at levels approximating a therapeutic dose based on glucose response. Compared with baseline values, there were no effects of insulin exposure on respiration rate, tidal volume, minute volume, lung resistance, or dynamic lung compliance.

Edwards et al. (1997) exposed rats by single inhalation exposure (10- to 20-min exposure; estimated inhaled dose not specified) to porous and nonporous particles containing insulin (species and formulation percentage were not specified), 50:50 poly (lactic acid co-glycolic acid) and rhodamine. Bronchioalveolar lavage conducted 48 h after exposure revealed a greater percentage of neutrophils

in the nonporous-treated group versus the porous-treated group; however, no vehicle-alone group was included in the study. Particles were found within phagocytes at the 48-h time point, indicating that the particles were poorly soluble.

Wolff et al. (2000) reported that no changes in pulmonary function parameters were observed in dogs inhaling insulin aerosols (2 μm mass median aerodynamic diameter [MMAD]) in a head-dome system either during acute exposure or in later repeated exposures (further details were not given). In a review of issues related to the safety of inhaled proteins, Wolff (1998) concluded that there is little potential for accumulation of inhaled proteins in the lungs and that the short residence time decreases the potential for adverse lung effects. Wolff further speculated that if there were to be a pathological response in the lungs, it would be expected to involve some type of cellular response to the inhaled proteins. Available data from the literature appear to be consistent with the conclusion stated by Wolff (1998).

Finally, a variety of *in vitro* experiments have been conducted with insulin, but these are not generally directed toward investigating the potential toxicity of insulin on lung cells. Farr and Taylor (1997), however, conducted 24-h exposures of rat type II alveolar cells to insulin, and measured detachment from substrate as a marker of toxicity. Insulin concentrations of up to 1000 μ*M* for 24 h did not result in toxicity.

A variety of nonclinical studies on the toxicology of insulin using other (i.e., noninhalation) routes of exposure have been published. These studies are pertinent to the safety of inhaled insulin, because (1) the lung is exposed to insulin circulating in the vascular bed, and (2) such studies often include high multiple doses relative to clinical doses and/or long-term dosing. Relative to the first point, lung uptake of circulating radioiodine-labeled insulin has been described (Iozzo et al., 2002). Relative to the second point, Low and Ferrill (1941) exposed rats daily for their life span via subcutaneous injection and observed no pulmonary abnormalities. More recently, no pulmonary effects were noted either from subcutaneously injected human insulin or the insulin analog glargine in both chronic and life-time carcinogenicity studies in Sprague–Dawley rats and NMRI mice, at doses constituting a maximum tolerated dose based on hypoglycemia (Stammberger et al., 2002). Thus, rodent lungs appear to tolerate chronic, high insulin concentrations through exposure via the vasculature.

As noted above, several clinical development programs have previously been conducted or are underway. Given the requirement that clinical trials be supported by nonclinical toxicology studies, it is clear that additional studies have been conducted, but not yet reported in the scientific literature. In this regard, Cefalu et al. (2001) reported that 1- and 6-month studies of intrapulmonary insulin had been conducted in rats and monkeys and that daily inhalation of powdered insulin was well tolerated and did not produce airway or pulmonary lesions. No specific methods or details were provided.

It is not possible to draw integrated conclusions from the scientific literature regarding the safety of chronic inhalation administration of insulin, because of (1) the limited exposure durations reported; (2) the variety of insulins and formulations used; (3) the nonphysiological nature of most animal exposure techniques; and (4) the incomplete reporting of study details. It is evident that in the only studies in which toxic effects were observed, the effects were attributed to the formulation characteristics, the presence of persistent particles, or the inclusion of absorption enhancers. The relevance of the available literature to a particular inhaled insulin development program, however, should be evaluated on a case-by-case basis.

25.3.2 Safety/Toxicology Studies of Other Inhaled Macromolecules

As noted above, inhalation delivery of a variety of proteins intended for systemic delivery has been evaluated from a pharmacological/pharmacokinetic perspective. Regarding safety, recent reports have described the safety of recombinant human growth hormone (hGH) (Carfagna et al., 2003) and human interferon beta-1A (IFN beta-1) (Demady et al., 2003).

Absorption of growth hormone from the lungs has been under investigation for some time (Patton et al., 1989–90). Carfagna et al. (2003) more recently described the inhalation toxicity of a dry-powder formulation of hGH in single-dose and 4-week studies in rhesus monkeys. Standard toxicology end points plus respiratory function, bronchioalveolar lavage parameters, and serum levels of immunoreactive growth hormone and growth hormone-specific antibodies were monitored in both studies. Bioavailability relative to subcutaneously injected hGH was approximately 10%. A moderate immunogenic response was elicited by 4 weeks, but there was no evidence of neutralizing antibodies or an anaphylactic response upon single-dose rechallenge after the 4-week exposure. There were no histopathological or other toxicological effects of the hGH formulation. It was concluded that no adverse effects were noted in monkeys exposed daily to inhaled doses up to 5.5 mg of hGH/kg body weight for 4 weeks, and that the data provided margins of safety well above the recommended clinical doses of hGH.

Demady et al. (2003) evaluated the safety and absorption of a dry-powder aerosol formulation of interferon (IFN) beta-1 in rhesus monkeys at inhaled doses up to 4.5 mg of IFN beta-1 per week, with safety assessments conducted at 1 month, 3 months, and 6 months (with a 4-week recovery) of treatment. Serum IFN beta-1 and the biological response marker neopterin showed dose-dependent concentration increases. Neutralizing titers of anti-IFN beta-1 antibodies were observed, beginning at the third week of dosing. The study also showed that IFN beta-1 was not associated with any significant changes in safety parameters. In the lung, histological changes consisted of minimal to mild subchronic alveolitis, which increased slightly with dose, but did not progress over 6 months of exposure. The investigators concluded that the chronic pulmonary administration of IFN beta-1 was generally safe.

Wolff (1998) reviewed issues surrounding safety of inhaled proteins for therapeutic use. The author reviewed safety data available at that time and found that, in the animal studies, toxicological responses to inhaled proteins were either absent or when present were relatively low. It was further noted that the relevance to humans was difficult to assess in cases where the human protein differs considerably from the animal protein.

25.4 CONCLUSIONS

A number of studies in both animals and humans have demonstrated the feasibility of insulin delivery to the respiratory tract, leading to the absorption of biologically active insulin into the circulation, resulting in its well-known biological action to lower blood glucose concentrations. Animal studies have played a crucial role, and continue to do so, in (1) demonstrating the importance of deep-lung deposition in increasing absorption; (2) demonstrating the role of enzymatic activity in the lung in modulating insulin absorption; and (3) characterizing the effects of formulation changes and/or insulin modifications on insulin absorption. Regarding safety, no long-term toxicology studies have been reported in the literature, and the available shorter-term exploratory data are consistent with the conclusion that insulin itself does not appear to be associated with harmful respiratory tract effects.

REFERENCES

Byron, P.R. and Patton J.S., Drug delivery via the respiratory tract, *J. Aerosol Med.*, 7, 49–75, 1994.

Carfagna, M.A., Clarke, D.O., Shaw, M.E., and Baker, Evaluation of a somatropin inhalation powder in a single dose and 4-week monkey inhalation toxicology study, *The Toxicologist*, 72 (S-1), Abstr. 240, 2003.

Cefalu, W.T., Rosenstock, J., and Bindra, S., Inhaled insulin: a novel route for insulin delivery, *Expert Opin Investig Drugs*, 11, 687–691, 2002.

Cefalu, W.T., Skyler, J.S., Kourides, I.A., Landschulz, W.H., Balagtas, C.C., Cheng, S.-L., and Gelfand, R.A., Inhaled human insulin treatment in patients with type 2 diabetes mellitus, *Ann. Intern. Med.*, 134, 203–207, 2001.

Cherrington, A.D., Hobbs, C.H., Leach, C., Neal, D.W., Edgerton, D.S., and Strack, T.R., Inhalation of insulin in dogs: assessment of insulin levels, presented at Sixty-third Meeting of the American Diabetes Association, New Orleans, LA, Abstr. 441-P, 2003.

Choi, W.S., Murthy, G.G.K., Edwards, D.A., Langer, R., and Klibanov, A.M., Inhalation delivery of proteins from ethanol suspensions, *Proc. Natl. Acad. Sci. USA*, 98, 11103–11107, 2001.

Colthorpe, P., Farr, S.J., Taylor, G., Smith, I.J., and Wyatt, D., The pharmacokinetics of pulmonary-delivered insulin: a comparison of intratracheal and aerosol administration to the rabbit, *Pharm Res*., 9, 764–768, 1992.

Crandall, E.D. and Matthay, M.A., Alveolar epithelial transport. Basic science to clinical medicine, *Am. J. Respir. Crit. Care Med*., 163, 1021–1029, 2001.

Demady, D.R., Hutto, D.L., Lane, J.L., and Osawa, Y., Safety and absorption of pulmonary delivered human interferon beta-1A in the non-human primate, *The Toxicologist*, 72 (S-1), Abstr. 239, 2003.

Driscoll, K.E., Costa, D.L., Hatch, G., et al., Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: uses and limitations, *Toxicol. Sci*., 55, 24–35, 2000.

Duckworth, W.C., Bennett, R.G., and Hamel, F.G., Insulin degradation: progress and potential, *Endocr. Rev*., 19, 608–624, 1998.

Edwards, D.A., Hanes, J., Caponetti, G., et al., Large porous particles for pulmonary delivery, *Science*, 276, 1868–1871, 1997.

Farr, S.J. and Taylor, G., Insulin inhalation, in *Inhalation delivery of therapeutic peptides and proteins. Lung Biology and Health and Disease*, Vol. 107, Adjei, A.L. and Gupta, P.K., Eds., Marcel Dekker, Inc, New York, 1997, pp. 371–386.

Folkesson, H.G., Matthay, M.A., Westrom, B.R., Kim, K.J., Karlsson, B.W., and Hastings, R.H., Alveolar epithelial clearance of protein, *J. Appl. Physiol*., 80, 1431–1445, 1996.

Forbes, B., Wilson, C.G., and Gumbleton, M., Temporal dependence of ectopeptidase expression in alveolar epithelial cell culture: implications for study of peptide absorption, *Int. J. Pharm*., 180, 225–234, 1999.

Gansslen, M., Uber Inhaledalation von Insulin, *Klin. Wochenschr.*, 4, 71, 1925.

Garcia-Contreras, L., Sarubbi, D., Flanders, E., O'Tode, D., Smart, J., Newcomer, C., and Hicke, A.J., Immediate and short-term cellular and biochemical responses to pulmonary single-dose studies of insulin and H-MAP, *Pharm. Res*., 18, 1685–1693, 2001.

Heinemann, L., Pfutzner, A., and Heise, T., Alternative routes of administration as an approach to improve insulin therapy: update on dermal, oral, nasal and pulmonary insulin delivery, *Curr. Pharm. Des*., 7, 1327–1351, 2001.

Hinds, K.D. and Kim, S.W., Effects of PEG conjugation on insulin properties, *Adv. Drug Deliv. Rev.*, 54, 505–530, 2002.

Hsu, M.C. and Bai, J.P., Investigation into the presence of insulin-degrading enzyme in cultured Type II alveolar cells and the effects of enzyme inhibitors on pulmonary bioavailability of insulin in rats*, J. Pharm. Pharmacol*., 50, 507–514, 1998.

Hussain, A., Yang, T., Zaghloul, A.A., and Ahsan, F., Pulmonary absorption of insulin mediated by tetradecyl-beta-maltoside and dimethyl-beta-cyclodextrin, *Pharm. Res*., 20, 1551–1557, 2003.

Iozzo, P., Osman, S., Glaser, M., et al., In vivo imaging of insulin receptors by PET: preclinical evaluation of iodine-125 and iodine-124 labelled human insulin, *Nucl. Med. Biol*., 29, 73–82, 2002.

Jendle, J., Karlberg, B.E., Persliden, J., Franzen, L., and Arborelius, M., Delivery and retention of an insulin aerosol produced by a new jet nebulizer, *J. Aerosol Med*., 8, 243–254, 1995.

Kim, K.J., Yamahara, H., Lehr, C.M., Lee, V.H.L., and Grandall, E.D., Fate of insulin during alveolar epithelial transit (Abstr.), *FASEB J*., 7, A436, 1993.

Komada, F., Iwakawa, S., Yamamoto, N., Sakakibara, H., and Okumura, K., Intratracheal delivery of peptide and protein agents: absorption from solution and dry powder by rat lung, *J. Pharm. Sci*., 83, 863–867, 1994.

Kuo, W.L., Montag, A.G., and Rosner, M.R., Insulin-degrading enzyme is differentially expressed and developmentally regulated in various rat tissues, *Endocrinology*, 132, 604–611, 1993.

Laube, B.L., Benedict, G.W., and Dobs, A.S., Time to peak insulin level, relative bioavailability, and effect of site of deposition of nebulized insulin in patients with noninsulin-dependent diabetes mellitus, *J. Aerosol Med*., 11, 153–173, 1998.

Leach, C.L., Kuo, M.-C., Perkins, K.M., Bueche, B., Guo, L., Bentley, M.D., and Patton, J.S., Dry Powder formulation of inhaled PEG-insulin yields prolonged systemic activity in dogs, presented at the Sixty-third Meeting of the American Diabetes Association, New Orleans, LA, Abstr. 451-P, 2003.

Low, F.N. and Ferrill, H.W., Chronic insulin injection in five successive generations of white rats: report of gross and histological findings, *Endocrinology*, 29, 1027–1028, 1941.

Mathias, N.R., Yamashita, F., and Lee, V.H.L., Respiratory epithelial cell culture models for evaluation of ion and drug transport, *Adv. Drug Deliv. Rev*., 22, 215–249, 1996.

Mitra, R., Pezron, I., Li, Y., and Mitra, A.K., Enhanced pulmonary delivery of insulin by lung lavage fluid and phospholipids, *Int. J. Pharm*., 217, 25–31, 2001.

Okumura, K., Iwakawa, S., Yoshida, T., et al., Intratracheal delivery of insulin. Absorption from solution and aerosol by rat lung, *Int. J. Pharm*., 88, 63–73, 1992.

Patton, J.S., Mechanisms of macromolecule absorption by the lungs, *Adv. Drug Deliv. Rev*., 19, 3–36, 1996.

Patton, J.S., Bukar, J., and Nagarajan, S., Inhaled insulin, *Adv. Drug Deliv. Rev*., 35, 235–247, 1999.

Patton, J.S., Bukar, J.G., and Eldon, M.A., Clinical pharmacokinetics and pharmacodynamics of inhaled insulin, *Clin. Pharmacokinet*., 43, 781–801, 2004.

Patton, J.S., McCabe, G.J., Hansen, S.E., Daugherty, A.L., Absorption of human growth hormone from the rat lung, *Biotechnol. Ther*., 1, 213–228, 1989–1990.

Pillai, R.S., Hughes, B.L., Wolff, R.K., Heisserman, J.A., and Dorato, M.A., The effect of pulmonary-delivered insulin on blood glucose levels using two nebulizer systems, *J. Aerosol Med*., 9, 227–240, 1996.

Schlesinger, R.B., Deposition and clearance of inhaled particles, in *Concepts in Inhalation Toxicology*, McClellan, R.O. and Henderson, R.F., Eds., Taylor and Francis, Washington, DC, 1995.

Selam, J.L., Inhaled insulin for the treatment of diabetes: projects and devices, *Expert Opin. Pharmacother*., 4, 1373–1377, 2003.

Shen, A., Zhang, Q., Wei, S., and Nagai, T., Proteolytic enzymes as a limitation for pulmonary absorption of insulin: in vitro and in vivo investigations, *Int. J. Pharm*., 192, 115–121, 1999.

Stammberger, I., Bube, A., Durchfeld-Meyer, B., Donaubauer, H., and Troschau, G., Evaluation of the carcinogenic potential of insulin glargine (LANTUS) in rats and mice, *Int. J. Toxicol*., 21, 171–179, 2002.

Todo, H., Okamoto, H., Iida, K., and Danjo, K., Effect of additives on insulin absorption from intratracheally administered dry powders in rats, *Int. J. Pharm*., 220, 101–110, 2001.

Welty-Wolf, K.E., Simonson, S.G., Huang, Y.C., et al., Aerosolized manganese SOD decreases hyperoxic pulmonary injury in primates. II. Morphometric analysis, *J. Appl. Physiol*., 83, 559–568, 1997.

Wolff, R.K., Safety of inhaled proteins for therapeutic use, *J. Aerosol Med*., 11, 197–219, 1998.

Wolff, R.K., Allen, D.L., Hughes, B.L., et al., The case for route-dependent toxicology comparisons: Using large animal models to speed clinical development of inhaled proteins, *Respir. Drug Del. VII Proc*., 163–170, 2000.

Part III

Inhalation Toxicology of Materials

26 Inhalation Toxicology of Alcohol/Gasoline Fuels—Current Status

Raymond Poon

CONTENTS

26.1 INTRODUCTION

26.1.1 General Description and Usage

In this chapter the term "alcohol/gasoline" refers to fuels that are composed of mixing methanol or ethanol with unleaded gasoline in various proportions. The resultant blends are suitable for use as alternative automotive fuels that address, to some extent, environmental concerns and regulatory requirements. They are sometimes referred to in abbreviations, e.g., E20 is a fuel consisting of a mixture of 20% ethanol and 80% gasoline by volume; M85 is a mixture of 85% methanol and 15% gasoline; and MEG refers to a mixture containing 33% methanol, 60% ethanol, and 7% gasoline. In addition, "gasohol" is marketed in Europe and North America as a mixture of up to 10% ethanol in gasoline.

As early as 1917, Charles Kettering and Thomas Midgely reported that grain alcohol mixed with gasoline produced an automotive fuel that reduced engine knocks (Nadim et al., 2000). It was not until the early 1970s that alcohol/gasoline fuels received widespread international attention for a combination of environmental, economical, and technical reasons. The need for alternative and economical, energy supplies is a major impetus. Ethanol is a renewable energy source that can be generated from sugar and corn and methanol is produced from natural gas. In countries such as Brazil, where sugar cane is in abundant supply, most automobiles are run on ethanol, or ethanol/gasoline blends (Ahmed, 2001; Coelho and Goldemberg, 2004). In Canada, ethanol/gasoline blends (gasohol) containing up to 10% ethanol are available in many distribution centers (NRCan, 1996). In the United States, Alternative Fuel Vehicles are routinely run on E85 fuels (Alternative Fuel News, 2003). With rapid biotechnological innovations, production of alcohols from biomasses such as cellulose and softwoods likely will become economically feasible in the near future. Concern about air quality and health effects is a major factor contributing to the increased use of alcohol/gasoline. The U.S. Clean Air Act Amendments of 1990 mandated the requirements to change the formulation of gasoline, specifically to reduce carbon monoxide emission, and also the requirement that in regions where the carbon monoxide standard was exceeded, oxygenated fuels containing at least 2.7% oxygen by weight must be used. The list of oxygenates included methyl *tert*-butyl ether (MTBE), ethanol, and methanol, among others. However, with the gradual withdrawal of MTBE from the market because of concern over reports of adverse reactions and groundwater contamination, use of ethanol as the replacement oxygenate is expected to increase with time.

26.1.2 Exposed Populations

Over one hundred million people in the United States are exposed to gasoline vapor during refueling activities (Wixtron and Brown, 1992). Although the number of people exposed specifically to alcohol/gasoline vapor is not known, it has been estimated that, in the United States, 70 million people live in areas where oxygenated fuels are used (Health Effects Institute [HEI], 1996). In Brazil, where ethanol and ethanol/gasoline are used exclusively as automobile fuels, exposure to ethanol/gasoline vapor is nationwide. Ethanol and ethanol/gasoline are used to varied extents in numerous countries in Africa, Asia, Australia, and Europe (Coelho and Goldemberg, 2004) although statistics on exposed populations are not available.

26.2 COMPOSITION

26.2.1 Liquid

Ethanol and methanol components within the alcohol/gasoline mixtures are individual, well-defined chemicals. Recent analysis of anhydrous ethanol stock used to prepare ethanol/gasoline blends

TABLE 26.1 Composition Ranges of European Gasoline[a] by Hydrocarbon Type

Composition	% by volume
Alkanes	30–90
Cycloalkanes	1–35
Alkenes	0–20
Aromatics	5–55

[a] May contain up to 0.15 g/l lead.

Source: Data adapted from CONCAWE (1992).

TABLE 26.2 Composition of Unleaded Gasoline (API 94-02) and Unleaded Gasoline Vapor Condensate

Composition	Liquid, vol %	Vapor, vol %
N-Alkanes	12.3	28.5
Total alkanes	48.4	79.5
Cycloalkanes	5.6	2.3
Total alkenes	9.7	14.1
Total aromatics	34.4	4.0
Benzene	1.4	1.0
Toluene	8.0	2.0
Carbon number		
4	5.1	18.9
5	16.8	46.4
6	18.6	23.6
7	19.4	7.8
8	20.7	3.1
9	11	0.2
10	5.2	0.0
11	2.1	0.0
12+	1.0	0.0
Total	99.9	100

Source: Adopted from Roberts et al. (2001).

detected some paraffins, aromatic hydrocarbons, aldehydes, ketones, and other alcohols (Vilar et al., 2003). In comparison, the gasoline component of the mixture is much more complex. Gasoline consists of mostly hydrocarbons whose composition varies widely depending on the sources of crude oil, the refinery processes, the product specification, and seasonal adjustment (Wixtrom and Brown, 1992). At least 150 compounds have been identified in gasoline and, theoretically, 1200–1500 hydrocarbons are possible (Domask, 1984). The wide range of variation in hydrocarbon types is illustrated in the analysis of the composition of European gasoline (Table 26.1). There is significant variation in organics even from different batches of gasoline (Kumarathasan et al., 1996). Therefore, any toxicological study of alcohol/gasoline must bear in mind that the toxic expressions result from the exposure to, and the interaction of, a complex mixture whose composition varied with sources and batches. The presence of lead could be a confounding factor. Although North American gasoline is unleaded (<0.013 g/l), lead may be present in gasoline sold in Europe at a concentration of <0.15 g/l. In this chapter, only the toxicology of alcohol/unleaded gasoline vapor is considered.

26.2.2 Vapor

The composition of gasoline vapor is governed by the boiling points of the individual liquid components and is influenced by ambient temperature. As shown in Table 26.2, the relative concentration of a hydrocarbon type is more or less inversely proportional to the carbon number. Thus, C4/C5 compounds such as *n*-butane, isobutane, *n*-pentane, and isopentane are the dominant constituents found in gasoline vapor at gasoline-loading facilities and service stations (Halder et al., 1986a; Mckee et al., 2000; Roberts et al., 2001). Benzene, a C-6 compound and a regular constituent of gasoline, is also

TABLE 26.3 Molecular Weight, Normal Boiling Point, and Vapor Density of Gasoline and Alcohols

		Gasoline	Isopentane	Benzene	Ethanol	Methanol
Molecular weight		108[a]	72.1	78.1	46.1	33.1
Boiling point, °C	Initial	39	30	80.1	78.3	64.9
	After 10% distilled	60				
	After 50% distilled	110				
	After 90% distilled	170				
	Final	204				
Vapor density, g/ml		3–4	2.48	2.77	1.6	1.11
Vapor pressure, kPa		62–103	79	12.7	7.9	6.8

[a] Average molecular weight.

Source: Data adapted from ATSDR, 1995, and von Burg, 1989.

present in the vapor phase in significant concentration. The mixing of a variable amount of alcohols with gasoline will of course add to the complexity of an already complex mixture and in turn the complexity of its vapor. However, based on a comparison of the critical physicochemical properties of ethanol and methanol with known volatiles in gasoline such as isopentane and benzene (Table 26.3), it can be predicted that, depending on the alcohol-to-gasoline ratio, these alcohols will form a substantial portion of an alcohol/gasoline vapor. In the only published study of vapor composition of an alcohol/gasoline fuel, Tsai and Weisel (2000) reported that the vapor composition in a garage due to evaporative emission from an M85-fueled vehicle varied from 0.015 ppm for benzene and 0.78 ppm for methanol at an average fuel tank temperature of 23°C, to 0.21 ppm for benzene and 1.2 ppm for methanol at 37°C . The higher proportion of methanol to benzene in the vapor certainly reflected the percent methanol in the M85 fuel, and also the lower normal boiling point of methanol. The vapor density of ethanol and methanol is much lower than that of benzene and gasoline (Table 26.3), which can affect the vapor composition in a well-ventilated area.

26.3 STUDIES ON HUMAN AND NONHUMAN PRIMATES

Benzene, a regular component of gasoline, is classified a group 1 agent, i.e., a human carcinogen (International Agency for Research on Cancer [IARC], 1987). Gasoline is classified as a group 2B agent, i.e., a possible human carcinogen (IARC, 1989). Although alcoholic beverages are considered as carcinogenic to humans (group 1) (IARC, 1988), ethanol and methanol in liquid or vapor phase have not been evaluated for carcinogenicity. No carcinogenicity data are available for alcohol/gasoline.

Concerns over the possible health effects have been raised with the realization of the possibility of a widespread use of alcohols as oxygenates and alternate fuels (HEI, 1987, 1996; Costantini, 1993; Caprino and Togna, 1998; Ahmed, 2001). However, only a few preliminary exposure and health effect studies of alcohol/gasoline have been conducted on humans. An initial study reported that the presence of 10% ethanol significantly lowered the odor-detection threshold for one brand of gasoline but not for another brand with a different aromatic content (Zhao et al., 1995). Backer et al. (1997) analyzed the gasoline-related volatile organic compounds (VOCs) in the blood and in the personal breathing zone of persons refueling regular unleaded gasoline or gasoline containing 10% ethanol (E10). They found a similar pattern of VOCs in the personal breathing zone for the two groups, and no significant differences in the VOCs in their blood. Tsai and Weisel (2000) demonstrated that evaporative emissions from an M85-fueled automobile increased the indoor VOCs represented by benzene, toluene, and methanol. Based on observations conducted on controls and exposed groups of limited sizes, Gattàs et al. (2001) reported that gas station operators exposed to a

mixed fuel called MEG (33% methanol, 60% ethanol, and 7% gasoline) had significantly increased frequency of oral mucosa micronuclei, suggesting a mutagenic hazard of MEG.

More studies in humans and monkeys are available on individual methanol and ethanol vapors. A summary report (New Energy Development Organization [NEDO], 1987) of a study on monkeys exposed to methanol vapor for 21 days indicated the following effects: abnormal cage-side neurobehavior at 5000 ppm; acidosis, probably due to accumulation of formic acid, at 5000–7000 ppm; increased white blood cell and changes in electroencephalogram at 7000 ppm; and histopathological changes in the brain at 5000 ppm. No increase in blood formate, the proximal toxic metabolite during acute methanol poisoning, was found in human volunteers following a 6-h inhalation exposure to 200 ppm methanol (Lee et al., 1992). Osterloh et al. (1996) observed a rapid increase in serum and urinary methanol but no significant increase in formate in humans inhaling methanol vapor at 200 ppm for 4 h. The overall elimination half-life for serum methanol was 3.2 h. Batterman et al. (1998) studied volunteers exposed to methanol vapor at 800 ppm for 0.5 to 2 h and estimated the half-life in blood, urine, and breath to be approximately 1.5 h. A physiologically based pharmacokinetic (PBPK) model constructed by Pastino et al. (1997) indicated that inhalation of ethanol at or above the concentrations expected to occur upon refueling resulted in minimal increase in blood ethanol concentration and was unlikely to result in toxicity. Similarly, employing a biologically based dynamic model, Bouchard et al. (2001) estimated that an 8-h inhalation of 500–2000 ppm methanol vapor is needed to significantly increase blood and urinary formate above the background level. A 4-h exposure to methanol vapor at 200 ppm was found to have little effect on several measures of neurobehavioral performance (Chuwers et al., 1995) and only a slight excitatory effect (Muttray et al., 2001) on humans. Mann et al. (2002) observed that interleukin-1β and interleukin-8 in nasal secretions were increased in healthy volunteers following exposure to 200 ppm of methanol vapor, suggesting a subclinical irritating effect.

Reproductive and developmental studies were conducted on monkeys exposed to 200–1800 ppm methanol for 2.5 h/d during the premating and mating period and during pregnancy (Burbacher et al., 1999a, 1999b). The mean duration of pregnancy was reduced in all methanol-treated groups. Two females in the 1800-ppm group demonstrated a wasting syndrome and were sacrificed. Some possible neurobehavioral effects in the form of reduced performance with the Visually Directed Reaching Test and a reduced novelty preference (Fagan Test of Infant Intelligence) were detected in infants prenatally exposed to methanol. A follow-up study on the exposed infants using a Simple and Choice Reaction Time test and movement time did not reveal a treatment effect (Cardenas et al., 2000). As with the human study noted above, there was a small increase in maternal blood methanol but not formate throughout the exposure. Dorman et al. (1994) showed that short-term (2-h) exposure to 900 ppm of methanol vapor did not result in elevation of blood formate above those presented endogenously.

26.4 TOXICOLOGICAL STUDIES ON ANIMALS

26.4.1 Technical Considerations and Dose Ranging

26.4.1.1 Generation of Vapors

Because of the complexity of the gasoline constituents and the wide ranges of boiling points (Table 26.3), inhalation toxicity of gasoline and alcohol/gasoline vapors are critically influenced by the methods of vapor generation. There are at least six modes of generating gasoline or alcohol/gasoline vapors for the test animals to inhale. They are summarized below with comments.

1. **Closed atmosphere system** (Gargas et al., 1986). A 9.0-l desiccator served as the exposure chamber into which filtered air was recirculated as a closed system. Test chemicals were introduced as liquids upstream of the chamber and the chamber atmosphere monitored by gas-liquid chromatography. Carbon dioxide was removed with barium hydroxide-lime.

Water vapor was removed by means of a cold trap. Oxygen was replenished to maintain a concentration of 19–21%. This system is suitable for experiments designed for small numbers of animals.

2. **Atomization** (Kuna and Ulrich, 1984). Gasoline was atomized into a small chamber, mixed with incoming air, and drawn by negative pressure into the exposure chamber. With this method, incomplete vaporization of gasoline and entry of aerosolized materials into the exposure chamber were experimental concerns. Deposition of aerosals on the skin may result in entry of test chemicals through the oral and dermal routes in addition to the desired inhalation route.
3. **Total vaporization** (MacFarland et al., 1984; Tilbury et al., 1993). In this method, gasoline was delivered from a metering pump to a heated countercurrent vaporization column or a heated stainless steel J-tube containing glass beads and completely volatilized. Dry nitrogen or air was used to carry the vapor into the exposure chamber. A drawback of this method was that the temperature of the heated vaporization unit was not reported and presumably not tightly controlled.
4. **Vapor recovery unit condensate** (VRU) (McKee et al., 2000). Some gasoline distribution terminals have installed VRUs, and the condensate from such units was deemed representative of the volatile materials to which workers and consumers are exposed during normal production and refueling of gasoline. The condensate was metered into a flask warmed with an electric mantle and the vapor was mixed with dilution air before introduction into the exposure chamber. It was not known if highly volatile constituents of the vapor were lost during the vapor collection and condensation process.
5. **Gasoline distillates**. In the methods of Roberts et al. (2001) gasoline was heated to a temperature of 65.5°C, resulting in a vapor temperature of 54°C. The vapor was condensed by passing through a series of two receiving vessels chilled with cold water and dry ice, respectively, then through additional vapor traps chilled in dry ice/isopropyl alcohol. The combined condensate represented 10.4% of the initial gasoline weight. In another method (Benson et al., 2003) the light fraction gasoline that consisted of all the components volatilized at 130°C was used for vapor generation.
6. **Controlled-temperature vaporization**. There are a variety of methods for generating vapors from whole gasoline at a controlled temperature. A dynamic flow unit specifically designed for alcohol/gasoline mixtures (Kumarasathan et al., 1996) is briefly described here. The heart of the unit is a J-tube filled partially with glass beads (Miller et al., 1980) and warmed with an electrical tape. Methanol and gasoline are delivered into the J-tube through a metering device while high-efficiency particulate air (HEPA)-filtered carrier air, warmed to 75°C, is introduced into the J-tube at a flow rate of 25 l/min. The volume delivered per unit time to generate the desired vapor concentration at 1 atmosphere in the exposure chamber is calculated as follows:

$$V_1 = \frac{C \times \mathrm{MW} \times V_2 \times 273\ ^\circ\mathrm{C}}{22.4\ \mathrm{l/mol} \times 10^6 \times d \times (273 + T)\ ^\circ\mathrm{C}}$$

Where V_1 (mL) is the volume of chemical evaporated, V_2 (L) is the final volume of the chamber, C is the vapor concentration (ppm) in the inhalation chamber, d is the density of the chemical in g/ml, T is the chamber temperature in °C, 22.4 l/mol is the ideal gas constant, and MW is molecular weight.

The methanol/gasoline vapor is mixed with HEPA-filtered dilution air (580 l/min) and introduced into a 2.5-m^3 inhalation chamber. The gasoline and vapor composition of the vapor within the inhalation chamber was monitored by gas chromatography-flame ionization detection (GC-FID) and further characterized by gas chromatography-mass spectrometry (GC-MS). The condition and dynamic parameters of the inhalation chamber are summarized in Table 26.4.

TABLE 26.4 Inhalation Chamber Conditions and Parameters for a Toxicity Study on Methanol/Gasoline Vapor

Parameters	Values
Temperature of carrier gas	75°C
Temperature of vapor (within J-tube)	60°C
Chamber volume, V	2.5 m^3
Chamber temperature	23 ± 2°C
Chamber humidity	45–50%
Flow rate, F (HEPA-filtered air)	
Vapor carrier	25 l/min
Chamber inlet	580 l/min
Number of air changes per hour[a]	14
Calculated t99[b]	13 min
Actual time to approach equilibrium	15–20 min
Agreement of measured with nominal methanol levels (5000–50 ppm)	±1%–7%

[a] Number of air change = 60 min/(V/F).

[b] Time to achieve 95% of the equilibrium concentration, $t_{99} = 4.605 \times V/F$ (MacFarland, 1986).

26.4.1.2 Dose Range and Exposure Levels

The choice of dose levels is a critical consideration in the reliable and realistic assessment of the toxicity of alcohol/gasoline vapors. Thus, methanol vapor as high as 20,000 ppm had been employed in developmental toxicity studies for maternal exposure (Nelson et al., 1985). For short-term and subchronic studies, the highest level of gasoline and methanol used has not exceeded 3000 ppm (McFarland et al., 1984; Halder et al., 1984, Kuna and Ulrich, 1984; Andrews et al., 1987; Poon et al., 1994, 1995a, 1998). To study the effect of occupational and industrial exposure, the dose range should be adjusted to reflect the likely exposure levels. For example, the mean gasoline vapor concentrations can be as high as 400 ppm during truck loading (Smith et al., 1993). Presented as the 8-h time-weighted average, the mean gasoline exposure level was 5.0 ppm for refinery workers, 7.7 ppm for service station attendants, and 13.2 ppm for truck drivers (Weaver, 1988). The current industrial exposure guidance levels should also be taken into account, e.g., the threshold limit value-time-weighed average (TLV-TWA) for ethanol, methanol, and gasoline are set at 1000 ppm, 200 ppm, and 300 ppm, respectively (American Conference of Governmental Industrial Hygienists [ACGIH], 2003). When the effects of long-term, low-level exposure are investigated, the dose levels should be pertinent to the levels present in the environment, households, and consumer settings. A person may be exposed to gasoline vapor as low as 0.26 ppm at the vicinity of a service station, and from 0.3 to 100 ppm during refueling (Halders et al., 1986a; Roberts et al., 2001). There are only limited data on alcohol/gasoline exposure. U.S. EPA has estimated a worst-case scenario of 16–44 ppm methanol emitted by a methanol-fueled vehicle in a parking garage (HEI, 1987). Gold and Moulis (1998) estimated that the ambient methanol concentration among hot engines being turned off, 10% of which are malfunctioning, to be as high as 114 ppm. On the other hand, Tsai and Weisel (2000) reported that the evaporative emission of methanol from an M85-fueled vehicle in the garage and an adjacent area was 1.2 and 0.28 ppm, respectively. Backer et al. (1997) found that volatiles such as benzene and toluene in a E10 fuel can reach 1 ppm in the personal breathing zone during refueling.

26.4.1.3 Exposure Modes

The whole-body inhalation exposure mode has been used in most of the studies on gasoline vapor. In small-scale and acute-exposure studies, the closed-chamber system was often used (see section 26.4.1.1). For studies involving a larger number of animals and longer exposure periods, specially constructed inhalation chambers with dynamic airflow and real-time monitoring of test atmosphere should be employed. For regulatory testing, various guidance documents have been promulgated to ensure the quality and proficiency of the results. For studies of alcohol/gasoline vapors, the pertinent specifications include: number of changes of air (10–15 per hour), total volume of test animals (< 5% of test chamber volume), oxygen content (at least 19%), continuous or intermittent in-chamber measurement of the test atmosphere, and uniform distribution of test vapor (U.S. EPA, 1998; Organization for Economic Co-operation and Development [OECD], 2002).

With the increasing availability and validation of the multiport systems (Pauluhn, 1994), the nose/head-only mode of exposure will be increasingly used in the study of the toxicity of gasoline and alcohol vapors. This type of system is particularly useful for studies in which the supply of test materials is limited and when the inhalation route alone is needed to characterize the disposition, metabolism, and pharmacokinetic parameters. A nose-only exposure system, for example, was used to study the metabolism of ^{14}C-MTBE vapor alone and in combination with gasoline vapor (Benson et al., 2003) and for the physiologically based pharmacokinetic model prediction of inhaled ethanol (Pastino et al., 1997).

A lung-only method has also been used for the precise delivery of ^{14}C-methanol to Cynomolgus monkeys for pharmacokinetic studies (Dorman et al., 1994).

26.4.2 Animal Toxicity

26.4.2.1 Alcohol/Gasoline and Gasoline Vapors

Neurobehavioral testing

Because of the neurobehavioral effects of gasoline and alcohols at high vapor concentrations (Reese and Kimbrough, 1993; Ritchie et al., 2001), neurobehavior testing should be an integral part of any comprehensive toxicity study on alcohol/gasoline vapors. A large array of functional observation battery (FOB) tests, including cage-side observations, open-field tests, and manipulative tests, is now available for this purpose (Moser, 1988; Moser et al., 1998). In the subchronic studies where rats were exposed to methanol/gasoline vapor at 570/3200 and 2500/3200 ppm (Poon et al., 1995a) and ethanol/gasoline vapor at 6130/500 ppm (Chu et al., 2005), a limited suite of cage-side behaviors was employed : arousal, ataxia, clonic movement, gait score, handling reactivity, home-cage posture, chromodacryorrhoea, salivation, cyanosis, alopecia, piloerection, and vocalization. Subjective ratings of 1 (frequent), 2 (occasional), 3 (infrequent), and 4 (not observed) were used to assess the presence or absence of an effect. No remarkable treatment-related changes were observed.

In a subchronic study in which rats inhaled methanol/gasoline vapor at a maximum concentration of 5000/300 ppm (Poon et al., 1998), cage-side observations were done daily, and a limited number of quantitative and semiquantitative neurobehavioral screening tests (Moser, 1988), open-field activity, righting reflex, grip strength, were performed at the termination of the treatment period. Except for a small but significant decrease in the forelimb grip strength in females exposed to the high-dose methanol/gasoline, no other treatment-related changes were detected. Decreased forelimb or hindlimb strength has been reported in a number of studies after exposure to alkylbenzene, benzene, and hexane (Dempster et al., 1984; Tegeris and Balster, 1994; Spencer et al., 1980). All three compounds are regular constituents of gasoline. Thus, although acute exposure to high levels of gasoline vapor is known to produce severe neurological and behavioral effects in humans (Poklis and Burkett, 1977), cage-side and neurobehavioral tests so far employed, with the possible exception of forelimb and hindlimb grip strength, have not been able to detect a treatment effect following repeated exposure to gasoline/alcohol vapors.

Body weight gain

Male but not female rats exposed to gasoline at 3200 ppm gasoline or methanol/gasoline at 570/3200 ppm and 2500/3200 ppm had reduced food consumption and depressed body weight gain over the 4-week exposure period. On the other hand, no significant change in growth curves were observed in rats of both sexes exposed to methanol vapor alone at 2500 ppm for 4 weeks (Poon et al., 1995a) or methanol/gasoline vapor at 5000/300 ppm for 13 weeks (Poon et al., 1998). In another study depressed body weight gain was observed in female rats exposed for 4 weeks to ethanol/gasoline at 6130/500 ppm, but not to 500 ppm gasoline or 6130 ppm ethanol alone (Chu et al., 2005). Earlier studies also reported depressed weight gain in male rats exposed to 20,000 mg/m^3 (670 ppm) for 2 months (Vyskočil et al., 1988) and in rats of both sexes at 2056 ppm for 103–113 weeks (MacFarland et al., 1984), but no changes at 300 ppm for 50 weeks (Short et al., 1989). Exposure to methanol at 5000 ppm had no effect on body weight gain (Andrews et al., 1987). Taken together, high levels of gasoline vapor appeared to be the primary cause of depressed body weight gain. At lower levels of gasoline vapor of 500 ppm or less, depressed weight gain was seen only when a high level of ethanol was also present.

Organ weights

Both male and female rats had increased relative liver weight following a 4-week exposure to gasoline at 3200 ppm alone or in the presence of 570 ppm or 2500 ppm methanol (Poon et al., 1995a). On the other hand, hepatomegaly was not observed in rats of both sexes when exposed to a vapor mixture of higher methanol and lower gasoline concentrations (5000/300 ppm) (Poon et al., 1998). In male rats inhaling a mixture of ethanol gasoline at 6130/500 ppm, the relative liver weight was significantly decreased but recovered after a 4-week recovery period (Chu et al., in press). Increased relative liver weight was also observed in male rats exposed to 570 ppm gasoline vapor for 30 days (Vyskočil et al., 1988) and in mice to 2056 ppm gasoline for 13 weeks (Tilbury et al., 1993, Standeven et al., 1994).

Male but not female rats showed increased kidney weights following a 13-week exposure to gasoline vapor at 500 ppm, whereas ethanol vapor at 6130 ppm had no effect on male and female kidney weights (Chu et al., 2005). This observation is consistent with the well-known male-specific renal toxicity of gasoline (Halder et al., 1984; McFarland et al., 1984, Short et al., 1989). Relative kidney weights were increased in rats of both sexes exposed to methanol/gasoline vapor at 570/3200 and 2500/3200 ppm but not to 2500 or 3000 methanol alone (Poon et al., 1994, 1995a). It is, therefore, possible that a combination of methanol and gasoline may have an effect on the kidney that is independent of a male-specific effect that is associated with hyaline-droplet nephropathy (see below).

Histopathology

The most common histological alteration following inhalation of methanol, gasoline, methanol/gasoline, methanol/toluene, or ethanol/gasoline was the mild and reversible changes occurring at the epithelial lining of the nasal cavity and upper respiratory tract (Poon et al., 1994, 1995a, 1998; Chu et al., 2005; Andrews et al., 1987; NEDO, 1987). In humans, there is a biochemical indication of irritation to the nasal passage even though histopathological evidence is lacking (Mann et al., 2003). Figure 26.1 depicts typical inflammatory changes occurring at the nasal cavity after methanol/toluene exposure, which can also be detected in milder degrees following alcohol/gasoline inhalation.

Hyaline droplet nephropathy in male rats is a typical treatment effect of gasoline. The etiology of this male-specific effect has been described. The liver of young and mature male rats produces a large amount of $\alpha_{2\mu}$-globulin, which is reabsorbed in the proximal tubules and phagocytized and digested by lysosomes. Branch-chain paraffin components of gasoline interfered with this process. For example, 2,2,4-trimethylpentane, a typical branch-chain paraffin, is metabolized to 2,4,4-trimethyl l-2-pentanol, which binds to $\alpha_{2\mu}$-globulin and interferes with the lysosomal catabolism of this protein. Abnormal accumulation of this protein complex caused the lysosomes to disintegrate, inducing cytotoxicity, cell death, and eventually the typical hyaline-droplet nephropathy. With chronic exposure, renal neoplasia may be the ultimate adverse expression (Swenberg et al., 1989). In a series of

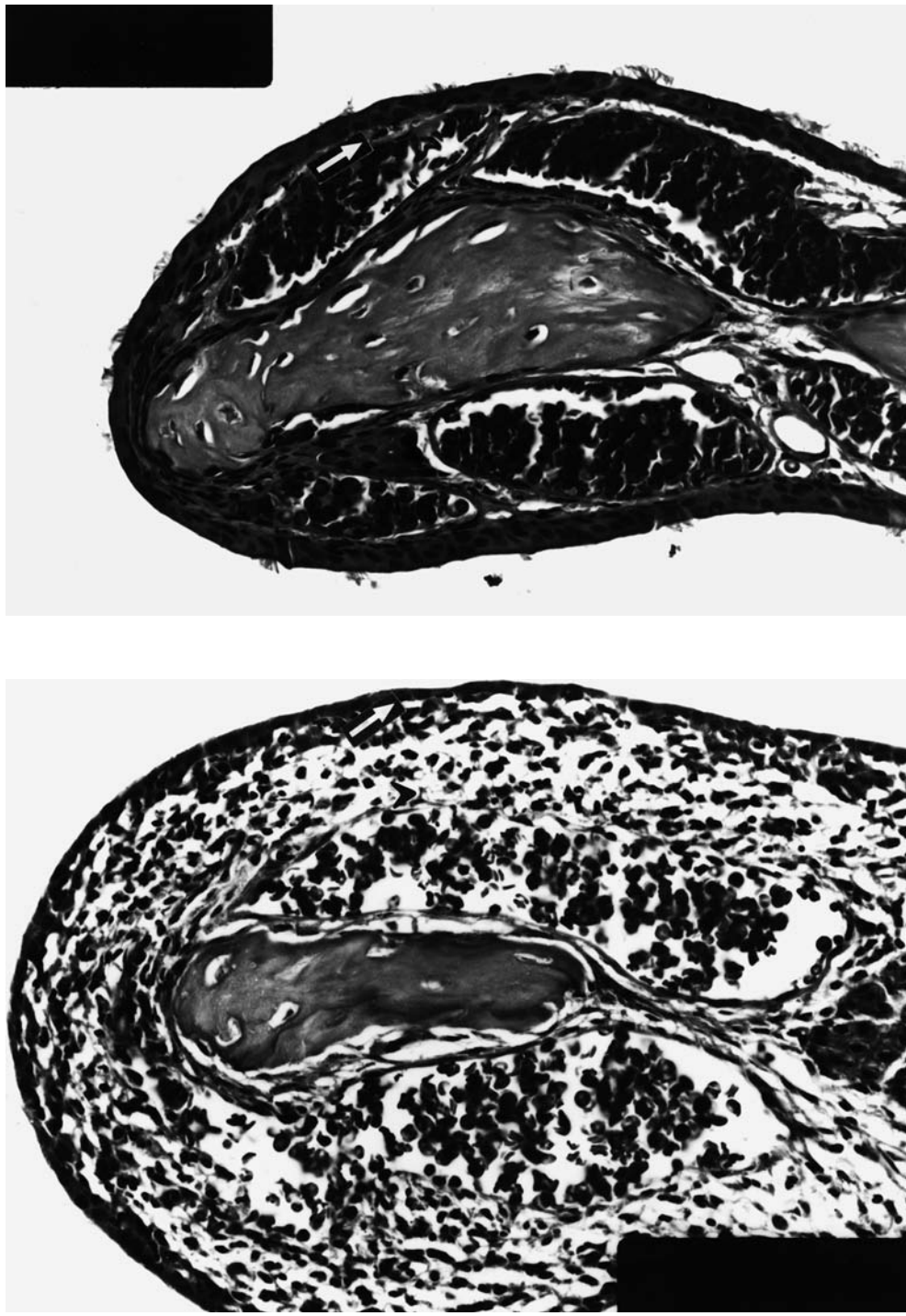


FIGURE 26.1 Nasoturbinate, level B of female rats exposed to 300 ppm methanol (upper panel) showing normal submucosal structures consisting of thin-walled veins and no subepithelial lymphoid proliferation; and to 300 ppm methanol and 30 ppm toluene (lower panel) showing laminar thickening of the submucosa due to edema and infiltration of mononuclear cells lying between the respiratory epithlelium and the large venous sinuses.

studies (Poon et al., 1994, 1995a, 1998; Chu et al., in press) this typical histopathological change in the proximal tubules was not a prominent observation in the male rats exposed to vapors of gasoline, gasoline/methanol, or gasoline/ethanol, even though subtle male-specific changes in terms of elevated urinary excretion of albumin and $ß_2$-microglobulin were evident at the time (Poon et al., 1998). In contrast, hyaline-droplet nephropathy was readily induced in male rats in other inhalation studies (Halders et al., 1984; Short et al., 1989) and when gasoline was administered orally (Garg et al., 1989). This difference in toxic effect is likely related to the concentrations of isoparaffins delivered to the rats. When gasoline is administered orally or a test atmosphere is generated at such a temperature that 2,2,4-trimethypentane (b.p. 98–99°C) is vaporized, a high concentration of isoparaffin will be delivered to the animals, producing the typical kidney effects. When vapor is generated under a control temperature of 60°C, much lower concentrations of isoparaffins will be present in the test atmosphere and kidney effects will be minimal. The critical role of vapor composition on male rat-specific nephropathy was first demonstrated by Halders et al. (1986a, 1986b).

Exposure to gasoline (3200 ppm) and methanol/gasoline (570/3200 ppm) appears to produce a subtle change in the rat uteri in the form of reduced submucosal and myometrial eosinophilia, suggesting a perturbed estrus cycle (Poon et al., 1995). Uterine changes consisting of decreased severity of cystic endometrial hyperplasia and increased incidence of uterine atrophy have been observed in mice following lifetime inhalation of gasoline vapor at 2056 ppm (MacGregor et al., 1993). Decreased uterine weight was observed in mice exposed to approximately 2000 ppm of two different unleaded gasoline vapors for as little as 3 d (Moser et al., 1996). Enhanced clearance of estrogen due to increased hepatic metabolism has been proposed as a possible mechanism for the observed uterine changes (Standeven et al., 1994; Moser et al., 1996). A cross-sectional retrospective study of over 3000 women who worked in a petrochemical company suggested a significant association between benzene exposure and abnormal menstrual cycle length (Thurston et al., 2000).

Hematology and clinical chemistry

The hematology parameters appeared not to be a major target of unleaded gasoline (Kuna and Ulrich, 1984; MacFarland et al., 1984) or alcohol/gasoline. Decreased serum glucose and cholesterol were found in male rats inhaling gasoline (3200 ppm) with or without methanol at 570 and 2500 ppm (Poon et al., 1995), but the values were within the normal physiological variation.

Hepatic enzymes

Both gasoline at 3200 ppm and gasoline/methanol at 3200/570 and 3200/2500 ppm increased the activities of hepatic ethoxyresorufin-O-deethylase, aniline hydroxylase, aminopyrine demethylase, and UDP-glucuronosyltransferase (Poon et al., 1995). Gasoline is responsible for these treatment effects because methanol alone did not produce changes in any of the enzymes (Poon et al., 1994, 1995), while increased activities in hepatic ethoxyresorufin O-deethylase, pentoxyresorufin dealkylase, and UDP-glucuronosyltransferase were commonly observed in rodents treated with gasoline (Standeven et al., 1994; Moser et al., 1996; Chu et al., 2005).

Bronchoalveolar fluid analytes

Analysis of the bronchoalveolar lavage fluid is a useful tool in the study of changes affecting the lung, specifically the bronchoalveolar lining (Henderson, 1984; Henderson et al., 1988). Table 26.5 describes a procedure routinely used for the preparation of cells and cell-free bronchoalveolar fluids. Cells and biochemical analytes that were affected at early stages of injury to the lung and bronchoalveolar lining are also summarized in the table. A limited amount of data is available pertaining to the effect of gasoline and alcohol/gasoline vapors. Le Mesurier et al. (1980) reported that rats inhaling extremely high concentration (74 g/l or approximately 24,900 ppm[1]) of a leaded gasoline 8 h/d for 15 days had decreased pulmonary surfactants. White et al. (1983) examined the lung tissues and cells and analytes from bronchoalveolar fluids of rats after inhalation of 10,000 ppm of methanol vapor

[1] Gasoline vapor concentration in mg/m^3 is converted to ppm by dividing with a factor of 2.967 (Reese and Kimbrough, 1993; IARC, 1989).

for up to 6 weeks and detected no significant changes. Exposure to methanol/gasoline at 570/3200 and 2500/3200 ppm produced a small decrease in ascorbic acid in male but not female rats. No other treatment-related changes were observed in the bronchoalveolar fluid analytes, including protein, lactic dehydrogenase, γ-glutamyltranspeptidase, and alkaline phosphatase (Poon et al., 1995).

Brain neurotransmitters

Male rats inhaling gasoline at 330 ppm for 2 months had increased hypothalamic noradrenaline (Vyskcoil et al., 1988). A 4-week exposure to ethanol (6130 ppm) or gasoline (500 ppm) resulted in altered levels of neurotransmitters which were brain-region and sex specific (Chu et al., 2005). Ethanol decreased mediodorsal thalamus 5-hydroxy indoleacetic acid and hippocampal serotonin,

TABLE 26.5 Bronchoalveolar Lavage Fluid—Procedure and Analysis

Lavage Procedure

1. Anesthetize animal with pentobarbital (50 mg/kg) and keep animal warm under a tungsten lamp.
2. Completely bleed animal by withdrawing blood from the abdominal aorta.
3. Cut open thoracic cavity, expose lung and trachea.
4. Cut loose the tongue to expose the laryngeal opening.
5. Insert a silicon-tipped animal-fleeding needle (20 gauge) into the trachea and ligate.
6. Instill saline (6.5 ml/100 g rat) at 37°C into the lungs. The lungs are massaged by gently pressing and releasing on the ribcage five times over a 10 sec period. The lavage fluid is slowly withdrawn. This step is repeated three times. Care must be taken to avoid contamination with blood.
7. The lavage step is repeated two more times with the same volume of fresh warm saline.
8. The pooled lavage fluid is centrifuged at 500 ´ g for 5 min.
9. The supernatant is collected as the cell-free bronchoalveolar lavage fluid and stored at–70°C until further biochemical analysis.
10. The cells are resuspended in phosphate-buffered saline or Dulbecco's modified Eagle's medium and used immediately for cell counts.

Biochemical Analysis

N-Acetylglucosaminidase
Albumin
Alkaline phosphatase
Al-antitrypsin
Ascorbic acid
Glucose
Lactic dehydrogenase
Pulmonary surfactant
Cytokines
Total protein

Cellular analysis

Total cell count
Mononuclear cell count
Polymorphonuclear cell count

Sources: Le Mesurier et al. (1980); White et al. (1983); Henderson et al. (1984, 1988); Hatch et al. (1986); Nambu et al. (1991); Poon et al. (1995b); Nomiyama (1995); Xie et al. (1995).

and gasoline increased entorhinal serotonin in female rats only. On the other hand, coexposure with ethanol/gasoline (6130/500 ppm) appeared to decrease dopamine in nucleus accumbens of both male and female rats.

In studies where leaded gasoline was used, there was added complexity in interpreting the neurotoxicity because of the presence of organic lead, a human neurotoxin (Robinson, 1978; Seeber et al.,1990). However, many components within unleaded gasoline, e.g., benzene and hexanes, are also known neurotoxins (see reviews by Burbacher, 1993; Ritchie et al., 2001). As described in section 26.4.2.2, inhaled ethanol also modulated the level of neurotransmitters in the brain. With such paucity of data, future investigations on the effect of alcohol/gasoline on neurotransmitters and their correlation to neurotoxicity, addictive behavior, etc., should be an area of fruitful research.

Urinary biomarkers

Urinary biomarkers are among the most sensitive biomarkers of gasoline and alcohol/gasoline effects. Increased urinary albumin excretion was detected following inhalation exposure to methanol/gasoline vapor at levels from 50/3 ppm to 5000/300 ppm (Figure 26.2) (Poon et al., 1998). The albumin effect was male specific and was likely related to the male-specific, gasoline-induced hyaline-droplet nephropathy (see section 26.4.2.1, Histopathology). Urinary albumin is a more sensitive biomarker of renal effect because the increases occurred in the absence of microscopic evidence of accumulation of hyaline droplets in the renal tubular cells. Increased urinary ascorbic acid excretion in rats of both sexes is also a sensitive biochemical marker of exposure to gasoline, whether by the inhalation or oral route (Poon et al., 1995a, 1998, 2001; Chu et al., in press). The toxicological significance of this increase is not yet established but is likely related to a xenobiotic activation of the hepatic glucuronic acid pathway (Burns et al., 1960; Poon et al., 1995b). Urinary hippuric acid (Ogata and Taguchi, 1987; Poon et al., 1995a; Chu et al., 2005), and *trans*,*trans*-muconic acid (Popp et al., 1994; Hotz et al., 1997) have been proposed for use as biomarkers of exposure to the toluene and benzene components, respectively, of gasoline.

Developmental and reproductive toxicity

No data are available for alcohol/gasoline vapors. Female rats inhaling gasoline vapor up to 9000 ppm during gestation days 6–19 did not produce any evidence of developmental toxicity (Roberts et al., 2001). Although histological examination pointed to subtle uterine changes in rodents exposed to gasoline vapor (section 26.4.2.1, Histopathology), a two-generation study on rats indicated that exposure to gasoline vapor at up to 20,000 mg/m^3 did not affect the reproductive parameters measured and the offspring survival and growth (McKee et al., 2000).

26.4.2.2 Methanol and Ethanol Vapors

A 52-week continuous inhalation study on rats and mice (NEDO, 1987) suggested that the no-observed-effect-level (NOEL)[2] for methanol is 100 ppm. At high methanol concentrations (10,000 ppm) methanol but not ethanol produced maternal toxicity and congenital malformations in rat fetuses (Nelson et al., 1985). Stanton et al. (1995) observed little effect on a broad battery of behavioral tests in offspring of rats exposed to 15,000 ppm of methanol vapor. On the other hand, Stern et al. (1997) indicated that exposure to 4500 ppm methanol vapor was associated with subtle behavioral changes in both neonates and adults. Developmental toxicity was also observed in mice following maternal exposure to methanol from 2000 ppm to 15,000 ppm (Bolon et al., 1993; Rogers et al., 1993; Dorman et al., 1995). Exposure to 800 ppm methanol vapor for 13 weeks had no effect on testicular morphology on 8-week old rats regardless of whether the rats were folate reduced or not (Lee et al., 1991). Decreased serum testosterone was observed in mature male rats following exposure to 200 ppm methanol vapor for 2 to 6 weeks (Cameron et al., 1984), but acute exposure to

[2] No-observed-effects level (NOEL) is the highest dose level at which no statistically significant effects of any kind were detected in the treated animals as compared with the control. In regulatory toxicology, this value is used together with the appropriate safety factors to calculate the minimal risk level or tolerable daily intake.

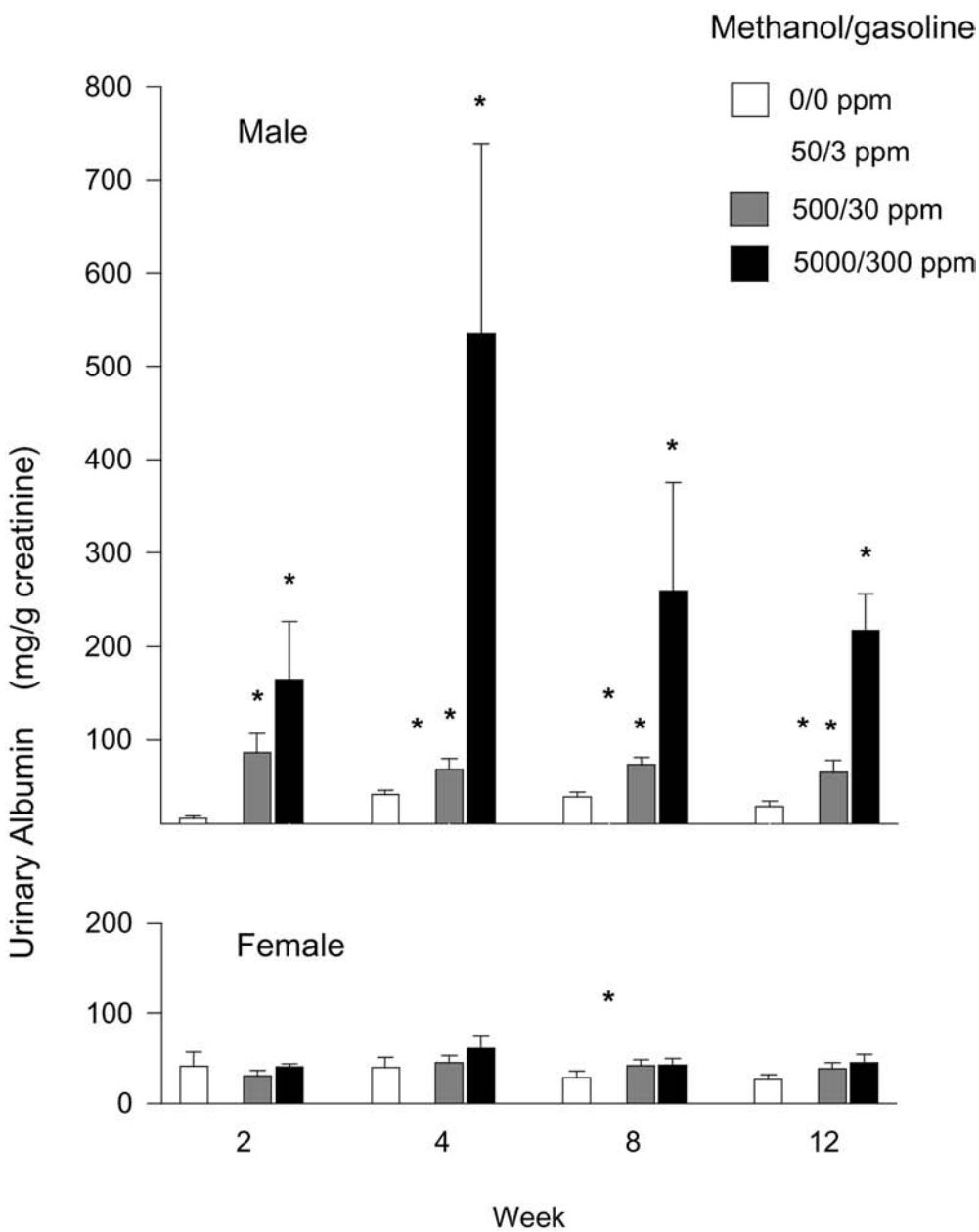


FIGURE 26.2 Effect of methanol/gasoline vapor on urinary albumin. Mean ± SD of five rats; * denotes signficant difference from the control at $p < 0.05$. From Poon et al. (1998).

5000 ppm methanol yielded an increase in serum prolactin but no changes in testosterone (Cooper et al., 1992). Andrews et al (1987) subjected rats to up to 5000 ppm methanol vapors for 4 weeks and found no effects on body and organ weights and no changes upon histopathological and ophthalmoscopic examinations.

Many of the studies on ethanol vapor were focused on neurological effects. Administration of ethanol to weanling rats by inhalation to achieve an average blood alcohol level of 239 mg/dl resulted in loss of Purkinje and granule cells in the cerebellar cortex (Bauer-Moffett and Altman, 1977). Significant changes in brain neurotransmitters were observed in offspring of rats after prenatal or

paternal exposure to 10,000 and 16,000 ppm ethanol vapor (Nelson et al., 1988). On the molecular biology level, prolonged ethanol inhalation resulted in decreased gamma-aminobutyric acid A receptor alpha subunit mRNAs in the rat cerebral cortex (Montpied et al., 1991).

26.4.3 Interactive Effects

The biological effects of gasoline are dependent largely on its composition, which is complex and variable in its own right. Investigating the *in vivo* biological interaction of alcohol and gasoline will no doubt involve a level of complexity that is beyond that of a simple binary mixture. A study in humans reported that the presence of 10% ethanol significantly lowered the odor detection threshold of one brand of gasoline but not another brand (Zhao et al., 1995). In rat studies, inflammation of the nasal passage was more prevalent in those inhaling methanol/toluene vapor than in those exposed to individual vapors (Poon et al., 1994). Mixture effects may also be present because exposure to ethanol/gasoline, but not to ethanol or gasoline alone, produced decreased liver weight in male rats and decreased body weight gain in female rats (Chu et al., 2005). Coexposure with gasoline vapor has been found to reduce tissue disposition and enhance metabolism of inhaled methyl tertiary-butyl ether (MTBE) in rats (Benson et al., 2003).

Although *in vivo* studies have so far failed to provide unequivocal evidence of the presence of an interactive effect between alcohol and gasoline, there are a number of studies on volatile hydrocarbons that have strongly indicated interactions between alcohol and individual hydrocarbons present in gasoline. Studies with rodents have clearly demonstrated that ingested ethanol increased hematotoxicity and myelotoxicity of inhaled benzene (Baarson et al., 1982; Nakajima et al., 1985; Marrubini et al., 2003). This enhancement of toxicity can be attributed to ethanol as a potent inducer of hepatic cytochrome P450 2E1, which is responsible for the conversion of benzene to its toxic metabolites (Kalf, 1987; Koop et al., 1989; Guengerich et al., 1991; Medinsky et al., 1994). Inhibition of cytochrome P450 2E1 in rodents markedly increased the concentration of exhaled hexane, suggesting that this cytochrome may be intimately involved in the metabolism of hexane (Mathews et al., 1997). Inhaled methanol has also been reported to increase the metabolism of *n*-hexane to the neurotoxic metabolite 2-hexanol (Aarstad et al., 1984), and the interactive effect has been attributed to an inducer effect of methanol on cytochrome P450 (Aarstad et al., 1984; Pankow and Jagielki, 1993; Allis et al., 1996). Methanol vapor has also been reported to reduce the biological half life of toluene in blood (Ishidao et al., 2000). Figure 26.3 is a schematic representation of the possible role played by enzyme induction in metabolic interaction by which a mechanism of toxicological interaction between alcohols and gasoline may be explored.

All these studies were conducted with test compounds that were administered orally or as vapors at concentrations ranging from 200 to 10,000 ppm. However, coexposure of mice with liquid ethanol and benzene vapor at 0.44 ppm for 6 weeks did not produce changes in immunotoxicity markers in the spleen. Further coexposure with liquid ethanol and benzene at 4.4 ppm for 11 weeks did not elicit significant alterations in the bone marrow and spleen cell genotoxic markers (Daiker et al., 2000). The disparate results obtained in the studies above are likely related to the levels of exposure

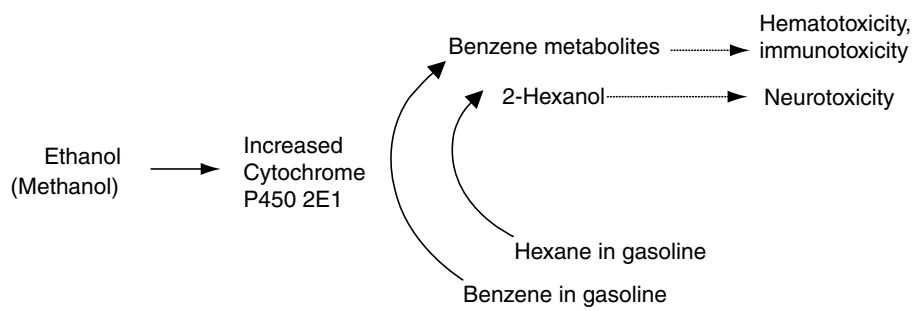
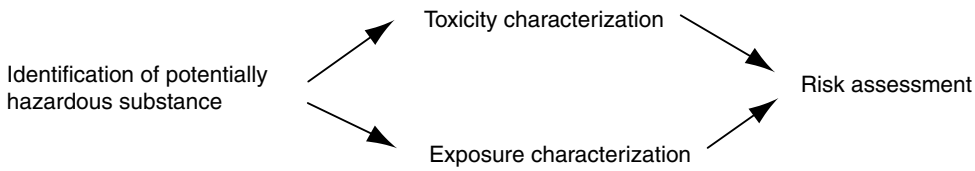


FIGURE 26.3 A proposed mechanism of interaction between alcohol and gasoline vapors.

and, therefore, highlighted the importance of the selection of doses levels that are environmentally and occupationally relevant in future studies.

26.5 RISK ASSESSMENT—EXISTING INFORMATION AND DATA GAP

The risk assessment of alcohol/gasoline follows a model process applicable to environmental contaminants:



The study of alcohol/gasoline vapors as an environmental hazard is a relatively recent phenomenon and there is not enough toxicity and exposure data to afford a comprehensive and in-depth risk assessment. Following is a list of data gaps and points to consider.

26.5.1 Identification of Alcohol/Gasoline in Use

The first step in risk assessment is the identification and characterization of the hazardous substances. It is uncertain at this time which alcohol/gasoline will be the alternate fuels of choice and hence the dominant presence in the environment. E85, M85, and E10 are the fuels currently in use in Brazil, the United States, Canada, and Europe, although other fuel mixtures such as methanol/isobutanol/gasoline (Kushneva et al., 1986), and methanol/ethanol/gasoline (Gattas et al., 2001) have been explored as alternate fuels. Although the complexity of gasoline is well known, there is very little information concerning the impurities in the methanol or ethanol blending stocks. A recent study indicated that anhydrous ethanol stock derived from sugar canes contained a variety of impurities such as saturated and aromatic hydrocarbons, aldehydes, ketones, and other alcohols (Vilar et al., 2003).

26.5.2 Characterization of Alcohol/Gasoline Vapors

It is clear from section 26.4.1.1 that there are many methods for generating vapors. It must be emphasized that the condition of vapor generation is a critical determinant of toxic expressions. Alcohol/gasoline delivered by atomization and aerosol results in the availability of almost all components of alcohol/gasoline for inhalation and hence their related toxic expressions. Higher temperature of vapor generation results in the presence of higher-molecular-weight compounds in the vapor, e.g., aromatics. Vapors generated below 65–75°C contain hydrocarbons of C8 or less (Table 26.2).

26.5.3 Long-Term Low-Level Exposure

Much of the available toxicity data are derived from short-term studies conducted at vapor levels that are many folds higher than those encountered occupationally or in the environment. Comprehensive chronic studies involving exposure to vapor concentrations from about 0.01 ppm (service station exposure settings) to 200 ppm (present occupational exposure level) are needed.

26.5.4 Species Differences

Rodents (males only) are highly susceptible to gasoline-induced hyaline droplet nephropathy. As discussed in section 26.4.2.1, Histopathology, this is a species- and sex-related effect due to the presence of high concentrations of $\alpha_{2\mu}$-globulin in male rodents but not in female rodents or in humans.

Therefore, this particular gasoline-induced effect bears very little weight in the risk assessment of alcohol/gasoline in humans.

Humans, nonhuman primates, and pigs are more susceptible to acute methanol poisoning than rodents. The typical symptoms of methanol poisoning involve metabolic acidosis, ocular toxicity, and, in severe cases, coma and death. Formic acid has been identified as the proximal toxicant, and the species difference in susceptibility can be attributed to the more efficient folate-dependent formate oxidation pathway of the rodents (Makar et al., 1990; Tephly, 1991). Studies with humans and monkeys indicated that exposure to methanol vapor commonly encountered in the consumer and occupational settings will most likely not result in significantly elevated blood formic acid levels (section 26.3). However, it is not known if acute exposure to methanol/gasoline, as in the case of gasoline sniffing, can achieve a formic acid level high enough to cause acidosis and ocular damage in humans. Future pharmacokinetic and epidemiological studies may provide some answers in this area.

The first enzymic step for methanol metabolism involved alcohol dehydrogenase in humans and nonhuman primates, and catalase in rodents (Tephly, 1991). Although an earlier review has suggested that the initial metabolic step proceeds at similar rates in nonhuman primates and rats (Kavet and Nauss, 1990), more recent pharmacokinetic modeling predicts that the ability to metabolize inhaled methanol was in the order mouse $>$ rat $>>$ human (Perkins et al., 1995a, 1995b). The comparative toxicokinetics of clearance of inhaled methanol and gasoline in various species is an important area of study because the data will complement findings on the differential toxicity and susceptibility exhibited by different species.

26.5.5 Mechanisms of Toxicity

Very little is known about the underlying basis of observed alcohol/gasoline toxicity. Several studies have explored possible associations between the ability of gasoline vapor to promote preneoplastic lesions in mouse liver to induction of the microsomal drug-metabolizing enzyme CYP2B (Standeven and Goldsworthy, 1993).

Uterine effects of gasoline on mice (MacGregor et al., 1993) and gasoline and methanol/gasoline on rats (Poon et al., 1995a) remain unexplained, although the study of Standeven et al. (1994) has suggested that a direct antiestrogenic effect of gasoline may not be the cause.

It is well established that acute exposure to methanol through the oral route produces metabolic acidosis and ocular toxicity in humans and that the proximal toxicant in this case is formic acid. There is debate as to whether methanol or formic acid is the proximal toxicant responsible for the developmental toxicity observed in rodents following methanol inhalation (Dorman et al., 1995; Medinsky and Dorman, 1995; NTP-CERHR, 2002; Clary, 2003) or methanol treatment of whole-embryo culture (Andrews, et al., 1995). In addition, a recent study comparing the activities of alcohol dehydrogenase, formaldehyde dehydrogenase, and catalase activity in mouse conceptuses has raised the possibility that the ability to remove formaldehyde may play a role in the sensitivity of mouse embryos to methanol (Harris et al., 2003). Studies incorporating a broad dose range, pharmacokinetic and tissue distribution of methanol, formaldehyde and formic acid in dams and offspring, and general and developmental toxicity end points are needed to resolve this issue.

A biochemical mechanism of the ocular toxicity of methanol involves the inhibition of cytochrome oxidase activity by formic acid, thereby interrupting the electron transport process and mitochondrial function (Liesivuori and Savolainen, 1991). This inhibitory mechanism has been proposed as a cause of the mitochondrial swelling observed in the retina and optic nerves of methanol-intoxicated rats (Eells et al., 1996, 2000). Similarly, depletion of ATP after methanol administration has been proposed as the mechanism behind Müller cell (retinal glial cell) toxicity (Garner et al., 1995). Another proposed mechanism was that optic neuropathies observed following chronic methanol administration were associated with accumulation of aspartate, an excitotoxic amino acid, in the optic nerve (Gonzalez-Quevedo et al., 2002).

26.5.6 Vulnerable Populations

Acetaldehyde, the immediate metabolite of ethanol, is further converted to acetic acid by aldehyde dehydrogenases (ALDHs), with ALDH2 being the most active isozyme. Genetic polymorphism in ALDH2 leads to partial or complete loss of enzyme function in persons carrying the heterozygote (ALDH2*1/2) and homozygote (ALDH*2/2) variants in comparison with the wild type (ALDH2*1/1). The results of the enzyme deficiency include a buildup of acetaldehyde in the blood and a flush reaction following ethanol intake. The role of ALDH2 polymorphism in human risk assessment, including that of cancer risk, is currently under review (Vasiliou and Pappa, 2000; Ginsberg et al., 2002). Because of the potential widespread exposure of the population to ethanol/gasoline vapor, effort should be directed at studying the effect of inhalation exposure on persons that are deficient in ALDH2.

In rodent studies, there is ample evidence that folic acid deficiency, achieved chemically or by dietary means, increased the accumulation of formic acid and enhanced methanol-induced toxicity (Eells et al., 1981, 1996; Lee et al., 1994; Sakanashi et al., 1996; Aziz et al., 2002). There is also some indication that monkeys that were folic acid deficient (Dorman et al., 1994; Eells et al., 1983) had significantly higher blood formate levels. Much toxicological study remains to be done to characterize the potential risk of methanol/gasoline vapor posed on the part of the population that are folic acid deficient.

REFERENCES

Aarstad, K., Zahlsen, K., and Nilsen, O.G., Induction of microsomal enzymes after inhalation of methanol, *Arch. Toxicol.,* Suppl. 7, 295–297, 1984.

ACCGIH (American Conference of Governmental Industrial Hygienists), 2003 TLVs and BEIs. Threshold limit Values for Chemical Substances and Physical Agents, Biological Indices, American Conference of Government Industrial Hygienists, Cincinnati, OH, 2003.

Ahmed, F.E., Toxicology and human health effects following exposure to oxygenated or reformulated gasoline, *Toxicol. Lett*., 123, 89–113, 2001.

Allis, J.W., Brown, B.L., Simmons, J.E., Hatch, G.E., McDonald, A., and House, D.E., Methanol potentiation of carbon tetrachloride hepatotoxicity: the central role of cytochrome P450, *Toxicology,* 112, 131–140, 1996.

Alternative Fuel News, Office of Energy Efficiency and Renewable Energy, U.S. Department of Energy, Vol. 6, No.3, 4–7, 2003.

Andrews, L.S., Clay, J.J., Terill, J.B., and Bolte, H.F., Subchronic inhalation toxicity of methanol*, J. Toxicol. Environ. Health,* 20, 117–124, 1987.

Andrews, J.E., Ebron-McCoy, M., Kavlock, R.J., and Rogers, J.M., Developmental toxicity of formate and formic acid in whole embryo culture: a comparative study with mouse and rat embryos, *Teratology,* 51, 243–251, 1995.

ATSDR (Agency for Toxic Substances and Disease Registry), Toxicology Profile for Gasoline, U.S. Department of Health and Human Services, Atlanta, GA, 1995.

Aziz, M.H., Agrawal, A.K., Adhami, V.M., Ali, M.M., Baig, M.A., and Seth, P.K., Methanol-induced neurotoxicity in pups exposed during lactation through mother: role of folic acid, *Neurotoxicol. Teratol.,* 24, 519–527, 2002.

Baarson, K.A., Snyder, C.A., Green, J.D., Sellakumar, A., Goldstein, B.D., and Albert, R.E., The hematotoxic effects of inhaled benzene on peripheral blood, bone marrow, and spleen cells are increased by ingested ethanol*, Toxicol. Appl. Pharmacol.,* 64, 393–404, 1982.

Backer L.C., Egeland, G.M., Ashley, D.L., Lawryk, N.J., Weisel, C.P., White, M.C., Bundy, T., Shortt, E., and Middaugh, J.P.. Exposure to regular gasoline and ethanol oxyuel during refueling in Alaska, *Environ. Health Perspect.,* 105, 850–855, 1997.

Batterman, S.A., Franzblau, A., D'Archy, J.B., Sargent, N.E., Gross, K.B., and Shreck, R.M., Breath, urine, and blood measurement as biological exposure indices of short-term inhalation exposure to methanol, *Int. Arch. Occup. Environ. Health,* 71, 325–335, 1998.

Bauer-Moffett, C. and Altman, J., The effect of ethanol chronically administered to preweanling rats on cerebellar development: a morphological study, *Brain Res.,* 119, 249–268, 1977.

Benson, J.M., Tibbetts, B.M., and Barr, E.B., The uptake, distribution, metabolism, and excretion of methyl tertiary-butyl ether inhaled alone and in combination with gasoline vapor, *J. Toxicol. Environ. Health Part A,* 66, 1029–1052, 2003.

Bolon, B., Dorman, D.C., Janszen, D., Morgan, K.T., and Welsch, F., Phase-specific developmental toxicity in mice following maternal methanol inhalation, *Fundam. Appl. Toxicol.,* 21, 508–516, 1993.

Bouchard, M., Brunet, R.C., Droz, P-O., and Carrier, G., A biologically based dynamic model for predicting the disposition of methanol and its metabolites in animals and humans, *Toxicol. Sci.,* 64, 169–184, 2001.

Burbacher, T.M., Neurotoxic effects of gasoline and gasoline constituents, *Environ. Health Perspect.,* 101 (Suppl.6), 133–141, 1993.

Burbacher, T., Shen, D., Grant, K., Sheppard, L., Damian, D., Ellis, S., and Liberato, N., Reproductive and Offspring Developmental Effects Following Maternal Inhalation Exposure to Methanol in Nonhuman Primate, HEI Research Report 89,. Part I. Methanol Disposition and Reproductive Toxicity in Adult Females, Health Effects Institute, Cambridge, MA, 1999a.

Burbacher, T., Grant, K., Shen, D., Damian, D., Ellis, S., and Liberato, N., Reproductive and Offspring Developmental Effects Following Maternal Inhalation Exposure to Methanol in Nonhuman Primate, HEI Research Report 89, Part II. Developmental Effects in Infants Exposed Prenatally to Methanol, Health Effects Institute, Cambridge, MA, 1999b.

Burns, J.J., Conney, A.H., Dayton, P.G., Evans, C., Martin, G.R., and Taller, G., Observations on the drug induced synthesis of D-glucuronic, L-gulonic and L-ascorbic acids in rats*, J. Pharmacol. Exp. Ther.,* 129, 132–138, 1960.

Cardenas, A.M., Liberato, N.A., Grant, K.S., and Burbacher, T.M., The effect of prenatal methanol exposure on Simple and Choice Reaction Time in nonhuman primates, *Neurotoxiocl. Teratol.,* 22, 454, 2000.

Cameron, A.M., Nilsen, O.G., Haug, E., and Eik-Nes, K.B., Circulating concentrations of testosterone, luteinizing hormone and follicle stimulating hormone in male rats after inhalation of methanol, *Arch. Toxicol.,* 7 (Suppl.), 441–443, 1984.

Caprino, L. and Togna, G.I., Potential health effects of gasoline and its constituents: a review of current literature (1990–1997) on toxicological data*, Environ. Health Perspect.,* 106, 115–125, 1998.

Chu, I., Poon, R., Valli, V.E, Yagminas, A., Bowers, W.J., Seegal, R., and Vincent, R., Effects of an ethanol-gasoline mixture: results of a four-week inhalation study in rats*, J. Appl. Toxicol.,* 25, 193–199, 2005.

Chuwers, P., Osterloh, J., Kelly, T., D'Alessandro, A., Quinlan, P., and Becker, C., Neurobehavioral effects of low-level methanol vapor exposure in healthy human volunteers, *Environ. Res.,* 71, 141–150, 1995.

Clary, J.J., Methanol, is it a developmental risk to humans*? Regul. Toxicol. Pharmacol.,* 37, 83–91, 2003.

Coelho, S.T. and Goldemberg, J., Alternative transportation fuels: contemporary case studes, in *Encyclopedia of Energy*. Vol. I, Cleveland, C.J., Ed., Elsevier Academic Press, Boston, MA, 2004, pp. 67–80.

CONCAWE, Gasolines, European Oil Company Organisation for Environment, Health and Safety, Brussels, Belgium, Product dossier 92/103, 1992.

Cooper, R.L., Mole, M.L., Rehnberg, G.L., Goldman, J.M., McElroy, W.K., Hein, J., and Stoker, T.E., Effect of inhaled methanol on pituitary and testicular hormones in chamber acclimated and non-acclimated rats, *Toxicology,* 71, 69–81, 1992.

Costantini, M.G., Health effects of oxygenated fuels*, Environ.Health Perspect.,* 101 (Suppl. 6), 151–160, 1993.

Daiker, D.H.., Shipp, B.K., Schoenfeld, H.A., Klimpel, G.R., Witz, G., Moslen, M.T., AND Ward, J.B., Jr., Effect of CYP2E1 induction by ethanol on the immunotoxicty and genotoxicity of extended low-level benzene exposure*, J. Toxicol. Environ. Health Part A*, 59, 181–196, 2000.

Dempster, A.M., Evans, H.L., and Snyder, C.A., The temporal relationship between behavioral and hematological effects of inhaled benzene*, Toxicol. Appl. Pharmacol.,* 76, 195–203, 1984.

Domask, W.G., Introduction to petroleum hydrocarbons Chemistry and composition in relation to petroleum-derived fuels and solvents, in *Advances in Modern Environmental Toxicology*, Vol. VII. *Renal Effects of Petroleum Hydrocarbons*, Mehlman, M.A., Hemstreet, G.P., Thorpe, J.J., and Weaver, N.K., Princeton Scientific Publishers, Princeton, NJ, 1984, pp 1–26.

Dorman D.C., Bolon, B., Struve, M.F., LaPerle, K.M.D., Wong, B.A., Elswick, B., and Welsch, F., Role of formate in methanol-induced exencephaly in CD-1 mice, *Teratology,* 52, 30–40, 1995.

Dorman, D.C., Moss, O.R., Farris, G.M., Janszen, D., Bond, J.A., and Medinsky, M.A., Pharmacokinetics of inhaled [14C] methanol and methanol derived [14C] formate in normal and folate-deficient cynomolgus monkeys, *Toxicol. Appl. Pharmacol.,* 128, 229–238, 1994.

Eells, J.T., Black, K.A., Tedford, C.E., and Tephly, T.R., Methanol toxicity in the monkey: effects of nitrous oxide and methionine, *J. Pharmacol. Exp. Ther.*, 227, 349–353, 1983.Eells, J.T., Henry, M.M., Lewandowski, M.F., Seme, M.T., and Murray, T.G., Development and characterization of a rodent model of methanol-induced retinal and optic nerve toxicity, *Neurotoxicology,* 21, 321–330, 2000.

Eells, J.T., Makar, A.B., Noker, P.E., and Tephly, T.R., Methanol poisoning and formate oxidation in nitrous oxide treated rats, *J. Pharmacol. Exp. Ther.*, 217, 57–61, 1981.

Eells, J.T., Salzman, M.M., Lewandowski, M.F., and Murray, T.G., Formate-induced alterations in retinal function in methanol-intoxicated rats, *Toxicol. Appl. Pharmacol.,* 140, 58–69, 1996.

Garg, B.D., Olson, M.J., Li, L.C., and Roy, A.K., Phagolysome alterations induced by unleaded gasoline in epithelial cells of the proximal convoluted tubales of male rats: effects of close and treatment duration, *J. Toxicol. Environ. Health,* 26, 101–118, 1989.

Gargas, M.L., Andersen, M.E., and Clewell, H.J., III, A physiological based simulation approach for determining metabolic constants from gas uptake data, *Toxicol. Appl. Pharmacol.,* 86, 341–352, 1986.

Garner, C.D., Lee, E.W., and Louis-Ferdinand, R.T., Muller cell involvement in methanol-induced retinal toxicity, *Toxicol. Appl. Pharmacol.,* 130, 101–107, 1995.

Gattàs, G.J.F., Cardoso, Lde A., Medrado-Faria, Mde A., and Saldanha, P.H., Frequency of oral mucosa micronuclei in gas station operators after introducing methanol, *Occup. Med.,* 51, 107–113, 2001.

Ginsberg, G., Smolenski, S., Hattis, D., and Sonawane, B., Population distribution of aldehyde dehydrogenase-2 genetic polymorphism: implications for risk assessment, *Regul. Toxicol. Pharmacol.,* 36, 297–309, 2002.

Gold, M.D. and Moulis, C.E., Effects of emission standards on vehicle-related ozone, formaldehyde and methanol exposures, in Proceedings of the 81st Air Pollution Control Association, Detroit, MI, 81, 30, 1998.

Gonzalez-Quevedo, A., Obregon, F, Urbina, M., Rousso, T., and Lima, L., Effect of chronic methanol administration on amino acids and monoamines in retina, optic nerve, and brain of the rat, *Toxicol. Appl. Pharmacol*., 185, 77–84, 2002.

Guengerich, F.P., Kim, D.H., and Iwasaki, M., Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects, *Chem. Res. Toxicol.,* 4, 168–179, 1991.

Halder, C.A., Van Gorp, G.S., Hatoum, N.S., and Warne, T.M., Gasoline vapor exposures, Part I. Characterization of workplace exposures, *Am. Ind. Hyg. Assoc. J.,* 47, 164–172, 1986a.

Halder, C.A., Van Gorp, G., Hatoum, N.S., and Warne, T.M., Gasoline vapor exposures: part II, evolution of nephrotoxicity of the major Cu/C5 hydrocarbon components, *Am. Ind. Hyg. Assoc. J.,* 47, 173–175, 1986b.

Halder, C.A., Warne, T.M., and Hatoum, N.S., Renal toxicity of gasoline and related petroleum naphthas in male rats, in *Advances in Modern Environmental Toxicology*, Vol. VII. *Renal Effects of Petroleum Hydrocarbons*, Mehlman, M.A., Hemstreet, G.P., Thorper, J.J., and Weaver, N.K., Eds., Princeton Scientific Publishers, Inc., Princeton, NJ, 1984, pp. 73–88.

Harris, C., Wang, S.W., Lauchu, J.J., and Hansen, J.M., Methanol metabolism and embryotoxicity in rat and mouse conceptuses: Comparisons of alcohol dehydrogenase (ADH1), formaldehyde dehydrogenase (ADH3), and catalase, *Reprod. Toxicol.,* 17, 349–357, 2003.

Hatch, G.E., Slade, R., Stead, A.G., and Graham, J.A., Species comparison of acute inhalation toxicity of ozone and phosgene, *J. Toxicol. Environ. Health,* 19, 43–53, 1986.

HEI (Health Effects Institute), Automotive Methanol Vapors and Human Health: An Evaluation of Existing Scientific Information and Issues for Future Research, Special Report, Cambridge, MA, 1987.

HEI (Health Effects Institute), Potential Health Effects of Oxygenates Added to Gasoline. Special Report of the Institute's Oxygenate Evaluation Committee, Cambridge, MA, 1996.

Henderson, R.F., Use of bronchoalveolar lavage to detect lung damage, *Environ. Health Perspect.,* 56, 115–129, 1984.

Henderson, R.F., Pickrell, J.A., Jones, R.K., Sun, J.D., Benson, J.M., Mauderly, J.L., and McClellan, R.O., Response of rodents to inhaled diluted diesel exhaust: Biochemical and cytological changes in bronchoalveolar lavage fluid and in lung tissue, *Fundam. Appl. Toxicol.,* 11, 546–567, 1988.

Hotz, P., Carbonnelle, P., Haufroid, V., Tschopp, A., Buchet, J.P., and Lauwerys, R., Biological monitoring of vehicle mechanics and other workers exposed to low concentration of benzene, *Int. Arch. Occup. Environ. Health,* 70, 29–40, 1997.

IARC (International Agency for Research on Cancer), IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Suppl. 7, International Agency for Research on Cancer, WHO, Lyon, France, 1987, pp 120–122.

IARC (International Agency for Research on Cancer), IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Alcoholic Drinking, Vol. 44, International Agency for Research on Cancer, WHO, Lyon, France, 1988.

IARC (International Agency for Research on Cancer), IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Occupational Exposure in Petroleum Refining: Crude Oil and Major Petroleum Fuels, Vol. 45, International Agency for Research on Cancer, WHO, Lyon, France, 1989.

Ishidao, T., Ishimatsu, S., Arashidani, K., amd Hori, H., Effect of repeated exposure to methanol and toluene vapor on the metabolism of rats, Indust. Health, 38, 4005–407, 2000.

Kalf, G.F., Recent advances in the metabolism and toxicity of benzene, *Crit. Rev. Toxicol.,*18, 141–159, 1987.

Kavet, R. and Nauss, K.M., The toxicity of inhaled methanol vapors, *Crit. Rev. Toxicol.,* 21, 21–50, 1990.

Koop, D.R., Laethem, C.L., and Schnier, G.G., Identification of ethanol-inducible P450 isozyme 3a (P450IIE1) as a benzene and phenol hydroxylase,. *Toxicol. Appl. Pharmacol*., 98, 278–288, 1989.

Kumarathasan, P., Vincent, R., Tardif, R., Potvin, M., Bjarnason, S., Poon, R., Moir, D., and Chu, I., Experimental atmosphere monitoring in gasoline vapor inhalation studies, *Toxicol. Methods*, 6, 65–82, 1996.

Kuna, R.A. and Ulrich, C.E., Subchronic inhalation toxicity of two motor fuels, *J. Am. Coll. Toxicol.,* 3, 217–229, 1984.

Kushneva, V.S., Kotunova, I.G., and Koloskova, G.A., Toxicological characteristics of a new motor fuel—A benzomethanol mixture, *Gig. Sanit.,* 2, 11–13, 1986.

Lee, E., Brady, A.N., Brabec, M.J., and Fabel, T., Effects of methanol vapors on testosterone production and testis morphology in rats, *Toxicol. Ind. Health,* 7, 261–275, 1991.

Lee, E.W., Garner, C.D., and Terzo, T.S., Animal model for the study of methanol toxicity: Comparison of folate-reducing rat responses with published monkey data, *Toxicol. Environ. Health*, 41, 71–82, 1994.

Lee, E.W., Terzo, T.S., D'Arcy, J.B., Gross, K.B., and Schreck, R.M., Lack of blood formate accumulation in humans following exposure to methanol vapor at the current permissible exposure limit of 200 ppm*, Am. Ind. Hyg. Assoc. J.,* 53, 99–104, 1992.

Le Mesurier, S.M., Lykke, A.W., and Stewart, B.W., Reduced yield of pulmonary surfactant : Patterns of response following administration of chemicals to rats by inhalation*, Toxicol. Lett.,* 5, 89–93, 1980.

Liesivuori, J. and Savolainen, H., Methanol and formic acid toxicity: biochemical mechanisms, *Pharmacol. Toxicol.,* 59, 157–163, 1991.

MacFarland, H.N., Design and operational characteristics of inhalation exposure equipment, in *Inhalation Toxicology,* Salem, H., Ed., Marcel Dekker, Inc., New York, 1986, pp. 93–120.

MacFarland, H.N., Ulrich, C.E., Holdsworth, C.E., Kitchen, D.N., Halliwell, W.H., and Blum, S.C., A chronic inhalation study with unleaded gasoline vapor, *J. Am. Coll. Toxicol.,* 3, 231–248, 1984.

MacGregor, J.A., Richter, W.R., and Magaw, R.I., Uterine changes in female mice following lifetime inhalation of wholly vaporized unleaded gasoline: a possible relationship to observed liver tumors? *J. Am. Coll. Toxicol*., 12, 119–126, 1993.

Makar, A.B., Tephly, T.R., Sahin, G., and Osweiler, G., Formate metabolism in young swine, *Toxicol. Appl. Pharmacol*., 105, 315–320, 1990.

Mann, W.J., Muttray, A., Schaefer, D., Klimek, L., Faas, M., and Konietzko, J., Exposure to 200 ppm of methanol increases the concentrations of interleukin-1β and interleukin-8 in nasal secretions of healthy volunteers*, Ann. Otol. Rhinol. Laryngol.,* 111, 633–638, 2002.

Marrubini, G., Castoldi, A.F., Coccini, T., and Manzo, L., Prolonged ethanol ingestion enhances benzene myelotoxicity and lowers urinary concentrations of benzene metabolite levels in CD-1 male mice, *Toxicol. Sci***.,** 75, 16–24, 2003.

Mathews, J.M., Raymer, J.H., Etheridge, A.S., Velez, G.R., and Bucher, J.R., Do endogenous volatile organic chemicals measured in breath reflect and maintain CYP2E1 levels in vivo? *Toxicol. Appl. Pharmacol.*, 146, 255–260, 1997.

McKee, R.H., Trimmer, G.W., Whitman, F.T., Nessel, C.S., Mackerer, C.R., Hagemann, R., Priston, R.A., Riley, A.J., Cruzan, G., Simpson, B.J., and Urbanus, J.H., Assessment in rats of the reproductive toxicity of gasoline from a gasoline vapor recovery unit, *Reprod. Toxicol*., 14, 337–353, 2000.

Medinsky, M.A. and Dorman, D.C., Recent developments in methanol toxicity, *Toxicol. Lett.,* 82/83, 707–711, 1995.

Medinsky, M.A., Schlosser, P.M., and Bond, J.A., Critical issues in benzene toxicity and metabolism: the effect of interactions with other organic chemicals on risk assessment, *Environ. Health Perspect.,* 102, 119–124, 1994.

Miller, R.R., Letts, R.L., Potts, W.J., and McKenna, M.J., Improved methodology for generating controlled test atmospheres, *Am. Ind. Hyg. Assoc. J.,* 41, 844–846, 1980.

Montpied, P., Morrow, A.L., Karanian, J.W., Ginns, E.I,, Martin, B.M., and Paul, S.M., Prolonged ethanol inhalation decreases gamma-aminobutyric acidA receptor alpha subunit mRNAs in the rat cerebral cortex, *Mol. Pharmacol.,* 39, 157–163, 1991.

Moser, V.C., Screening approach to neurotoxicity: a functional observational battery, *J. Am. Coll. Toxicol.,* 8, 85–93, 1988.

Moser, V.C., Becking, G.C., MacPhail, R.C., and Kulig, B.M., The ICPS Collaborative study on neurobehavioral screening methods, *Fundam. Appl. Toxicol.,* 35, 143–151, 1997.

Moser, G.J., Wong, B.A., Wolf, D.C., Moss, W.R., and Goldsworthy, T.L., Comparative short-term effects of methyl tertiary butyl ether and unleaded gasoline vapor in female B6C3F1 mice, *Fundam. Appl. Toxicol.,* 31, 173–183, 1996.

Muttray, A., Kurten, R., Jung, D., Schicketanz, K.H., and Konietzko, J., Acute effects on the human EEG after an external exposure to 200 ppm methanol*, Int. Arch. Occup. Environ. Health,* 74, 43–48, 2001.

Nadim, F., Zack, P., Hoag, G.E., and Liu, S., United States experience with gasoline additives, *Energy Policy,* 29, 1–5, 2000.

Nakajima, T., Okuyama, S., Yonekura, I., and Sato, A., Effects of ethanol and Phenobarbital administration on the metabolism and toxicity of benzene*, Chem. Biol. Interact.,* 55, 23–38, 1985.

Nambu, Z., Ichikawa, I., and Yokoyama, E., Neutral sugar accumulation in lung lavage fluid following ozone exposure—a sensitive indicator of ozone-induced pulmonary damage, *Toxicol. Ind. Health,* 7, 465–471, 1991.

NEDO (New Energy Development Organization), Toxicological Research of Methanol as a Fuel for Power Station: Summary Report on the Tests with Monkeys Rats and Mice, Alcohol Biomass Energy Department, Tokyo, Japan, 1987.

Nelson, B.K., Brightwell, W.S., MacKenzie, D.R., Khan, A., Burg, J.R., Weigel, W.W., and Goad, P.T., Teratological assessment of methanol and ethanol at high inhalation levels in rats, *Fundam. Appl. Toxicol.,* 5, 727–736, 1985.

Nelson, B.K., Brightwell, W.S., MacKenzie-Taylor, D.R., Burg, J.R., and Massari, V.J., Neurochemical, but not behavioral, deviations in the offspring of rats following prenatal inhalation or paternal inhalation exposure to ethanol, *Neurotoxicol. Teratol.,* 10, 15–22, 1988.

Nomiyama, T., Inhalation toxicity of diborane in rats assessed by bronchoalveolar lavage examination*, Arch. Toxicol.,* 70, 43–50, 1995.

NRCan (Natural Resources Canada), Alternative Transportation Fuels "Ethanol," NRCan, Cat. M27-74/31-1996-1, 1996.

NTP-CERHR (National Toxicology Program—Center for the Evaluation of Risk to Human Reproduction), Expert Panel Report on the Reproductive and Developmental Toxicity of Methanol, Center for the Evaluation of Health and Human Services, Research Triangle Park, NC, 2002., Available at http:/cerhr.niehs.nih.gov

OECD (Organization for Economic Co-operation and Development), Acute inhalation toxicity—fixed concentration procedure, draft guideline 433, Environment Directorate, Organization for Economic Co-operation and Development, Paris, October, 2002.

Ogata, M. and Taguchi, T., Quantitation of urinary metabolites of toluene, xylene, styrene, ethylbenzene, benzene and phenol by automated high performance liquid chromatography, *Int. Arch. Occup. Environ. Health,* 59, 263–272, 1987.

Osterloh, J.D., D'Alessandro, A., Chuwers, P., Mogadeddi, H., and Kelly, T.J., Serum concentrations of methanol after inhalation at 200 ppm, *J. Occup. Environ. Med.,* 36, 571–576, 1996.

Pankow, D. and Jagielki, S., Effect of methanol or modification of the hepatic glutathione concentration on metabolism of dichloromethane to carbon monoxide in rats, *Hum. Exp. Toxicol.,* 12, 227–231,1993.

Pastino, G.M., Asgharian B., Roberts, K., Medinsky, M.A., and Bond, J.A., A comparison of physiologically based pharmacokinetic model predictions and experimental data for inhaled ethanol in male and female B6C3F1 mice, F344 rats, and humans, *Toxicol. Appl. Pharmacol.,* 145, 147–157, 1997.

Pauluhn, J., Validation of an improved nose-only exposure system for rodents, *J. Appl. Toxicol.,* 14, 55–62, 1994.

Perkins, R.A., Ward, K.W., and Pollack, G.M., A pharmacokinetic model of inhaled methanol in humans and comparison to methanol disposition in mice and rats, *Environ. Health Perspect.*, 103, 726–733, 1995a.

Perkins R.A., Ward, K.W., and Pollack, G.M., Comparative toxicokinetics of inhaled methanol in the female CD-1 mouse and Sprague-Dawley rat, *Fundam Appl Toxicol.,* 28, 245–254, 1995b.

Poklis, A. and Burkett, C.D., Gasoline sniffing: a review, *Clin. Toxicol.,* 11, 35–41, 1977.

Poon, R., Chu, I., Bjarnason, S., Potvin, M., Vincent, R., Miller, R.B., and Valli, V.E., Inhalation toxicity study of methanol, toluene, and methanol/toluene mixtures in rats: effects of 28-day exposure, *Toxicol. Ind. Health,* 10, 231–245, 1994.

Poon, R., Chu, I., Bjarnason, S., Vincent, R., Potvin, M., Miller, R.B., and Valli, V.E., Short-term inhalation toxicity of methanol, gasoline, and methanol/gasoline in the rat, *Toxicol. Ind. Health,* 11, 343–361, 1995a.

Poon, R., Park, G., Viau, C., Chu, I., Potvin, M., Vincent, R., and Valli, V.E., Inhalation toxicity of methanol/gasoline in rats: effects of 13-week exposure, *Toxicol. Ind. Health,* 14, 501–520, 1998.

Poon R., Vu, D., and Vincent, R., N-Acetyl-β-D-glucosaminidase activity in bronchoalveolar fluid : characterization and response to ozone exposure*, Inhalation Toxicol.,* 71, 1195–1206, 1995b.

Poon, R., Yagminas, A., Singh, A., Valli, V.E., and Chu, I., Short-term oral toxicity of gasohol in female rats, *J. Appl. Toxicol.,* 21, 461–467, 2001.

Popp, W., Rauscher, D., Muller, G., Angerer, J., and Norpoth, K., Concentrations of benzene in blood and S-phenylmercapturic and t,t-muconic acid in urine in car mechanics, *Int. Arch. Occup. Environ. Health.,* 66, 1–6, 1994.

Reese, E. and Kimbrough, R.D., Acute toxicity of gasoline and some additives, *Environ. Health Perspect.,* 101 (Suppl. 6), 115–131, 1993.

Ritchie, G.D., Still, K.R., Alexander, W.K., Nordholm, A.F. Wilson, C.L., Rossi III, J., and Mattie, D.R., A review of the neurotoxicity risk of selected hydrocarbon fuels, *J. Toxicol. Environ. Health (Part b),* 4, 223–312, 2001.

Roberts, L., White, R., Bui, Q., Daughtrey, W., Koschier, F., Rodney, S., Schreiner, C., Steup, D., Breglia, R., Rhoden, R., Schroeder, R., and Newton, P., Developmental toxicity evaluation of unleaded gasoline vapor in the rat, *Reprod. Toxiol.,* 15, 487–494, 2001.

Robinson, R.O., Tetraethyl lead poisoning from gasoline sniffing, *J. Am. Med. Assoc.,* 240, 1373–1374, 1978.

Rogers, J.M., Mole, M.L., Chernoff, N, Barbee, B.D., Turner, C.I., Logsdon, T.R., and Kavlock, R.J., The developmental toxicity of inhaled methanol in the CD-1 mouse, with quantitative dose-response modeling for estimation of benchmark doses, *Teratology,* 47, 175–188, 1993.

Sakanashi, T.M., Rogers, J.M., Fu, S.S., Connelly, L.E., and Keen, C.L., Influence of maternal folate status on the developmental toxicity of methanol in the CD-1 mouse, *Teratology*, 54, 198–206, 1996.

Seeber, A., Kiesswetter, E., Neidhart, B., and Blaszkewicz, M., Neurobehavioral effects of a long-term exposure to tetraalkyllead, *Neurotoxicol Tetratrol*., 12, 653–655, 1990.

Short, B.G., Burnett, V.L., and Swenberg, J.A., Elevated proliferation of proximal tubule cells and localization of accumulated α2u-globulin in F344 rats during chronic exposure to unleaded gasoline or 2,4,4-trimethylpentane*, Toxicol. Appl. Pharmacol.,* 101, 414–431, 1989.

Smith, T.J., Hammond, S.K., and Wong, O., Health Effects of gasoline exposure. I. Exposure assessment for U.S. distribution workers, *Environ. Health Perspect.,* 101 (Suppl. 6), 13–21, 1993.

Spencer, P.S., Couri, D., and Schaumburg, H.M., n-Hexane and methyl n-butyl ketone, in *Experimental and Clinical Neurotoxicology*, Spencer, P.S. and Schaumburg, H.H., Eds., Williams and Wilkins, Baltimore, MD, 1980, pp. 456–475.

Standeven A.M., Blazer, D.G., III, and Glodsworthy, T.L., Investigation of antiestrogenic properties of unleaded gasoline in female mice, *Toxicol. Appl. Pharmacol.,* 127, 233–240, 1994.

Standeven, A.M. and Goldsworthy, T.L., Promotion of preneoplastic lesions and induction of CYP2B by unleaded gasoline vapor in female B6C3F1 mouse liver, *Carcinogenesis,* 14, 2137–2141, 1993.

Standeven, A.M., Wolf, D.C., and Goldsworthy, T.L., Promotion of hepatic preneoplastic lesions in male B6C3F1 mice by unleaded gasoline*, Environ. Health Perspect.,* 103, 696–700, 1995.

Stanton, M.E., Crofton, K.M., Gray, L.E., Gordon, C.J., Boyes, W.K., Mole, M.L., Peele, D.B., and Bushnell, P.J., Assessment of offspring development and behavior following gestational exposure to inhaled methanol in the rat*, Fundam. Appl. Toxicol.,* 28, 100–110, 1995.

Stern, S., Cox, C., Preston, R., Sharma, A., Inglis, G.B., Balys, M., and Weiss, B., Perinatal methanol exposure in the rats. II. Behavioral effects in neonates and adults, *Fundam. Appl. Toxicol.,* 36, 163–176, 1997.

Swenberg, J.A., Short, B., Borghoff, S., Strasser, J., and Charbonneau, M., The comparative pathobiology of α2u-globulin nephropathy, *Toxicol. Appl. Pharmacol.,* 97, 35–46, 1989.

Tegeris, J.S. and Balster, R.L., A comparison of the acute behavioral effects of alkylbenzenes using a functional observational battery in mice, *Fundam. Appl. Toxicol.,* 22, 240–250, 1994.

Tephly, T.R., The toxicity of methanol, *Life Sci.,* 48, 1031–1041, 1991.

Thurston, S.W., Ryan, L., Christiani, D.C., Snow, R., Carlson, J., You, L., Cui, S., Ma, G., Wang, L., Huang, Y., and Xu, X., Petrochemical exposure and menstrual disturbances, *Am. J. Ind. Med.,* 38, 555–564, 2000.

Tilbury, L., Butterworth, B.E., Moss, O., and Goldsworthy, T.L., Hepatocyte cell proliferation in mice after inhalation exposure to unleaded gasoline vapor, *J. Toxicol. Environ. Health,* 38, 293–307, 1993.

Tsai, P.Y. and Weisel, C.P. (2000). Penetration of evaporative emissions into a home from an M85-fueled vehicle parked in an attached garage. J. Air & Waste Manage. Assoc. **50,** 371–377.

U.S. EPA (U.S. Environmental Protection Agency), Health Effects Test Guidelines OPPTS 870.1300—Acute Inhalation Toxicity, U.S. Environmental Protection Agency, Prevention, Pesticides and Toxic Substances, 1998. Available at www.epa.gov/opptsfrs/OPPTS_Harmonized/ 870_Health_ Effects_ Test_Guidelines/ Series/

Vasiliou, V. and Pappa, A., Polymorphisms of human aldehyde dehydrogenases. Consequences for drug metabolism and disease, *Pharmacology,* 61, 192–198, 2000.

Vilar, R.B., da Silva, R., Schossler, P., Cataluna Veses, R., Piatnicki, C., Samios, D., and Caramão, E.B., Preliminary characterization of anhydrous ethanol used in Brazil as automotive fuel, *J. Chromatogr. A,* 985, 367–373, 2003.

Von Berg, R., Toxicity update gasoline, *J. Appl. Toxicol.,* 9, 203–210, 1989.

Vysko**č**il, A., Tušl, M., Obršál, J., and Zaydlar, K., A subchronic inhalation study with unleaded petrol in rats, *J. Appl. Toxicol***.,** 8, 239–242, 1988.

Weaver, N.K., Gasoline toxicology. Implication for human health, *Ann. N. Y. Acad. Sci.,* 534, 441–451, 1988.

White, L.R., Marthinsen, A.B., Richards, R.J., Eik-Nes, K.B., and Nilsen, O.G., Biochemical and cytological studies of rat lung after inhalation of methanol vapor, *Toxicol. Lett.,* 17, 1–5, 1983.

Wixtrom, R.N. and Brown, S.L., Individual and population exposures to gasoline, *J. Expos. Anal. Environ. Epidemiol.,* 2, 23–78, 1992.

Xie, J., Kolls, J., Bagby, G., and Greenberg, S.S., Independent suppression of nitric oxide and TNF alpha in the lung of conscious rats by ethanol,. *FASEB J.,* 9, 253–261, 1995.

Zhao, X., Smith, S.L., and Duffy, L.K., Effects of ethanol as an additive on odor detection thresholds of Alaskan gasoline at sub-artic temperatures, *Chemosphere,* 31, 4531–4540, 1995.

27 Asbestos

David M. Bernstein

CONTENTS

27.1 INTRODUCTION

Asbestos has been known for centuries. The Romans used it in cremation cloths and lamp wicks, the Greeks also wove the material into cloth, and, in the Middle Ages, asbestos was used for insulating suits of armor. Asbestos was used because it is strong, insulates well, and resists fire and corrosion (Browne and Murray, 1990; Ross and Nolan, 2003).

It was not until the late 1800s, however, that asbestos was mined commercially, starting in Italy and England. In Canada, the first mine was opened in 1879 at Thetford, in the Quebec province. This was followed shortly thereafter with commercial asbestos mining in Russia.

The term "asbestos" is a generic term used to describe a group of mineral families that have overlapping properties. The two most common mineral families that the term asbestos is used to describe are the serpentines and the amphiboles. Although the distinction between these two minerals has been well described mineralogically since the 1930s, until more recently, little if any differentiation has been made in addressing the potential health effects of these two types of minerals.

The toxicological differences between chrysotile asbestos, a serpentine mineral, and the amphibole asbestos, such as amosite, crocidolite, and tremolite, have more recently been debated extensively. Although many studies have shown that chrysotile is not of the same potency as the amphiboles and is cleared from the lung more rapidly than amphibole, this issue still is mired in controversy.

Both serpentine and amphibole asbestos are naturally occurring minerals that are extracted from the earth in surface, open-pit, or underground mines. The fibers are intertwined with adjacent rock and are separated through crushing and milling with subsequent filtration/separation steps. Although the production of chrysotile asbestos has probably always exceeded that of amphibole asbestos by more than 10:1, the amphibole minerals are more abundant and are frequently constituents of igneous rocks and often major components of metamorphic rocks; they are thought

to account for approximately 20% of the shield area of the earth. However, fortunately for us, most of these rock-forming amphiboles are not at all asbestos like (Whittaker, 1979).

The serpentine minerals mainly occur in serpentinized ultramafic rocks, which have a wide distribution throughout the world, occurring in mountain chains, in Precambrian shield areas, in island arcs, and in midocean ridges. Although concentrations of chrysotile asbestos large enough to mine are rare, serpentinized ultramafic rocks almost inevitably contain chrysotile and some of this will be chrysotile asbestos (Wicks, 1979).

Most asbestos fibers were formed under unique conditions when the rock formations were undergoing intense deformation characterized by folding, faulting, shearing, and dilation. These deformations were often accompanied by the intrusion of magmatic fluids forming dikes and sills. The fibers crystallized in these high-strain environments, such as within folds, shear planes, faults, and dilation cavities and at intrusion boundaries (Ross and Nolan, 2003).

27.2 CHRYSOTILE CHARACTERISTICS

In 1930, Pauling (1930) reported that if serpentine had a kaolinite-type crystal structure, it would have a tendency to curve because of the misfit of the octahedral tetrahedral layers of the unit cell.

The chrysotile fiber is a sheet silicate, monoclinic in crystalline structure, and has a unique rolled form. The chemistry of chrysotile is composed of a silicate sheet of composition $(Si_2O_5)_n^{-2n}$, in which three of the O atoms in each tetrahedron are shared with adjacent tetrahedra and a non-silicate sheet of composition $[Mg_3O_2(OH)_4]_n^{+2n}$. In chrysotile the distances between apical oxygens in a regular (idealized) silicate layer are shorter (0.305 nm) than the O–O distances in the ideal Mg-containing layer (0.342 nm), which may account for the curling of the layers that results in the rolling up like a carpet to form concentric hollow cylinders (Skinner et al., 1988). This structure is illustrated in Figure 27.1 (adopted from Skinner et al., 1988) and transmission electron micrographs of chrysotile are shown in Figure 27.2 (Kiyohara, 1991). The Mg molecule is on the outside of the curl and is thus exposed to the surrounding environment. This layered construction of chrysotile is illustrated in Figure 27.3.

Table 27.1 summarizes the chemical composition of typical serpentine and amphibole asbestos. The chemical composition and the structure of chrysotile are notably different from that of amphiboles such as tremolite or amosite (Hodgson, 1979).

Commercial chrysotile is usually subdivided into groups by using the Canadian Quebec Screening Scale (QSS). These groups are determined by using an apparatus with a nest of four rotating trays superimposed one above the other. A known quantity of fiber is placed on the top tray

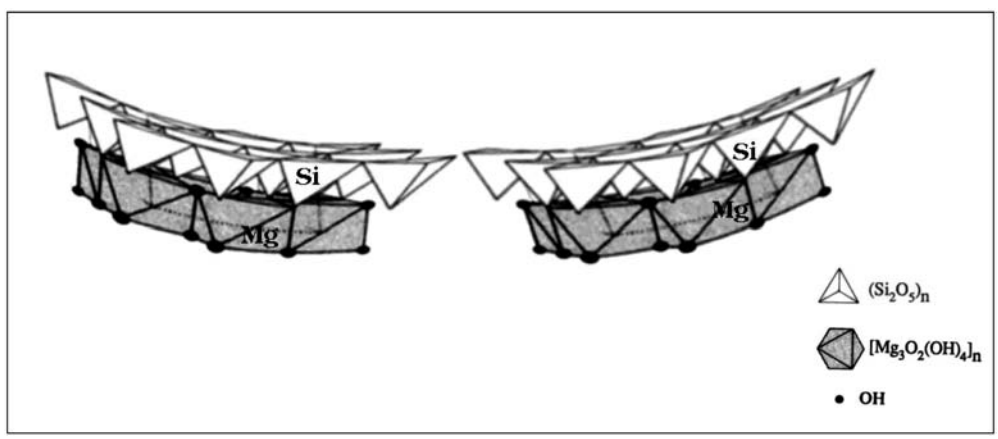


FIGURE 27.1 Schematic representation of the chemical structure of chrysotile showing the Mg molecule is on the outside of the curl. Adapted from Skinner et al. (1988).

and the trays are rotated for a fixed time to produce a sifting action. The longest/thickest fibers stay on the top screen (tray), which has the largest openings, and the shorter/thinner fibers fall though to lower screens. The grade is determined based on the weight fractions deposited on each screen and ranges from 3 to 9 with 3 being the longest (Cossette and Delvaux, 1979).

Nagy and Bates (1952), reporting on the stability of chrysotile, showed that it has a high solubility in hydrochloric acid. They also observed that chrysotile has a relatively low thermal stability compared with other hydrous silicate minerals. The heat of the electron beam of an electron microscope caused a very rapid change in the morphology of the fibers, and prolonged exposure to electron bombardment resulted in complete disintegration of the material (Noll and Kircher, 1952). Hargreaves and Taylor (1946) reported that if fibrous chrysotile is treated with dilute acid, the magnesium can be completely removed, and the hydrated silica remaining, though fibrous in form, completely lost the elasticity characteristic of the original chrysotile and gave an x-ray pattern of one or perhaps two diffuse broad bands, indicating that the structure is "amorphous" or "glassy" in type.

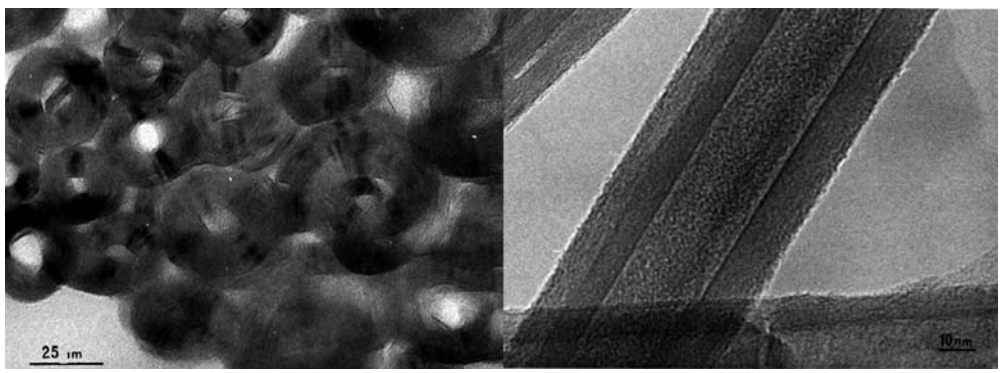


FIGURE 27.2 Transmission electron micrograph of chrysotile showing the curled sheet-like form of the fibers (Kiyohara, 1991).

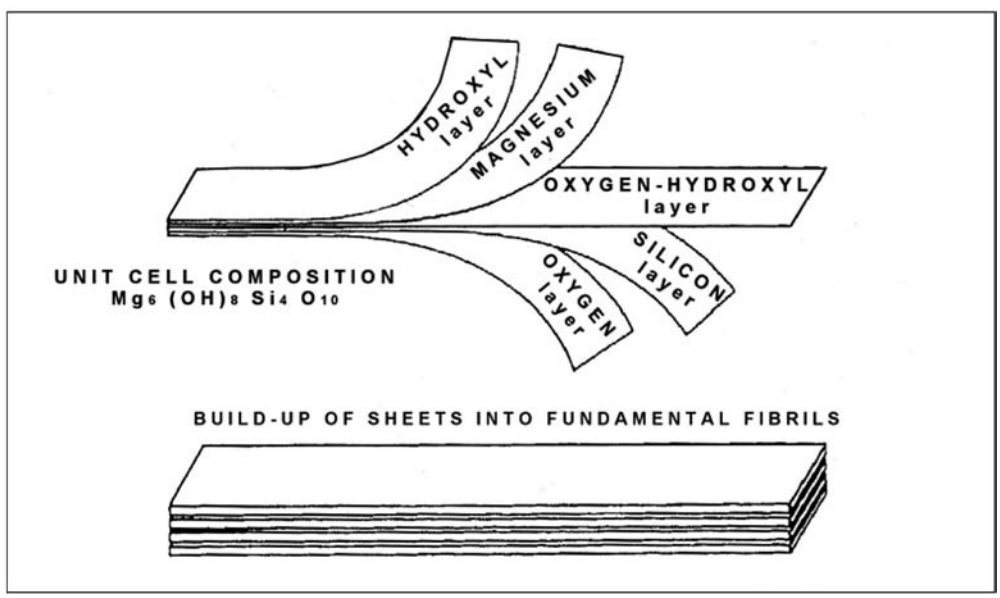


FIGURE 27.3 Illustration of the layered structure of chrysotile (reproduced from Speil and Leineweber, 1969).

TABLE 27.1 Typical Chemical Composition (Percent)

Compound	Chrysotile[a]	Tremolite[b]	Amosite[b]
SiO_2	40.6	55.10	49.70
Al_2O_3	0.7	1.14	0.40
Fe_2O_3	2.3	0.32	0.03
FeO	1.3	2.00	39.70
MnO	—	0.10	0.22
MgO	39.8	25.65	6.44
CaO	0.6	11.45	1.04
K_2O	0.2	0.29	0.63
Na_2O	—	0.14	0.09
H_2O^+	—	3.52	1.83
H_2	—	0.16	0.09
CO_2	0.5	0.06	0.09
Ignition loss	14.0	—	—
Total	100	99.93	100.26

[a] Typical chemical analysis of Canadian chrysotile from the Quebec Eastern Townships (LAB Chrysotile, Inc., Quebec, Canada).

[b] Hodgson (1979), pp. 80–81.

27.3 AMPHIBOLE CHARACTERISTICS

In contrast to chrysotile, with amphiboles, the basic structure is in the form of a double-silica chain, which appears as an I-beam with corner-linked $(SiO_4)^{-4}$ tetrahedra linked together in a double-tetrahedral chain that sandwiches a layer with the Ca_2Mg_5. These chains are paired, "back-to-back," with a layer of hydrated cations in between to satisfy the negative charges of the silica chains. The final structure is formed by stacking these sandwich ribbons in an ordered array (Speil and Leineweber, 1969). This is illustrated in Figure 27.4. Each of the blue boxes represents a double chain of tetrahedral (SiO_2). (The tetrahedra are illustrated in the middle chains.) With tremolite, the circles represent the magnesium and calcium cations that effectively glue one chain to its neighbor (Figure 27.4A).

Fewer shared cations bond the chains together along the broad sides of the chains than along the narrow sides, resulting in these broad surfaces being bonded less strongly. As shown in Figure 27.4B, it is along these weakly bonded surfaces, shown in red dashed lines, that the mineral will most likely break. With tremolite, these weak bonds are associated with the Mg. Figure 27.4C simplifies the picture and shows that the double-chain silicates can break into a set of fragments with a potentially regular shape.

Figure 27.4D shows the same situation in three dimensions. The potential breakages run along the chains and it can be seen how the fiber shape is formed. The chains themselves do not break easily because the bonds between the silica tetrahedra are very strong compared with the bonds gluing one chain to the next.

Depending on the type of amphibole, the principle cations are magnesium, iron, calcium, and sodium. The principle types are:

Crocidolite	$(Na_2Fe_3^{2+}Fe_2^{3+})Si_8O_{22}(OH)_2$
Amosite	$(Fe^{2+},Mg)_7Si_8O_{22}(OH)_2$
Tremolite	$Ca_2Mg_5Si_8O_{22}(OH)_2$
Actinolite	$Ca_2(Mg, Fe^{2+})_5Si_8O_{22}(OH)_2$
Anthophyllite	$(Mg, Fe^{2+})_7Si_8O_{22}(OH)_2$

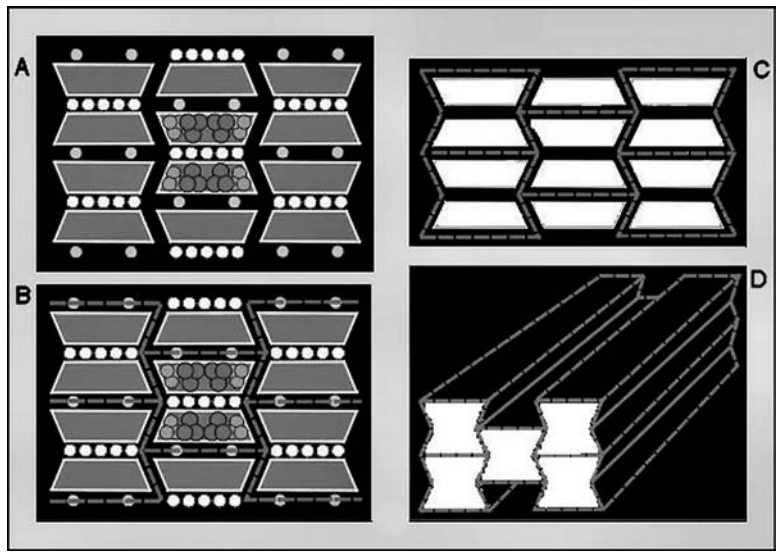


FIGURE 27.4 The structural formation of the double-chain silica tremolite asbestos is illustrated. (A) The amphiboles shown schematically with the slice directly across the chains. Each of the blue boxes represents a double chain of tetrahedral (SiO_2). (The tetrahedra are illustrated in the middle chains.) With tremolite, the circles represent the magnesium and calcium cations that glue one chain to its neighbor. Fewer shared cations bond the chains together along the broad sides of the chains than along the narrow sides. These broad surfaces are, therefore, bonded less strongly. (B) It is along these weakly bonded surfaces, shown in red dashed lines, that the mineral will most likely break. With tremolite, these weak bonds are associated with the Mg. (C) Simplification of the picture; the double-chain silicates can break into a set of fragments with potentially regular shape. (D) The same situation in three dimensions. The potential breakages run along the chains and it can be seen how the fiber shape is formed. The chains themselves do not break easily because the bonds between the silica tetrahedra are very strong compared with the bonds gluing one chain to the next.

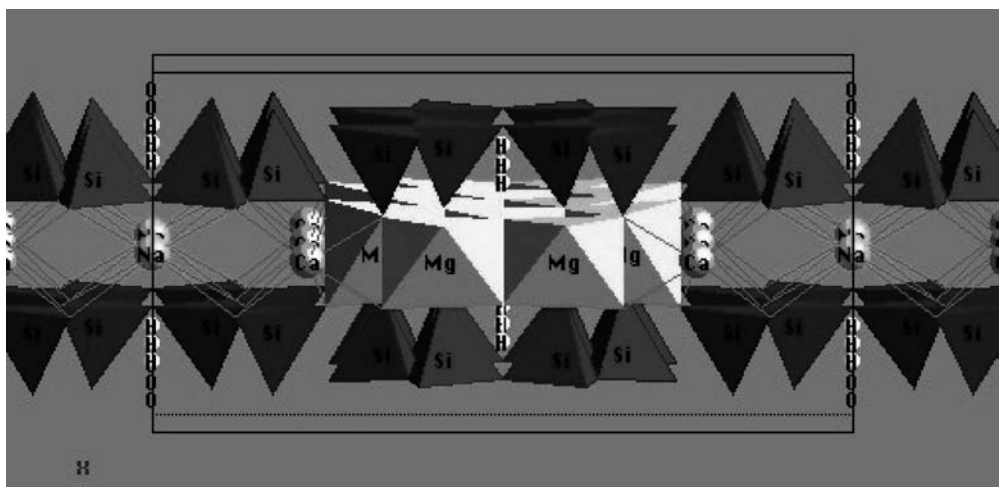


FIGURE 27.5 Schematic representation of the chemical structure of tremolite showing the Mg that is locked within the I-beam structure. Adapted with permission from: Department of Geology and Geophysics, University of Wisconsin, Crystal Structure Movies. Available at http://www.geology.wisc.edu.

The exterior surfaces of the amphiboles are tightly bound silica-based structures. This is illustrated with tremolite in Figure 27.5.

27.3.1 Factors Influencing Fiber Toxicology

Mineral fiber toxicology has been associated with three key factors: dose, dimension, and durability. The dose is determined by the fiber's physical characteristics/dimensions, how the fibrous material is used, and the control procedures that are implemented. In addition, the thinner and shorter fibers will weigh less and thus can remain suspended in the air longer than thicker and longer fibers. Most asbestos fibers are thinner than commercial insulation fibers; however, they are thicker than the new nanofibers that are currently being developed.

The fiber dimensions govern two factors, first, whether the fiber is respirable and, second, if it is respirable, whether the fiber will produce a response in the lung milieu once inhaled. Shorter fibers of the size that can be fully engulfed by the macrophage will be cleared by mechanisms similar to those for nonfibrous particles. These include clearance through the lymphatics and macrophage phagocytosis and clearance. It is only the longer fibers that the macrophage can not fully engulf, which if they are persistent will lead to disease.

This leads to the third factor, that of durability. Those fibers whose chemical structure renders them wholly or partially soluble once deposited in the lung are likely to either dissolve completely or dissolve until they are sufficiently weakened focally to undergo breakage into shorter fibers. The remaining short fibers can then be removed though successful phagocytosis and clearance.

These factors have been important determinants for synthetic mineral fibers (Hesterberg et al., 1998a, 1998 b; Miller et al., 1999; Oberdoester, 2000; Bernstein et al., 2001a, 2001b).

27.3.2 *In Vitro* Toxicology

In vitro toxicology studies are often very helpful in elucidating possible mechanisms involved in pathogenesis. However, as used in the assessment of fiber toxicology, they are difficult to interpret. This stems from several factors. The *in vitro* test system is a static system and thus is not sensitive to differences in fiber solubility. High doses of fibers are used to obtain a positive response and it is difficult to extrapolate from these large short-term cellular exposures to lower-dose chronic exposures that occur *in vivo*. In addition, the number of fibers and size distribution are often not quantified. Most important, however, is that these end points have not been validated as screening assays that are predictive of long-term pathological effects *in vivo*. Although *in vitro* tests may be useful tools to identify and evaluate possible mechanisms, with fibers, these *in vitro* test systems are of limited use in differentiating different fiber types (Olin et al., 2005).

27.3.3 Biopersistence

A fiber is unique among inhaled particles in that the aerodynamic diameter of fibers is largely related to three times the fiber diameter. Because of this, long thin fibers can penetrate into the deep lung, effectively bypassing the filtration that occurs for nonfibrous particles. Within the lung, fibers that can be fully engulfed by the macrophage can be removed as with any other particle. However, those fibers that are too long to be fully engulfed by the macrophage can not be cleared by this route.

Fibers less than 5 μm in length are effectively no different than nonfibrous particles and are cleared with kinetics and mechanisms similar to particles. Although longer fibers may also be cleared effectively by the macrophage and as a result not be different kinetically than particles, the 5-μm cutoff was chosen to mirror the use by the World Health Organization (WHO) of a 5-μm cutoff in their counting schemes for fibers. As is discussed later, recent reviews of these size fibers have concluded that they present very little or no risk to human health (ATSDR, 2003).

Fibers between 5 and 20 μm in length represent the transition range between those fibers that are cleared as particles and the longer fibers that the macrophage can not fully phagocytize. The actual

TABLE 27.2 Inhalation Biopersistence Clearance Half-Times of Natural and Synthetic Fibers (Length > 20 μm)

Fiber	Type	Weighted $t_{1/2}$ Fibers l > 20 μm (days)	Reference
Calidria chrysotile	**Serpentine asbestos**	**0.3**	**Bernstein et al., 2005b**
Brazilian chrysotile	**Serpentine asbestos**	**2.3**	**Bernstein et al., 2004**
Fiber B	B01.9	2.4	Bernstein et al., 1996
Fiber A	Glasswool	3.5	Bernstein et al., 1996
Fiber C	Glasswool	4.1	Bernstein et al., 1996
Fiber G	Stonewool	5.4	Bernstein et al., 1996
MMVF34	HT stonewool	6	Hesterberg et al., 1998
MMVF22	Slagwool	8	Bernstein et al., 1996
Fiber F	Stonewool	8.5	Bernstein et al., 1996
MMVF11	Glasswool	9	Bernstein et al., 1996
Fiber J	X607	9.8	Bernstein et al., 1996
Canadian chrysotile	**Serpentine asbestos**	**11.4**	**Bernstein et al., 2005a**
MMVF 11	Glasswool	13	Bernstein et al., 1996
Fiber H	Stonewool	13	Bernstein et al., 1996
MMVF10	Glasswool	39	Bernstein et al., 1996
Fiber L	Stonewool	45	Bernstein et al., 1996
MMVF33	Special purpose glass	49	Hesterberg et al., 1998a
RCF1a	Refractory ceramic	55	Hesterberg et al., 1998a
MMVF21	Stonewool	67	Hesterberg et al., 1998a
MMVF32	Special purpose glass	79	Hesterberg et al., 1998a
MMVF21	Stonewool	**85**	Bernstein et al., 1996
Amosite	**Amphibole asbestos**	**418**	**Hesterberg et al., 1998a**
Crocidolite	**Amphibole asbestos**	**536**	**Bernstein et al., 1996**
Tremolite	**Amphibole asbestos**	∞	**Bernstein et al., 2005b**

limit as to what length fiber can be fully phagocytized has been proposed for the rat as ranging from 15 μm (Miller, 2000) to 20 μm (Luoto et al., 1995; Morimoto et al., 1994).

In the lung, extensive work on modeling the dissolution of synthetic vitreous fibers (SVFs) by using *in vitro* dissolution techniques and inhalation biopersistence has shown that the lung has a very large fluid buffer capacity (Mattson, 1994). These studies have shown that an equivalent *in vitro* flow rate of up to 1 ml/min is required to provide the same dissolution rate of SVF as that which occurs in the lung. This large fluid flow within the lung results in the dissolution of the more soluble fibers. Recent publications have shown that the biopersistence of the fibers longer than 20 μm is an excellent predictor of the pathological response to fibers following chronic inhalation studies and chronic intraperitoneal studies (Bernstein et al., 2001a, 2001b; Hesterberg et al., 1998a, 1998b). The value of 20 μm is used as an index for fibers that can not be fully phagocytized and cleared by the macrophage. The protocol used in these biopersistence studies was developed by a working group for the European Commission and involves a 5-d inhalation exposure followed by analysis of the lungs at periodic intervals up to 1 year postexposure (Bernstein and Riego-Sintes, 1999).

For synthetic vitreous fibers, the clearance half-time of fibers longer than 20 μm ranges from a few days to less than 100 days. This is illustrated in Table 27.2. Highlighted in this table are

those studies performed on chrysotile and amphiboles with the same protocol. For synthetic vitreous fibers, the European Commission has established a Directive which states that if the inhalation biopersistence clearance half-time of a fiber is less than 10 days then it is not classified as a carcinogen.

Clearly there is a large difference in biopersistence between serpentine asbestos and amphiboles. In addition, because serpentine is a naturally occurring mined fiber, there seem to be some differences in biopersistence, depending on where it was mined. However, chrysotile lies on the soluble end of this scale and ranges from the least biopersistent fiber to a fiber with biopersistence in the range of glass and stonewools. It remains less biopersistent than ceramic and special purpose glasses and more than an order of magnitude less biopersistent than amphiboles.

The rapid clearance of chrysotile is thought to be characterized not by congruent dissolution as with many SVFs but rather with the loss of structural integrity of the serpentine sheet silicate and the subsequent disintegration into smaller pieces.

This difference between chrysotile and amphiboles is better illustrated with the actual lung burden data for the fibers longer than 20 μm from the inhalation biopersistence studies. In Figure 27.6, the number of fibers remaining in the rat's lungs is shown as a function of the time in days following cessation of the 5-d exposure (Bernstein et al., in press; 2005). Included are the two amphiboles, tremolite and amosite, a SVF fiber, HT, which has a clearance half-time of 6 days and which showed no tumors or fibrosis in a chronic inhalation toxicology study, and the three chrysotile fibers from Brazil, the United States (Calidria), and Canada. The inhalation exposure aerosol in terms of the number of fibers longer than 20 μm was in the range of 150–200 fibers (length $>$ 20 μm) per cm^3 for all fibers except the Brazilian chrysotile, which was 400 fibers (length $>$ 20 μm) per cm^3.

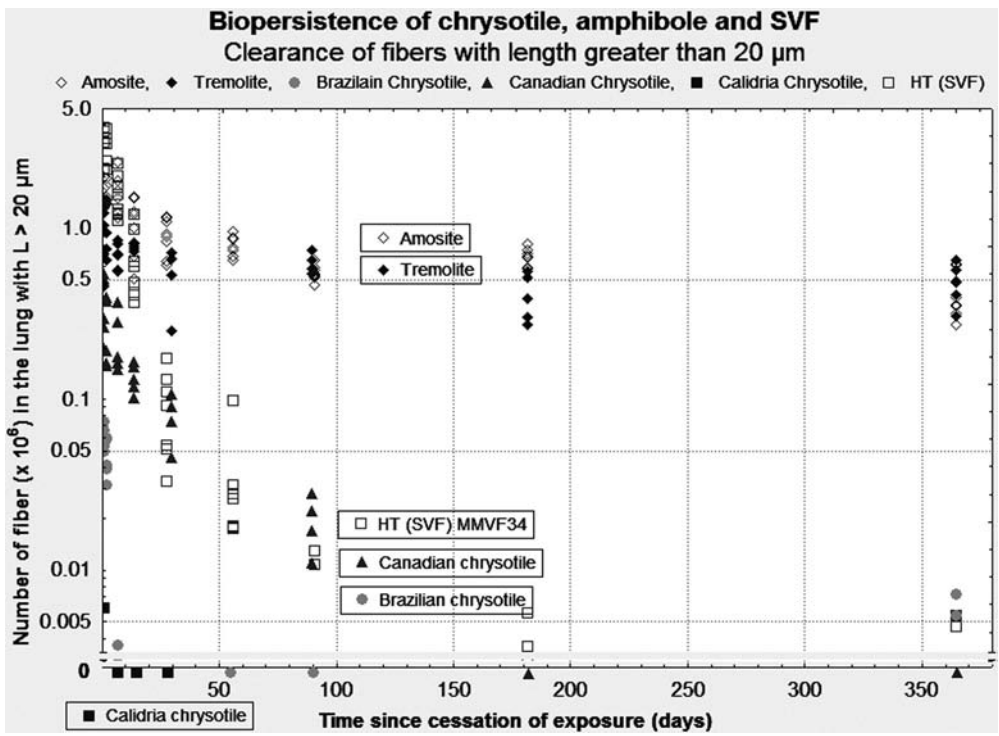


FIGURE 27.6 The number of fibers remaining in the rat's lungs is shown as a function of the time in days following cessation of the 5-day exposure (Bernstein et al., 2003a, 2005b). Included are the two amphiboles, tremolite and amosite, a soluble synthetic vitreous (SVF) fiber, HT, which has a clearance half-time of 6 days and which showed no tumors or fibrosis in a chronic inhalation toxicology study and the three chrysotile fibers from Brazil, the United States (Calidria), and Canada.

The amphiboles are very durable with only a small amount of clearance after cessation of exposure followed by virtually no further clearance. In the tremolite biopersistence study the histopathological response of the lungs was examined following the 5-day exposure. A pronounced inflammatory response with the rapid development of granulomas was seen at day 1 postexposure, followed by the development of fibrosis characterized by collagen deposition within these granulomas and by 90 days even mild interstitial fibrosis. In the same study, chrysotile showed no inflammatory or pathological response following the 5-d exposure (Bernstein et al., 2003b, 2005).

Although all the chrysotiles cleared relatively quickly, differences were observed among the three types studied. The Calidria chrysotile, which is known to be a short-fiber chrysotile, cleared the fastest with a clearance half-time for the fibers longer than 20 μm of 0.3 days.

The clearance half-time of the Brazilian chrysotile was 2.3 days. At the end of 12 months, two to three long fibers were measured following the lung digestion procedure. However, the exposure concentration for the Brazilian chrysotile was 400 fibers (length $>$ 20 μm) per cm^3 rather than the 150 to 200 fibers (length $>$ 20 μm) per cm^3 for the other fibers evaluated, thus resulting in a very high aerosol concentration of 7 million WHO fibers per cm^3 and more than 32 million total fibers per cm^3. It certainly is possible that this extremely high total exposure resulted in a response very different from what might be expected at lower exposure concentrations. Even so, the number of fibers observed at 12 months was not statistically different than that which was observed for the HT fiber, which had a 6-d clearance half-time for the long fibers.

For the Canadian chrysotile study textile-grade chrysotile was evaluated. This grade was chosen because it was specifically produced to have thin long fibers, which facilitated the production of textiles. The clearance of the Canadian chrysotile long fibers was 11.4 days. By 365 days there were no long Canadian chrysotile fibers remaining in the lung.

27.3.3.1 Clearance Mechanism of HT and Chrysotile

Kamstrup et al. (2002) described possible mechanisms that could account for the rapid clearance half-time of the long HT fibers. They stated that "The HT fiber is characterized by relatively low silica and high alumina content, with a high dissolution rate at pH 4.5 and relatively low rate at pH 7.4 (Knudsen et al., 1996). Apart from possible exposure to the acidic environment of the phagolysosomes within the macrophages (Oberdörster, 1991), measurements have shown that the microenvironment at the surface of activated macrophages is acidic with pH $<$ 5 between attached macrophages and a non-porous glass surface (Etherington et al., 1981). It is therefore probable that long HT fibers, highly soluble at pH 4.5, are subject to extracellular dissolution and consequent breakage when exposed to the acidic environment of attached macrophages without being engulfed completely."

As mentioned above, at acidic pH chrysotile also becomes less stable and a similar mechanism may help accelerate the clearance/disintegration of the long chrysotile fibers.

27.3.3.2 Short-Fiber Clearance

For all fiber exposures, there are many more shorter fibers less than 20 μm in length and even more less than 5 μm in length. The clearance of the shorter fibers has in these studies been shown to be either similar to or faster than the clearance of insoluble nuisance dusts (Stoeber et al., 1970; Muhle et al., 1987). In a recent report issued by the Agency for Toxic Substances and Disease Registry entitled "Expert Panel on Health Effects of Asbestos and Synthetic Vitreous Fibers: The Influence of Fiber Length," the experts stated that "Given findings from epidemiological studies, laboratory animal studies, and in vitro genotoxicity studies, combined with the lung's ability to clear short fibers, the panelists agreed that there is a strong weight of evidence that asbestos and SVFs (synthetic vitreous fibers) shorter than 5 μm are unlikely to cause cancer in humans" (ATSDR, 2003; U.S. Environmental Protection Agency [EPA], 2003). In addition, Berman and Crump (2003) in their technical support document to the EPA on asbestos-related risk also found that shorter fibers do not appear to contribute to disease.

27.3.4 Chronic Inhalation Toxicology Studies

The studies presented above indicate that a large difference exists in the biopersistence between the serpentine chrysotile and the amphiboles, tremolite, and amosite. These differences appear to be related to the differences in chemical structure between the serpentines and amphiboles and possibly the influence of the acidic pH associated with the macrophage on the chrysotile fiber. Yet when the chronic inhalation studies that have been performed on chrysotile and amphiboles are examined these differences are not always apparent.

Berman et al. (1995) and Berman and Crump (2003), in an analysis of 13 inhalation studies that have been performed on nine different types of asbestos, concluded that:

- short fibers (less than between 5 and 10 μm in length) do not appear to contribute to cancer risk;
- beyond a fixed, minimum length, potency increases with increasing length, at least up to a length of 20 μm (and possibly up to a length of as much as 40 μm);
- the majority of fibers that contribute to cancer risk are thin with diameters less than 0.5 μm and the most potent fibers may be even thinner. In fact, it appears that the fibers that are most potent are substantially thinner than the upper limit defined by respirability;
- identifiable components (fibers and bundles) of complex structures (clusters and matrices) that exhibit the requisite size range may contribute to overall cancer risk because such structures likely disaggregate in the lung. Therefore, such structures should be individually enumerated when analyzing to determine the concentration of asbestos;
- for asbestos analyses to adequately represent biological activity, samples need to be prepared by a direct-transfer procedure; and
- based on animal dose–response studies alone, fiber type (i.e., fiber mineralogy) appears to impart only a modest effect on cancer risk (at least among the various asbestos types).

Concerning the lack of differentiation seen in the dose–response studies, the authors stated that this may be due at least in part to the limited lifetime of the rat relative to the biodurability of the asbestos fiber types evaluated in these studies.

Perhaps more important in understanding these results are the specifics in the study design of theses studies in light of the more recent understanding of the effect of high concentrations of insoluble particles on the rat lung.

The chronic inhalation studies that have been performed on asbestos are summarized in Tables 27.3 and 27.4. The exposure regime was similar in most studies and ranged from 5 to 7 h/d, 5 days per week for either 12 months or 24 months. Although it is difficult to determine how this was derived, the exposure concentration was set for most studies based on mass concentration at 10 mg/m^3. Davis et al. (1978) referencing Wagner et al. (1974) states that 10 mg/m^3 was considered to be high enough to cause significant pathological change; however, no rationale is given in the Wagner paper as to why 10 mg/m^3 was chosen.

The issue of using equivalent fiber numbers for exposure was approached in a study reported by Davis et al. (1978) where chrysotile, crocidolite, and amosite were compared on an equal-mass and equal-number basis, but the fiber number was determined by phase-contrast optical microscopy (PCOM) and thus the actual number of especially the chrysotile fibers was probably greatly underestimated. As an example, by PCOM, the 10 mg/m^3 exposure to chrysotile was reported by PCOM as approximately 2000 fibers/cm^3 (length greater than 5 μm), whereas when a similar mass concentration of another chrysotile was measured by scanning electron microscopy (SEM) 10,000 fibers/cm^3 (length greater than 5 μm) were reported with a total fiber count of 100,000 fibers/cm^3 (Mast et al., 1995). There are few quantitative data presented in these publications on the nonfibrous particle concentration of the test substances to which the animals were exposed. Pinkerton et al. (1983) presented summary tables of length measurements of Calidria chrysotile by SEM in which the number of nonfibrous particles counted is stated; however, from the data presented, the aerosol

exposure concentration of nonfibrous particles can not be extracted. In all studies, the asbestos was ground prior to aerosolization, a procedure that would produce a lot of short fibers and dust. Most of the studies prior to Mast et al. (1995) used for aerosolization of the fibers an apparatus in which a rotating steal blade pushed/chopped the fibers off a compressed plug into the airstream. As some of the authors state, the steal used in the grinding apparatus and the aerosolization apparatus often wore, resulting in sometimes considerable exposure to the metal fragments as well. These factors contribute significantly to the difficulty in interpreting the results of the serpentine chrysotile and the amphibole inhalation exposure studies.

In these studies a tumorigenic response to amphiboles response is observed as would be expected from the biopersistence results; however, as mentioned, there is also a tumorigenic response to some of the chrysotile exposures even though the biopersistence results would suggest otherwise. Eastes and Hadley (1996) developed a model that related the dose of fibers in the lung to potential pathogenicity.

However, as many studies have now shown, in the rat another factor can also influence the inflammatory and pathological response. High concentrations of insoluble nuisance dusts have been shown to compromise the clearance mechanisms of the lung, cause inflammation and a tumorigenic response in the rat, a phenomenon often referred to as lung overload (Bolton et al., 1983; Muhle et al., 1988; Morrow, 1988; Oberdorster, 1995).

The biopersistence studies elucidate two kinetic patterns with chrysotile. They show that the long fibers are not biopersistent. From the fiber chemistry, the longer fibers are likely falling apart or disintegrating into the smaller pieces. The biopersistence studies also show that these smaller pieces clear at a rate that is similar to the rate of clearance of insoluble nuisance dusts. Chrysotile has also been shown to split longitudinally. In most of the chronic inhalation studies the total aerosol concentration was probably on the order of 10^6 particles and fibers per cm^3 and if the fibers upon contact with the lung begin to split and break apart, the effective dose in terms of the total number of particles would be increased even further.

With such a breakdown of chrysotile into shorter particles, the question remains as to whether the resulting concentration of particles can result in a nonspecific inflammatory reaction and an overload effect in the rat lung. In a recent study, Bellmann et al. (2003) reported on a calibration study to evaluate the end points in a 90-d subchronic inhalation toxicity study of man-made vitreous fibers with a range of biopersistence and amosite. One of the fibers was a calcium-magnesium-silicate (CMS) fiber for which the stock preparation, due to the method of preparation, had a large concentration of particulate material in addition to the fibers. The aerosol exposure concentration for the CMS fiber was 286 fibers/cm^3 (length $< 5\mu m$), 990 fibers/cm^3 (length $> 5\ \mu m$), and 1793 particles/cm^3, a distribution that is not observed in manufacturing. The total CMS exposure concentration was 3069 particles and fibers per cm^3. The authors point out that "The particle fraction of CMS that had the same chemical composition as the fibrous fraction seemed to cause significant effects." The number of polymorphonuclear leukocytes (PMNs) in the bronchoalveolar lavage fluid (BALF) was higher and interstitial fibrosis was more pronounced than had been expected on the basis of biopersistence data. In addition, interstitial fibrosis persisted through the 14-week recovery period following the 90-day exposure. In a separate study on X607, a fiber chemically similar to CMS, but with considerably fewer particles present in the aerosol, was evaluated in a chronic inhalation toxicity study and produced no lung tumors or fibrosis at any time point (Hesterberg et al., 1998b).

This effect attributed to particles in the rat CMS study was observed with an exposure concentration of 3069 particles and fibers per cm^3, 50% of which were particles or short fibers. It would follow directly from this and the many publications on overload to expect that a dramatically more pronounced effect would occur if the exposure concentration was 1,000,000 particles and fibers per cm^3, 90% of which were particles or short fibers, as was the case with chrysotile.

These discrepancies in study design put in question the value of especially the chrysotile studies listed in Table 27.3. The only well-designed multiple-dose study that was performed on any asbestos where particle and fiber number and length were controlled was that for amosite in the hamster (McConnell et al., 1999) as shown in Table 27.4. In this study the aerosol concentration ranged from

TABLE 27.3 Chronic Inhalation Toxicology Studies with Chrysotile and Amphibole Asbestos in Rats[a]

Fiber Type	Exposure Time h/d, d/wk, total months	Exposure Type, Exposure Concentration mg/m^3	Fiber Concentration f/cm^3 (determined by electron microscopy unless otherwise noted)	Type & Total No. of Rats	Number Pulmonary Tumours	% Pulmonary Tumours	Noumber of Meso-Theliomas	References
Chrysotile Canadian (Nickel, cobalt, chromium and lead contamination)	6, 5, 14	w.b. 86	Nd	NS, 41	10	24	1	Gross et al., 1967
Chrysotile UICC Canadian	7, 5, 24	w.b. 10	Nd	W, 21	10	48	1	Wagner et al., 1974
Chrysotile UICC Rhodesian	7, 5, 24	w.b. 10	Nd	W, 17	11	65	0	Wagner et al., 1974
Chrysotile Canadian 714-7D (friction linings)	5, 5, 24	w.b. 15	1.7×10^5 SEM 9978 > 5μm	W, 45	9	20	0	Le Bouffant et al., 1984-1987
SFA chrysotile	7, 5, 24	w.b. 10	430 > 5μm pcom 669 particles pcom	W, 22	8	36	0	Wagner et al., 1980
Grade 7 chrysotile	7, 5, 24	w.b. 10	1020 > 5μm pcom 745 particles pcom	W, 24	3	13	0	Wagner et al., 1980
UICC chrysotile	7, 5, 24	w.b. 10	3750 > 5μm pcom 338 particles pcom	W, 23	5	22	0	Wagner et al., 1980
Chrysotile Calidria	5, 5, 12	w.b. 6	241 131 > 5 μm reported as “thick bundles”	W, 50	0	0	0	Muhle et al., 1987
Chrysotile long	7, 5, 12	w.b. 10	1170 > 5μm pcom 33 > 20 μm pcom	W, 40	20	50	2	Davis et al., 1988
Chrysotile court	7, 5, 12	w.b. 10	5510 > 5μm pcom 670 > 20 μm pcom	W, 40	7	17	1	Davis et al., 1988
Chrysotile UICC A	7, 5, 12	w.b. 10	2560 > 5μm pcom	Included for comparative fiber numbers without animal exposure				Davis et al., 1988

Chrysotile NIEHS	6, 5, 24	n-o 10	1.02×10^5 SEM 1.06×10^4 >5μm	F, 69	13	18	1	Mast et al., 1995
Chrysotile	7, 5, 12	w.b. 10	1950 >5μm pcom 360 >20μm pcom	W, 40	15	38	0	Davis et al., 1978
Chrysotile	7, 5, 12	w.b. 2	390 >5μm pcom 72 >20μm pcom	W, 42	8	19	1	Davis et al., 1978
Crocidolite	7, 5, 12	w.b. 10	860 >5μm pcom estimated figure 34 >20μm pcom	W, 40	1	3	0	Davis et al., 1978
Crocidolite	7, 5, 12	w.b. 5	430 >5μm pcom 17 >20μm pcom	W, 43	2	5	1	Davis et al., 1978
Amosite	7, 5, 12	w.b. 10	550 >5μm pcom 6 >20μm pcom	W, 43	2	5	0	Davis et al., 1978
Chrysotile Calidria	7, 5, 12	w.b. 10	Nd	F, 51	2	4	0	Ilgren & Chatfield 1997, 1998; Pinkerton et al., 1983
Chrysotile Jeffrey	7, 5, 12	w.b. 10	Nd	F, 49	11	22	0	Ilgren & Chatfield 1997, 1998; Pinkerton et al., 1983
Chrysotile UICC/B	7, 5, 12	w.b. 10	Nd	F, 54	13	24	0	Ilgren & Chatfield 1997, 1998; Pinkerton et al., 1983
Tremolite Korean	7, 5, 12	w.b. 10	1600 pcom	39	18	46	2	Davis et al.
Amosite UICC	7, 5, 24	w.b. 10	Nd	W, 21	13	62	0	Wagner et al., 1974

(*Continued*)

TABLE 27.3 Chronic Inhalation Toxicology Studies with Chrysotile and Amphibole Asbestos in Rats[a] (Continued)

Fiber type	Exposure Time h/d, d/wk, total months	Exposure type, Exposure Concentration mg/m³	Fiber Concentration f/cm³ (determined by electron microscopy unless otherwise noted)	Type & Total no. of rats	Number Pulmonary Tumours	% Pulmonary Tumours	Noumber of meso-theliomas	References
Amosite long	7, 5, 12	w.b. 10	2060 > 5μm pcom 70 > 10 μm pcom	W, 40	11	28	3	Davis et al., 1986
Amosite short	7, 5, 12	w.b. 10	70 > 5μm pcom 12 > 10 μm pcom	W, 42	0	0	1	Davis et al., 1986
Amosite	—	w.b. 300	Nd	SD, 16	3	19	0	Lee et al., 1981
Crocidolite UICC	7, 5, 24	w.b. 10	Nd	W, 18	13	72	0	Wagner et al., 1974
Crocidolite	5, 5, 12	w.b. 2.2	2011 162 > 5 μm	W, 50	1	2	0	Muhle et al., 1987
Crocidolite UICC	6, 5, 24	w.b. 7	3000 90 > 10 μm	OM, 60	3	5	1	Smith et al., 1987
Crocidolite exposure truncated	6, 5, 10	n-o 10	1.6×10^4 > 5 μm SEM	F, 106	15	14	1	McConnell et al., 1994

[a] Exposure types: w.b., whole body; n-o, nose only. Type of rat: F, Fisher 344; OM, Osborne Mendel; SD, Sprague–Dawley; W, Wistar. ND, not determined; pcom, phase-contrast optical microscopy; SEM, scanning electron microscopy.

TABLE 27.4 Chronic Inhalation Toxicology Studies with Chrysotile and Amphibole Asbestos in Hamsters[a]

Fiber Type	Exp Time h/d, d/wk max months	Exposure Concentration mg/m^3	Fiber Concentration f/cm^3	Total Number of Hamsters	Number Pulmonary Tumours	% Pulmonary Tumours Adenoma, Carcinomes	Number of Meso-Theliomas	References
Amosite	—	w.b. 300	ND	7	0	0	—	Lee et al., 1981
Amosite –low	6, 5, 18	n-o 0.8	36 > 5 μm 10 > 20 μm	83	0	0	3	Hesterberg et al., 1999; McConnell et al., 1999
Amosite –mid	6, 5, 18	n.o 3.7	165 > 5 μm 38 > 20 μm	85	0	0	22	Hesterberg et al., 1999; McConnell et al., 1999
Amosite –high	6, 5, 18	n-o 7.1	263 > 5 μm 69 > 20 μm	87	0	0	17	Hesterberg et al., 1999; McConnell et al., 1999
Crocidolite UICC	6, 5, 18	w.b. 7	3000 90 > 10 μm	58	0	0	0	Smith et al., 1987
Chrysotile NIEHS	6, 5, 18	n-o 10	1.02×10^5 $1.06 \times 10^4 > 5\mu m$	??	0	0	0	Mast et al., 1994

[a] Exposure types: w.b., whole body; n-o, nose only.

10 to 69 fibers/cm^3 (longer than 20 μm) and was chosen based on a previous, multidose 90-day subchronic inhalation toxicology study (Hesterberg et al., 1999).

27.3.4.1 Fiber Length

In an analysis that provided the basis for the European Commission's directive on synthetic mineral fibers, Bernstein et al. (2001a, 2001b) reported that an excellent correlation exists for SVF between the biopersistence of fibers longer than 20 μm and the pathological effects following either chronic inhalation or chronic intraperitoneal injection studies. This analysis showed that it was possible using the clearance half-time of the fibers longer than 20 μm as obtained from the inhalation biopersistence studies to predict the number of fibers longer than 20 μm remaining after 24 months of chronic inhalation exposure. These studies, however, only included synthetic mineral fibers.

As mentioned above, Berman et al. (1995) analyzed statistically nine different asbestos types in 13 separate studies. Because of limitations in the characterization of asbestos structures in the original studies, new exposure measures were developed from samples of the original dusts that were regenerated and analyzed by transmission electron microscopy. The authors reported that although no univariate model was found to provide an adequate description of the lung tumor responses in the inhalation studies, the measure most highly correlated with tumor incidence was the concentration of structures (fibers) greater than or equal to 20 μm in length. However, by using multivariate techniques, measures of exposure were identified that do adequately describe the lung tumor responses.

The potency appears to increase with increasing length, with structures (fibers) longer than 40 μm being about 500 times more potent than structures between 5 and 40 μm in length. Structures <5 μm in length do not appear to make any contribution to lung tumor risk. As discussed above, this analysis also did not find a difference in the potency of chrysotile and amphibole toward the induction of lung tumors.

27.3.4.2 Purity of the Samples

In most inhalation studies on both amphiboles and serpentines, there was no analytical confirmation reported that the fibers that were aerosolized were uniquely of the type stated.

In addition, an issue that has been discussed at length is whether the presence of tremolite in the chrysotile samples can account for some of its carcinogenic potential as well. This is especially pertinent to the mesotheliomas that have been observed in some of the rat inhalation studies (Churg, 1994; McDonald et al., 1999; Roggli et al., 2002). Using microscopic analysis, Frank et al. (1998) reported the absence of tremolite in the UICC chrysotile sample that has often been used in the chronic studies. However, when present with chrysotile, tremolite is usually found in very low concentrations that could be missed when using microscopic analysis.

To resolve this issue of method sensitivity, Addison and Davies (1990) developed a method of chemical digestion of chrysotile in which the chrysotile is dissolved away by using an acid digestion, leaving behind the amphiboles such as tremolite. This method was applied to a sample UICC chrysotile obtained from Dr. Fred Pooley who has a repository of the original UICC preparation. In conjunction with Gesellschaft fuer Schadstoffmessung und Auftragsanalytik GmbH (GSA, Neuss, Germany), 2.13 g of UICC chrysotile were digested in acid after a procedure similar to that of Addison and Davies (1990). Following digestion, the bivariate size distribution was determined for all residual fibers by transmission electron microscopy and the chemical composition of each fiber determined by energy-dispersive analysis of x-rays (EDAX) to clearly identify it as amphibole, chrysotile, or other.

In the 2-mg sample analyzed, the results indicated that there were 3400 tremolite fibers per mg of UICC chrysotile. These fibers ranged in length from 1.7 to 14.4 μm and had a mean diameter of 0.65 μm. Forty-one percent of the fibers were longer than 5 μm with 1394 WHO tremolite fibers per mg of UICC chrysotile. These results indicate that tremolite is present in the UICC sample at low concentrations. Because no dose–response studies have been performed at low amphibole concentrations, quantification of the effect of these fibers is not possible in the rat. However, as discussed above,

amphibole asbestos fibers are very biopersistent in the lung and will persist once inhaled. Davis et al. (1985) performed a chronic inhalation toxicity study on tremolite to determine the effect of commercial tremolite in comparison with other asbestos types. The authors reported that tremolite was the most dangerous mineral that they have studied, producing 16 carcinomas and 2 mesotheliomas in a group of 39 animals. As described above, even short exposure to tremolite produces a notable response in the lung. Bernstein et al. (2003b) reported that after a 5-d exposure to tremolite, a pronounced inflammatory response was observed with the rapid development of granulomas, collagen deposition within these granulomas, and, by 90 days, even mild interstitial fibrosis.

27.3.5 Epidemiology

Many studies have shown that chrysotile is not of the same potency as the amphiboles and is cleared from the lung more rapidly than amphibole (Howard, 1984; Churg and DePaoli, 1988; Mossman et al., 1990; Morgan, 1994; Churg, 1994; McDonald et al., 1995, 1997, 1999, 2002, 2003; McDonald, 1998; Rodelsperger et al., 1999; Hodgson and Darnton, 2000; Berman and Crump, 2003). Still, other studies have stated the opposite.

Two studies have provided a quantitative review of the potency of chrysotile and amphiboles based on the statistical analysis of currently available epidemiology studies.

Hodgson and Darnton (2000) conducted a comprehensive quantitative review of potency of asbestos for causing lung cancer and mesothelioma in relation to fiber type. They concluded that amosite and crocidolite were, respectively, on the order of 100 and 500 times more potent for causing mesothelioma than chrysotile. They regarded the evidence for lung cancer to be less clear cut, but concluded nevertheless that amphiboles (amosite and crocidolite) were between 10 and 50 times more potent for causing lung cancer than chrysotile.

Berman and Crump (2003) have recently reviewed and analyzed as part of a technical support document for the U.S. EPA an epidemiology database consisting of approximately 150 studies of which approximately 35 contained exposure data sufficient to derive quantitative exposure/response relationships.

Using this database of epidemiological studies, the authors determined estimates of lung cancer or mesothelioma from asbestos exposure with mathematical models that express risk as a function of exposure. The models contain parameters (K_L for lung cancer and K_M for mesothelioma) that gauge the potency of asbestos for causing these health effects. Using their reanalysis of this dataset, they determined that for disease in humans the important fibers are longer than 10 μm and thinner than 0.4 μm. The authors concluded that the corresponding coefficients for pure fiber types were, for lung cancer, 0.6 and 3 for chrysotile or amphibole, respectively, and for mesothelioma, 0.04 and 30 for chrysotile or amphibole, respectively. They also noted that, without adjustments for fiber size, the lung cancer exposure–response coefficients (K_L values) estimated from 15 studies vary by a factor of 72 and these values are mutually inconsistent (based on nonoverlap of uncertainty intervals). However, when the studies were adjusted for fiber size and type, the overall variation in K_L values across these studies was reduced to a factor of 50. Similarly, without adjustments, the mesothelioma exposure–response coefficients K_M values varied by a factor of 1089, and that these values are likewise mutually inconsistent. However, when the studies were adjusted for fiber size and type, the overall variation in K_M values was reduced to a factor of 30.

Direct comparison between these two analyses is difficult because the Hodgson and Darnton (2000) potency estimates are based on WHO fibers, whereas the Berman and Crump (2003) potency estimates are based on fibers longer than 10 μm and thinner than 0.4 μm, which they found as the determinant fibers for disease. Berman and Crump (2004) also stated in their report that, based on their review, the supporting literature suggests that the optimum cutoff for increased potency occurs at a length that is closer to 20 μm than to 10 μm (the latter of which is the cutoff in the exposure index provided in this study). However, they found that not enough data currently exist to improve quantitatively on this latter cutoff.

27.4 SUMMARY

The mineralogy of the serpentine chrysotile fibers and amphiboles fibers shows distinct differences in the structure and chemistry of these two minerals. In contrast to the curled layered construction of the sheet silicate chrysotile, which appears to result in greater susceptibility to degradation, the amphibole fibers are rigid impermeable structures that are resistant to degradation. These differences are reflected in the inhalation biopersistence studies that clearly differentiate chrysotile from the amphiboles and show that longer chrysotile fibers rapidly disintegrate in the lung whereas the longer amphiboles, once deposited, remain. There is an excellent correlation between the biopersistence of the longer synthetic vitreous fibers and chronic toxicity data. Because of the difficulties in study design and the large particle/fiber exposure concentrations used, the chronic inhalation studies with asbestos are difficult to interpret due in part to the nonspecific effects of the very large particle concentrations in the exposure aerosols.

Recent quantitative reviews that analyzed the data of available epidemiological studies to determine potency of asbestos for causing lung cancer and mesothelioma in relation to fiber type also differentiated between chrysotile and amphibole asbestos. The most recent analysis also concluded that it is the longer, thinner fibers that have the greatest potency. The quantitative experimental results provide additional support for this differentiation.

There is no question that amphibole asbestos is highly carcinogenic. Both animal studies and epidemiology studies indicate the potency of amphibole asbestos. Recent studies on tremolite show that even short exposure can produce a pathogenic response in the lung.

With chrysotile asbestos, indeed, there is evidence that humans can and do develop lung cancer when the exposure is high and sustained for long periods. The weight of evidence suggests that at low exposure pure chrysotile is probably not hazardous. It also suggests that the hazard may be low if even high exposures were of short duration.

It would be most helpful if future studies on chrysotile and amphiboles, whether *in vitro* or *in vivo*, could be performed using size distributions and at doses approaching those to which humans have been exposed.

REFERENCES

Addison, J. and Davies, L.S., Analysis of amphibole asbestos in chrysotile and other minerals, *Ann. Occup. Hyg*., 34, 159–175, 1990.

ATSDR (Agency for Toxic Substances and Disease Registry), Report on the Expert Panel on Health Effects of Asbestos and Synthetic Vitreous Fibers: The Influence of Fiber Length, Atlanta, GA, Prepared for: Agency for Toxic Substances and Disease Registry, Division of Health Assessment and Consultation, 2003.

Bellmann, B., Muhle, H., Creutzenberg, O., Ernst, H., Muller, M., Bernstein D.M., and Riego Sintes, J.M., Calibration study on subchronic inhalation toxicity in man-made vitreous fibers in rats, *Inhalation Toxicol*., 15, 1147–1177, 2003.

Berman, D.W. and Crump, K.S., Technical Support Document for a Protocol to Assess Asbestos-Related Risk, Office of Solid Waste and Emergency Response, U.S. Environmental Protection Agency, Washington, DC, 2004.

Berman, D.W., Crump, K.S., Chatfield, E.J., Davis, J.M., and Jones, A.D., The sizes, shapes, and mineralogy of asbestos structures that induce lung tumors or mesothelioma in AF/HAN rats following inhalation, *Risk Anal*., 15, 181–195, 1995.

Bernstein, D.M., Chevalier, J., and Smith, P., Comparison of Calidria chrysotile asbestos to pure tremolite: inhalation biopersistence and histopathology following short-term exposure, *Inhalation Toxicol*., 15, 1387–1419, 2003.

Bernstein, D.M., Chevalier, J., and Smith,. P., Comparison of calidria chrysotile asbestos to pure tremolite: final results of the inhalation biopersistence and histopathology examination following short-term exposure, *Inhalation Toxicol*., 17, 427–449, 2005.

Bernstein, D.M., Morscheidt, C., Grimm, H.-G., The venaz, P., and Teichert, U., Evaluation of soluble fibers using the inhalation biopersistence model, a nine-fiber comparison, *Inhalation Toxicol*., 8, 345–385, 1996.

Bernstein, D.M., Riego Sintes, J.M., Ersboell, B.K., and Kunert, J., Biopersistence of synthetic mineral fibers as a predictor of chronic inhalation toxicity in rats, *Inhalation Toxicol*., 13, 823–849, 2001a.

Bernstein, D.M., Riego Sintes, J.M., Ersboell, B.K., and Kunert, J., Biopersistence of synthetic mineral fibers as a predictor of chronic intraperitoneal injection tumor response in rats, *Inhalation Toxicol*., 13, 851–875, 2001b.

Bernstein, D.M. and Riego-Sintes, J.M.R., Methods for the determination of the hazardous properties for human health of man made mineral fibers (MMMF), Vol. EUR 18748 EN, April 93, European Commission Joint Research Centre, Institute for Health and Consumer Protection, Unit: Toxicology and Chemical Substances, European Chemicals Bureau, 1999. Available at http://ecb.ei.jrc.it/DOCUMENTS/Testing-Methods/mmmfweb.pdf.

Bernstein, D.M., Rogers, R., and Smith, P., The biopersistence of Canadian crysotile asbestos following inhalation, *Inhalation Toxicol*., 15, 1247–1274, 2003.

Bernstein, D.M., Rogers, R., and Smith, P., The biopersistence of Canadian chrysotile asbestos following inhalation: final results through 1 year after cessation of exposure, *Inhalation Toxicol*., 17, 1–14, 2005.

Bolton, R.E., Vincent, J.H., Jones, A.D., Addison, J., and Beckett, S.T., An overload hypothesis for pulmonary clearance of UICC amosite fibres inhaled by rats, *Br. J. Ind. Med*., 40, 264–272, 1983.

Browne, K. and Murray, R., Asbestos and the Romans, *Lancet*, 336, 445, 1990.

Churg, A., Deposition and clearance of chrysotile asbestos, *Ann. Occup. Hyg*., 38, 625–633, 424–425, 1994.

Churg, A. and DePaoli, L., Clearance of chrysotile asbestos from human lung, *Exp. Lung Res*., 14, 567–574, 1988.

Cossette, M. and Delvaux, P., Technical evaluation of chrysotile asbestos ore bodies, in *Short Course in Mineralogical Techniques of Asbestos Determination*, Ledoux, R.C., Ed., Mineralogical Association of Canada, 1979.

Davis, J.M., Addison, J., Bolton, R.E., Donaldson, K., Jones, A.D., and Miller, B.G., Inhalation studies on the effects of tremolite and brucite dust in rats, *Carcinogenesis*, 6, 667–674, 1985.

Davis, J.M., Addison, J., Bolton, R.E., Donaldson, K., Jones, A.D., and Smith, T., The pathogenicity of long versus short fibre samples of amosite asbestos administered to rats by inhalation and intraperitoneal injection, *Br. J. Exp. Pathol*., 67, 415–430, 1986.

Davis, J.M., Beckett, S.T., Bolton, R.E., Collings, P., and Middleton, A.P., Mass and number of fibres in the pathogenesis of asbestos-related lung disease in rats, *Br. J. Cancer*, 37, 673–688, 1978.

Davis, J.M. and Jones, A.D., Comparisons of the pathogenicity of long and short fibres of chrysotile asbestos in rats, *Br. J. Exp. Pathol*., 69, 717–737, 1988.

Eastes, W., and Hadley, J.G., A mathematical model of fiber carcinogenicity and fibrosis in inhalation and intraperitoneal experiments in rats, *Inhalation Toxicol*., 8, 323–342, 1996.

EPA (Environmental Protection Agency), Report on the Peer Consultation Workshop to discuss a proposed protocol to assess asbestos-related risk. Prepared for U.S. Environmental Protection Agency, Office of Solid Waste and Emergency Response, Washington, DC, EPA Contract 68-C-98-148, Work Assignment 2003–05 by Eastern Research Group, Inc., Lexington, MA, Final Report May 30, 2003.

Etherington, D.J., Pugh, D., and Silver, I.A., Collagen degradation in an experimental inflammatory lesion: studies on the role of the macrophage, *Acta Biol. Med. Ger.*, 40, 1625–1636, 1981.

Frank, A.L., Dodson, R.F., and Williams, M.G., Carcinogenic implications of the lack of tremolite in UICC reference chrysotile, *Am. J. Ind. Med*., 34, 314–317, 1998.

Gross, P., DeTreville, R.T., Tolker, E.B., Kaschak, M., and Babyak, M.A., Experimental asbestosis. The development of lung cancer in rats with pulmonary deposits of chrysotile asbestos dust, *Arch. Environ. Health*, 15, 343–355, 1967.

Hargreaves, A. and Taylor, W.H., An X-ray Examination of decomposition products of chrysotile (asbestos) and serpentine, *Mineral. Mag*., 27, 204–216, 1946.

Hesterberg, T.W., Axten, C., McConnell, E.E., Hart, G.A., Miiller, W., Chevalier, J., Everitt, J., Thevenaz, P., and Oberdorster, G., Studies on the inhalation toxicology of two fiberglasses and amosite asbestos in the syrian golden hamster. Part I. Results of a subchronic study and dose selection for a chronic study, *Inhalation Toxicol*., 11, 747–784, 1999.

Hesterberg, T.W., Chase, G., Axten, C., Miller, W.C., Musselman, R.P., Kamstrup, O., Hadley, J., Morscheidt, C., Bernstein, D.M., and Thevenaz, P., Biopersistence of synthetic vitreous fibers and amosite asbestos in the rat lung following inhalation, *Toxicol. Appl. Pharmacol*., 151, 262–275, 1998a.

Hesterberg, T.W., Hart, G.A., Chevalier, J., Miiller, W.C., Hamilton, R.D., Bauer, J., and Thevenaz, P., The importance of fiber biopersistence and lung dose in determining the chronic inhalation effects of X607, RCF1, and chrysotile asbestos in rats, *Toxicol. Appl. Pharmacol*., 153, 68–82, 1998b.

Hodgson, A.A., Chemistry and physics of asbestos, in *Asbestos: Properties, Applications and Hazards*, L.M. and Chissick, S.S., Eds., John Wiley & Sons, New York, 1979.

Hodgson, J.T. and Darnton, A., The quantitative risks of mesothelioma and lung cancer in relation to asbestos exposure, *Ann. Occup. Hyg*., 44, 565–601, 2000.

Howard, J.K., Relative cancer risks from exposure to different asbestos fibre types, *N. Z. Med. J*., 97, 646–649, 1984.

Ilgren, E. and Chatfield, E., Coalinga fibre—A short, amphibole-free chrysotile. Evidence for lack of fibrogenic activity, *Indoor Built Environ*., 6, 264–276, 1997.

Ilgren, E. and Chatfield, E., Coalinga Fibre—A short, amphibole-free chrysotile part 2: evidence for lack of tumourigenic activity, *Indoor Built Environ*., 7, 18–31, 1998.

Kamstrup, O., Ellehauge, A., Collier, C.G., and Davis, J.M., Carcinogenicity studies after intraperitoneal injection of two types of stone wool fibres in rats, *Ann. Occup. Hyg.*, 46, 135–142, 2002.

Kiyohara, P.K., Estudo da interface crisotila-cimento Portland em compósitos de fibro-cimento por métodos óptico-eletrônicos, Tese de Doutorado, apres. EPUSP, São Paulo, 1991.

Le Bouffant, L., Daniel, H., Henin, J.P., Martin, J.C., Normand, C., Tichoux, G., and Trolard, F., Experimental study on long-term effects of inhaled MMMF on the lungs of rats, *Ann. Occup. Hyg*., 31, 765–790, 1987.

Lee, K.P., Barras, C.E., Griffith, F.D., Waritz, R.S., and Lapin, C.A., Comparative pulmonary responses to inhaled inorganic fibers with asbestos and fiberglass, *Environ. Res*., 24, 167–191, 1981.

Luoto, K., Holopainen, M., Kangas, J., Kalliokoski, P., and Savolainen, K., The effect of fiber length on the dissolution by macrophages of rockwool and glasswool fibers, *Environ Res*., 70, 51–61, 1995.

Mast, R.W., Hesterberg, T.W., Glass, L.R., McConnell, E.E., Anderson, R., and Bernstein, D.M., Chronic inhalation and biopersistence of refractory ceramic fiber in rats and hamsters, *Environ. Health Perspect*., 102 (Suppl. 5), 207–209, 1994.

Mast, R.W., McConnell, E.E., Anderson, R., Chevalier, J., Kotin, P., Bernstein, D.M., Thevenaz, P., Glass, L.R., Miller, W.C., and Hesterburg, T.W., Studies on the chronic toxicity (inhalation) of four types of refractory ceramic fiber in male Fischer 344 rats, *Inhalation Toxicol*., 7, 425–467, 1995.

Mattson, S.M., Glass fibres in simulated lung fluid: dissolution behavior and analytical requirements, *Ann. Occup. Hyg*., 38, 857–877, 1994.

McConnell, E.E., Axten, C., Hesterberg, T.W., Chevalier, J., Miiller, W.C., Everitt, J., Oberdorster, G., Chase, G.R., Thevenaz, P., and Kotin, P., Studies on the inhalation toxicology of two fiberglasses and amosite asbestos in the Syrian golden hamster. Part II. Results of chronic exposure, *Inhalation Toxicol*., 11, 785–835, 1999.

McConnell, E.E., Kamstrup, O., Musselman, R., Hesterberg, T.W., Chevalier, J., Miiller, W.C., and Thievenaz, P., Chronic inhalation study of size-separated rock and slag wool insulation fibers in Fischer 344/N rats, *Inhalation Toxicol*., 6, 571–614, 1994.

McDonald, J.C., Mineral fibre persistence and carcinogenicity, *Ind. Health*, 36, 372–375, 1998.

McDonald, J.C., Harris, J., and Armstrong, B., Cohort mortality study of vermiculite miners exposed to fibrous tremolite: an update, *Ann. Occup. Hyg*., 46 (Suppl 1), 93–94, 2002.

McDonald, J.C., Harris, J., and Armstrong, B., Mortality in a cohort of vermiculite miners exposed to fibrous amphibole in Libby, Montana, *Occup. Environ. Med*., 61, 363–366, 2004.

McDonald, J.C. and McDonald, A.D., Chrysotile, tremolite, and mesothelioma, *Science*, 267, 776–777, 1995.

McDonald, J.C. and McDonald, A.D., Chrysotile, tremolite and carcinogenicity, *Ann. Occup. Hyg*., 41, 699–705, 1997.

McDonald, J.C., McDonald, A.D., and Hughes, J.M., Chrysotile, tremolite and fibrogenicity, *Ann. Occup. Hyg*., 43, 439–442, 1999.

Miller, B.G., Jones, A.D., Searl, A., Buchanan, D., Cullen, R.T., Soutar, C.A., Davis, J.M., and Donaldson, K., Influence of characteristics of inhaled fibres on development of tumours in the rat lung, *Ann. Occup. Hyg*., 43, 167–179, 1999.

Miller, F.J., Dosimetry of particles: critical factors having risk assessment implications, *Inhalation Toxicol*., 12 (Suppl. 3), 389–395, 2000.

Morgan, A., The removal of fibres of chrysotile asbestos from lung, *Ann. Occup. Hyg*., 38, 643–646, 1994.

Morimoto, Y., Yamato, H., Kido, M., Tanaka, I., Higashi, T., Fujino, A., and Yokosaki, Y., Effects of inhaled ceramic fibers on macrophage function of rat lungs, *Occup. Environ. Med*., 51, 62–67, 1994.

Morrow, P.E., Possible mechanisms to explain dust overloading of the lungs, *Fundam. Appl. Toxicol*., 10, 369–384, 1988.

Mossman, B.T., Bignon, J., Corn, M., Seaton, A., and Gee, J.B., Asbestos: scientific developments and implications for public policy, *Science*, 247, 294–301, 1990.

Muhle, H., Bellman, B., and Heinrich, U., Overloading of lung clearance during chronic exposure of experimental animals to particles, *Ann. Occup. Hyg*., 32 (Suppl. 1), 141–147, 1988.

Muhle, H., Pott, F., Bellmann, B., Takenaka, S., and Ziem, U., Inhalation and injection experiments in rats to test the carcinogenicity of MMMF, *Ann. Occup. Hyg*., 31, 755–764, 1987.

Nagy, B. and Bates, T.F., Stability of chrysotile asbestos, *Am. Mineral*., 37, 1055–1058, 1952.

Noll, W. and Kircher, H., Veranderungen von Chrysotilasbest, *Elektronenmikroscop. Naturw*., (Historical Archive), 39, 188, 1952.

Oberdörster, G., Deposition, elimination and effects of fibers in the respiratory tract of humans and animals, *Ver. Dtsch. Ing. Ber*., 17–37, 1991.

Oberdorster, G., Lung particle overload: implications for occupational exposures to particles, *Regul. Toxicol. Pharmacol*., 21, 123–135, 1995.

Oberdorster, G., Determinants of the pathogenicity of man-made vitreous fibers (MMVF), *Int. Arch. Occup. Environ. Health*, 73 (Suppl.), S60–S68, 2000.

Olin, S., Testing of fibrous particles: short-term assays and strategies, Report of an ILSI Risk Science Institute Working Group, *Inhalation Toxicol*., 17, 497–537, 2005.

Pauling, L., The structure of chlorites, *Proc. Natl. Acad. Sci. USA*, 16, 578–582, 1930.

Pinkerton, K.E., Brody, A.R., McLaurin, D.A., Adkins, B., Jr., O'Connor, R.W., Pratt, P.C., and Crapo, J.D., Characterization of three types of chrysotile asbestos after aerosolization, *Environ. Res*., 31, 32–53, 1983.

Rodelsperger, K., Woitowitz, H.J., Bruckel, B., Arhelger, R., Pohlabeln, H., and Jockel, K.H., Dose-response relationship between amphibole fiber lung burden and mesothelioma, *Cancer Detect. Pre*v., 23, 183–193, 1999.

Roggli, V.L., Vollmer, R.T., Butnor, K.J., and Sporn, T.A., Tremolite and mesothelioma, *Ann. Occup. Hyg*., 46, 447–453, 2002.

Ross, M., and Nolan, R.P., History of asbestos discovery and use and asbestos-related disease in context with the occurrence of asbestos within ophiolite complexes, in *Ophiolite Concept and the Evolution of Geological Thought*, Dilek, Y. and Newcomb, S., Eds., Geological Society of America, Boulder, CO, 2003.

Skinner, H.C.W., Ross, M., and Frondel, C., Eds., *Asbestos and Other Fibrous Minerals*, Oxford University Press, Oxford, pp. 30–31.

Smith, D.M., Ortiz, L.W., Archuleta, R.F., and Johnson, N.F., Long-term health effects in hamsters and rats exposed chronically to man-made vitreous fibres, *Ann. Occup. Hyg*., 31, 731–754, 1987.

Speil, S. and Leineweber, J.P., Asbestos minerals in modern technology, *Environ. Res.*, 2, 166–208, 1969.

Stoeber, W., Flachsbart, H., and Hochrainer, D., Der Aerodynamische Durchmesser von Latexaggregaten und Asbestfassern, *Staub-Reinh. Luft*, 30, 277–285, 1970.

Wagner, J.C., Berry, G., Skidmore, J.W., and Pooley, F.D., The comparative effects of three chrysotiles by injection and inhalation in rats, *IARC Sci. Publ*., 30, 363–372, 1980.

Wagner, J.C., Berry, G., Skidmore, J.W., and Timbrell, V., The effects of the inhalation of asbestos in rats, *Br. J. Cancer*, 29, 252–269, 1974.

Whittaker, E.J.W., Mineralogy, chemistry and crystallography of amphibole asbestos, in *Short Course in Mineralogical Techniques of Asbestos Determination*, Ledoux, R.C., Ed., Mineralogical Association of Canada, Toronto, 1979.

Wicks, F.J., Mineralogy, chemistry and crystallography of chrysotile asbestos, in *Short Course in Mineralogical Techniques of Asbestos Determination*, Ledoux, R.C., Mineralogical Association of Canada, Toronto, 1979.

28 The Inhalation Toxicity of Benzene

Robert Snyder and Marcel Van Raaij

CONTENTS

28.1 INTRODUCTION

Benzene, which was discovered early in the nineteenth century, came into heavy use in Europe around 1870–1880 as a solvent for rubber used to make tires for bicycles and automobiles. The process involved dissolving rubber latex in benzene, pouring the solution into molds, and allowing the benzene to evaporate, thereby leaving the formed tire. The workers recognized the smell of benzene, which at low concentrations was aromatic and not necessarily unpleasant, without realizing the inherent dangers in exposure to the solvent.

Early reports of acute benzene toxicity were, in general, poorly documented with respect to the air concentrations of benzene that resulted in central nervous system (CNS) depression. There was better documentation of exposures leading to chronic toxicity. The first report describing the results of chronic industrial exposure to benzene was published by Santesson in 1897. However, little benzene was produced in this country and exposure to benzene was not significant in the United States during that time. Benzene was heavily used in Germany and to a lesser extent in Britain (Hamilton, 1922). Benzene required for specific industrial purposes was imported from Germany and was quite expensive. Therefore, petroleum-based solvents were used in the United States in preference to benzene. With the advent of World War I the picture changed dramatically. Benzene, along with toluene, which was required for the manufacture of munitions, was no longer available from Germany and large-scale production became a requirement.

Several reports appeared in the early literature that documented measurements of benzene in the air of factories where benzene was used as a solvent. Greenburg (1926a, 1926b) described a survey conducted by the U.S. Public Health Service that focused initially on 78 facilities of which ultimately 18 rooms in 12 plants were studied. The data in Table 28.1, selected from Greenburg (1926b), show examples of the amount of benzene used for various operations in each of several rooms per week. The air levels of benzene were estimated by a method in which room air was passed through

TABLE 28.1 Industrial Benzene Exposure and Abnormal Blood Findings in Early Twentieth Century Industry

Process	Gallons of Benzene Used per Week	Average Air Concentration of Benzene, ppm	No. of Workers Exposed	No. of Workers with Abnormal Blood Observations
Painting	4,200	340	6	2
Insulating Wire	300	210	12	6
Coating	4,200	2.640	1	1
Compound mixing	4,200	410	10	1
Cement mixing	10,000	860	9	6
Dry cleaning	1,800	4,140	—	—
Cementing	50	120	9	2

Source: Data taken from Greenburg (1926b).

charcoal-containing tubes at a defined rate and time. The vapors in the air were trapped on charcoal, and the trapped vapor was determined by weighing the charcoal tubes. In retrospect we can question the accuracy of the gravimetric technique. Furthermore, in some of the rooms other chemicals were used with benzene and would be trapped by the charcoal. Nevertheless, the table provides a general picture of the levels of exposure to benzene in several factories in the early twentieth century. Clearly, large amounts of benzene were used. Individual area concentrations ranged from zero to as high as 4140 ppm with mean values estimated at 70–1800 ppm. Of 81 workers studied 26 (32%) displayed evidence of decreases in circulating blood cells (a characteristic of benzene toxicity which will be covered when chronic benzene toxicity is discussed.

A readily available source of benzene and toluene was identified in the process by which coal was converted to coke. The production of steel involves the solution of pure carbon in molten iron. Coke is produced by heating coal to high temperatures in the absence of oxygen in an enclosed space called a coke oven. During the heating many components of coal other than carbon are either vaporized or melted. Molten chemicals derived from coal are collected at the bottom of the coke oven and processed to isolate many chemicals, including aniline and its derivatives, which were the basis of extensive industrial uses, such as the synthesis of dyes. The more volatile compounds, including benzene and the alkylbenzenes, were recovered from the emitted vapors in relatively pure form. With benzene then available in considerable quantities at more reasonable prices its use increased significantly in the United States, as did the incidence of benzene toxicity.

In this chapter we will examine the effects of acute exposure to high concentrations of benzene, which are expressed as CNS depression, and chronic exposure, which may result in depressed bone marrow function leading to aplastic anemia or myelodysplasia, a precursor to some forms of leukemia. We will also discuss some thoughts on the mechanism(s) by which benzene causes its adverse effects. Mechanistic studies should clarify the impact of benzene on fundamental cellular processes and should help in establishing acceptable limits to benzene exposure in various environments.

Agencies are often required to set acceptable exposure levels for benzene in various situations in the absence of a complete understanding of mechanism. For example, Table 28.2 (Agency for Toxic Substances and Disease Registry [ATSDR], 1995) shows threshold limit value (TLV)–time-weighted average (TWA) exposure for benzene for an 8-h work day and permissible short-term exposure limits (STEL) (15 min) established by the American Conference of Governmental Industrial Hygienists (ACGIH) and by the Occupational Health and Safety Administration (OSHA) of the U.S. Department of Labor. Note that the ACGIH and OSHA differ by a factor of 2. The Spacecraft Maximum Allowable Concentrations for Selected Airborne Contaminants (SMAC Committee) of the National

TABLE 28.2 TLV-TWA and SMAC Values for Benzene

Agency	TLV-TWA (8 his) ppm	STEL (short-term exposure limit) (15 min) ppm	Maximum Peak (10 min) ppm	SMAC ppm	Time
ACGIH	0.5	2.5	—	—	—
OSHA (For industry segments covered 29 CFR 1910 1028)	1	5	—	—	—
OSHA (For industry segments not covered 29 CFR 1910, 1028)	—	25	50	—	—
NASA	—	—	—	10	1 h
	—	—	—	3	24 h
	—	—	—	0.5	7 d
	—	—	—	0.1	30 d
	—	—	—	0.02	180 d

Research Council (NRC) designed exposure limits based on benzene's bone marrow toxicity and effects on the immune system, recognizing that exposure may extend to days, weeks, and months under conditions of microgravity. The SMAC values published by the NRC range from 10 ppm/1 h to 0.02 ppm/180 d. Thus, it is clear that the mode of exposure, the duration of exposure, and the atmosphere in which exposure occurs play key roles in determining acceptable exposure levels. Note the significant contrast in exposures to benzene between Tables 28.1 and 28.2. Here we will review key features of the toxicology of benzene that have been used to establish these and other acceptable exposure standards.

28.2 ACUTE TOXICITY OF BENZENE

28.2.1 Lethality

Because of the pattern of benzene production most of the early cases of acute benzene poisoning were reported in Germany. Greenburg (1926a) described a report by Dr. J. Rambousek at the 1910 International Congress of Industrial Hygiene, in which 22 cases of acute benzene poisoning due to inhalation were discussed. Many similar reports described acute lethality due to high concentrations of benzene. In some cases the deaths were thought to have been caused by asphyxiation. When workers entered tanks in which the air was virtually saturated with benzene vapor there may have been insufficient oxygen to support respiration. In other cases air may have been blown through the vessel to reduce the level of benzene vapor, but the residual air contained enough benzene to overcome the workers by virtue of its CNS-depressant activity.

It has been suggested (Bass, 1970; Reinhardt et al., 1971; Litovitz, 1988) that benzene may lead to fatal cardiac arrhythmias in workers exerting vigorous activity, such as attempting to remove a fallen comrade during a rescue operation, by sensitizing the myocardium to catecholamines. The mechanism is similar to the mechanism thought to cause cardiac arrhythmias by a number of halocarbons used as anesthetics. However, the data are insufficient to permit the conclusion that induction of cardiac arrhythmias by benzene is a demonstrated mechanism of acute benzene toxicity.

Hamilton (1922) described some examples of benzene-induced lethality in Germany in 1912. One man using a benzene-based paint was overcome by the vapors while painting the inside of a barrel. Another worked in a dry-cleaning establishment where benzene was used as the solvent. He climbed

into a washing machine and was overcome by vapors of residual benzene. A third was in charge of a distilling operation in which benzene was being collected. He succumbed when he neglected to turn on the cold water that fed the condenser, which resulted in accumulation of high levels of benzene vapors in his breathing zone. These were events in which a single individual was exposed because of a combination of lack of understanding of the dangers of benzene and lack of safety procedures in industry; they are examples of the many reports of acute benzene toxicity published in the early years of the twentieth century.

Flury (1928) estimated that in people exposure to benzene vapor at a concentration of 20,000 ppm for 5–10 min would be fatal. Exposure to 7500 ppm for 30–60 min would result in the toxicity described by Hunter (1962) as excitement, incoherent speech, flushed face, headache, giddiness, nervousness, insomnia, nausea, paresthesia of the hands and feet, and fatigue. Over the same period 3000 ppm was considered to be without immediate effects.

Lethality studies have been reported in rabbits (Carpenter et al., 1944), rats (Carpenter et al., 1944; Smyth et al., 1962; Drew and Fouts, 1974; Bonnet et al., 1982), and mice (Lazarew, 1929; Svirbely et al., 1943; Bonnet et al., 1982) (Table 28.3). Data sufficient to calculate LC_{50} values were reported by Drew and Fouts (1974) and Bonnet et al. (1982) in rats and by Lazarew (1929) and Bonnet et al. (1982) in mice. The exposure concentrations ranged from 4980 to 15,000 ppm and the exposure times from 4 to 7 h. Although performed in three different laboratories over a span of 30 years the resulting LC_{50} values, i.e., 13,700 and 9536 ppm in rats and 14,122 and 9980 ppm in mice, are reasonably consistent. (See Table 28.3.)

28.2.2 Acute Nonlethal Effects

Several types of nonlethal effects of benzene have been reported. Acute benzene exposure is able to induce various toxicologically important effects such as CNS depression, irritation of the eyes and airways, effects on the hematopoietic system, and genotoxic events. The most prominent effect of acute benzene exposure is CNS depression, a property shared by many other solvents. Although these effects have been recognized for over 100 years, detailed exposure–response data are limited. For example, in contrast to the rich database for toluene, no neurobehavioral studies in human volunteers exist with benzene. Gerarde (1960) estimated that 50–150 ppm for 5 h results in headache, lassitude, and weariness whereas 500 ppm for 1 h induces symptoms of illness. Fishbein (1984) suggested that CNS effects would be initially seen at exposures above 250 ppm. In a case study, one of four male workers showed major CNS effects after working in a confined space for five intermittent periods of 2–3 h spread over 2 d. Area levels up to 600–1500 ppm were measured in a simulation experiment (Drozd and Bockowski, 1967). Workers exposed to benzene vapors in fuel tanks for 1 d to 3 weeks, where their exposure averaged 60 ppm but may have reached a maximum of 653 ppm, reported dizziness/light headedness, nausea, headache, and drowsiness (Midzenski et al., 1992). CNS-related effects were also reported in occupational studies (Kellerova, 1985; Yin et al., 1987; Kraut et al., 1988; Greenburg, 1939), although in most of these occupational studies repeated benzene exposure

TABLE 28.3 LC_{50} Values for Benzene in Rats and Mice

Species	Range of Exposures ppm	Exposure Duration	LC50 ppm	Reference
Rat	11,500–5,500	4	13,700	Drew and Fouts (1974)
Rat	7,000–15,000	6	9,536	Bonner et al. (1982)
Mouse	7,000–15,000	6	14,122	Bonner et al. (1982)
Mouse	4,980–14,600	7	9,980	Svirbely et al. (1943)

may have resulted in accumulation in fat-rich tissues while coexposure to other solvents cannot be excluded.

Animal data related to CNS effects involve two categories: information on narcotic effects (e.g., Engelhardt and Estler, 1935; Carpenter et al., 1944) and neurobehavioral changes. Neurobehavioral changes follow a specific pattern for this type of solvent: at subnarcotic levels hyperreactivity and increased locomotor behavior is observed (similar to stage II anesthesia observed in humans) and at higher levels decreased activity and lethargy may result (e.g., Molnar et al., 1986; Evans et al., 1981). Changes in neurological responses (e.g., hind limb grip strength) were reported by Dempster et al. (1984) and Frantik et al. (1994).

Von Oettingen (1940) described a study by Lehman (1910) on the narcotic action of benzene in cats. The data permit an examination of "Haber's rule" with respect to the inhalation toxicology of benzene. Haber had postulated that for a number of war gases $C \times T = K$, i.e., the concentration of the gas multiplied by the time over which the gas was administered yields a constant value. For example, if the concentration was 100 ppm and gas was administered for 10 min the constant would be 1,000 ppm·min. If the concentration were reduced to 50 ppm and the time of administration increased to 20 min the product would again be 1000 ppm·min. Although the rule was intended to describe a limited number of gases the concept has been extrapolated in toxicology not only to other gases, but also to all forms of chemicals, given at a variety of routes and for longer periods than envisioned by Haber. Nevertheless, for short-term exposures to gases, ten Berge et al. (1986) proposed that the rule should be modified to

$$C^n \times T = K.$$

Lehman (1910) exposed animals at various exposure times (3 min to 7 h) and exposure concentrations (10–170 mg/l, equivalent to 3080–52,360 ppm) were performed to determine a time–concentration–response pattern for light narcosis and deep narcosis. From this experiment it was shown that for slight CNS depression, the exposure concentration is more important than exposure duration because the $C \times T$ product becomes greater with increasing duration of exposure. When the data are fitted to $C^n \times T = K$, the time–concentration–response curve for slight CNS depression yields an n-value of 2.2, light narcosis yields a n-value of 1.08, whereas the curve for deep narcosis yields an n-value of 0.93. Together, these results in cats indicate that the CNS-depressing effects of benzene within a time frame of 3 min to 7 h and concentrations up about 50,000 ppm can be described by using $n = 1$.

The CNS effects of benzene are normally rapidly reversible and persistent structural damage is not likely to occur. Benzene is less or about equipotent to toluene, xylenes, and other alkylbenzenes to induce CNS effects in animals (Molnar et al., 1986; Tegeris and Balster, 1994; Frantik et al., 1994). High concentrations of benzene are irritating to mucous membranes of the eyes, nose, and respiratory tract (Wolf et al., 1956; Gerarde, 1960). Skin burns and blistering may occur after acute exposure to vapors with high levels of benzene (Avis and Hutton, 1993). Lehman (1910) reported that exposure of three volunteers to about 3400–4900 ppm for 5–15 min caused airway irritation. Volunteers ($n = 23$) exposed to levels up to 110 ppm for 2 h reported no subjective symptoms (Srbova et al., 1950). In several occupational studies involving benzene exposure, irritative effects of the upper airways were reported (Yin et al., 1987; Kraut et al., 1988; Midzenski et al., 1992), but exposure levels in these cases were not known with certainty and coexposure to other organic substances could not be ruled out. Von Oettingen (1940) reported corneal damage in rabbits exposed to about 12,000 ppm for 1 h/d for several days. Grayish white turbidity of the cornea was apparent after 6–8 d but first changes were noted after 25 min of exposure. Furthermore, exposure of rats to 1000–2440 ppm (duration not provided) resulted in irritation of the mucous membranes.

Repeated benzene exposure causes bone marrow toxicity (see elsewhere in this chapter). However, a single inhalation exposure to benzene may induce effects on some circulating cells (Uyeki et al., 1977; Dempster et al. 1984; Cronkite et al., 1989). It is important to separate effects on circulating cells, progenitor cells, and the pluripotent stem cells. In quantitative terms the dose–effect relationship of

acute benzene exposure and hematotoxic effects is poorly described and no data are available to show what level of benzene is able to affect the pluripotent stem cells in a single dose.

Benzene is a genotoxic, clastogenic agent that induces chromosome aberrations, sister chromatid exchanges, and micronuclei (Zhang et al., 2002). In various animal studies, inhalation of benzene for short periods (30 min to 6 h) induced clastogenic effects (Ranaldi et al., 1998; Tice et al., 1980; Erexson et al., 1986; Styles and Richardson, 1984) at levels starting from about 10 ppm. The importance of such acutely induced genotoxic effects for the carcinogenic risk assessment is not yet clear. It appears that some of these effects are readily reversible (Cirranni et al., 1991; Fuije et al., 1992).

28.3 CHRONIC TOXICITY OF BENZENE

Unlike acute benzene toxicity, which is manifested as depression of the central nervous system, chronic benzene toxicity is a disease of the bone marrow. The literature is replete with examples of chronic benzene toxicity ranging from individual case studies to large-scale epidemiological examination of exposed cohorts. A limited sampling of some of those that have had a major impact on the study of benzene toxicity and/or on the regulation of benzene in the workplace will be presented here. Chronic exposure to benzene results in decreases in numbers of circulating blood cells as a result of damage to the bone marrow. Hemopathies have been described in many reports. Table 28.4 shows the results of blood studies on Finnish workers (Savilahti, 1956) who had been chronically exposed to benzene at 400 ppm. Of 107 workers 31 displayed decreases in red blood cells (anemia), white blood cells (leukopenia), and platelets (thrombocytopenia). When all three blood cell types are decreased the result is termed pancytopenia and is usually the result of bone marrow aplasia, i.e., a replacement of normal bone marrow with fat and scar tissue, and a nonfunctioning hematopoietic system. Other workers showed leukopenia and thrombocytopenia but not anemia. The table shows that any combination of these effects may be seen. Clearly, the more cell types that are affected, the more serious the disease. It is likely that the severity of the hemopathy is related to the dose and the time of exposure. Furthermore, the data suggest that removal from exposure results in some degree in the reversal from the effects of exposure. Reexposure can intensify the effects again. Furthermore, the bone marrow damage shown here may lead, after some latency period, which may extend from 5 to 20 years, to some forms of leukemia (see below).

The first report on the hematotoxic effects of benzene was presented at the Twelfth International Medical Congress in Moscow by Santessen (1897). He described nine cases of purpura hemorrhagica in young women who used a benzene-based cement in their work. The outstanding feature that he observed both in the four women who died and the five survivors was evidence of purpura, i.e., bleeding under the skin and in the internal organs, associated with inhibited blood clotting. He also reported decreases in circulating erythrocytes and leukocytes. He was unsuccessful in reproducing these effects in animals injected with benzene because the high doses he used killed the animals. However, he was the first to specifically claim that exposure to benzene in the workplace resulted in hematological abnormalities, referring to benzene as ". . . das wesentlich toxische Princip . . . " (i.e., the intrinsic toxic principle).

Selling (1910) of Johns Hopkins University reported that 14 girls between the ages of 14 and 16 worked in a room where benzene-based rubber cement was applied to seal cans and allowed to dry by the release of benzene into the air. Approximately 10 gallons of benzene evaporated into the air of the room each day. Three of the girls were admitted to Johns Hopkins Hospital displaying various degrees of purpura. Two died and were shown to have aplastic bone marrows. Blood studies in the fatal cases demonstrated severe depression of the numbers of circulating erythrocytes and leukocytes and dramatically impaired blood clotting. Selling (1916) went on to treat rabbits with benzene by injection and by using repeated doses, smaller than those employed by Santessen, and succeeded in reproducing the disease.

H.G. Weiskotten was a pathologist at Syracuse University Medical School who examined benzene toxicity in rabbits, in most instances injecting the benzene in oil subcutaneously. He followed

TABLE 28.4 Effect of Chronic Benzene Exposure (400 ppm) in Finnish Workers at Initial Examination (0) and at 1-, 3-, 6-, 9, and 12-Month Follow-Up Studies[a]

Hemopathy	0	1	3	6	9	12
Anemia, leukopnia, thrombocytopenia (i,e, pancytotenia)	31	30	22	15	13	11
lekopenia thrombocytopenia	8	6	3	2	2	1
Anemia thrombocytopenia	11	7	5	3[a]	3	2
Anemia leukopnia	1	1	1	1	1	1
Anemia	8	—	—	3	3	3
Leukopenia thrombocytopenia	7	—	—	2	2	1
Thrombocytopenia	41	17	2	3	2	2
Total	107	61	33	29	26	21

[a] One worker died.

Source: Data taken from Savilahti (1956).

decreases in erythrocytes, the various forms of leukocytes and thrombocytes during repeated daily administration of benzene (1320 or 1760 mg/kg) (Weiskotten et al., 1915–16, 1916–17; Weiskotten and Steensland, 1918–19; Brewer and Weiskotten, 1916; Weiskotten and Steensland, 1917; Weiskotten et al., 1924). Weiskotten et al. (1920) performed the first study of benzene toxicity in rabbits in which benzene vapor was administered by inhalation. Although the concentration of benzene was not reported the paper contains a detailed description of the inhalation apparatus, the amount of benzene used, and the flow rate. By using these data it was possible to demonstrate that the concentration of benzene in the air of the chamber was approximately 240 ppm (Snyder and Kocsis, 1976). Among a group of five animals in which mean total leukocytes equaled 11,892/μl prior to exposure, subsequent exposure for 10 h/d, for a mean time of 6.4 d, resulted in a mean decrease to 1768 μl. Two animals died but the remainder demonstrated total leukocyte levels of 6166/μl one year later, suggesting significant but incomplete recovery.

During the ensuing half-century numerous reports appeared demonstrating that exposure of workers to benzene vapor resulted in progressive decreases in erythrocytes, the various forms of leukocytes, and platelets. In its most severe form, aplastic anemia, the marrow ceased to produce mature circulating cells. One of the best studied cohorts in the pre-World War II era was New York City Rotogravure printers (Greenburg et al., 1939). Benzene was used to dissolve the inks used to print colored newspapers in high-speed printing presses because of its excellent solvent properties and the rapid rate at which it evaporated, leaving a dry newspaper. The evaporated benzene created a benzene-laden atmosphere in the printing plant and all the symptoms of benzene toxicity could be observed among the workers. The concentration of benzene in the air varied upon the location and the operation in which the workers were involved. Greenburg estimated that exposure ranged between 11 and 1060 ppm. His study group included 81 controls and 235 exposed printers. He examined a number of hematological end points, of which two will be cited here. Assuming that mild anemia can be defined as having fewer than 4.49 million red blood cells/μl of blood, 2.4% of the controls displayed mild anemia, whereas 48% of the benzene-exposed workers ranged from mildly to

severely anemic. Assuming that total leukocyte levels below 5000/μl define mild leukopenia, among the controls 8.6% were deficient in white blood cells, whereas among the benzene-exposed workers 31% displayed mild to severe leukopenia.

Before 1955 rubber cement used in shoe making in Turkey was prepared by dissolving rubber in petroleum-based solvents. Benzene replaced petroleum solvents because it was cheaper. Subsequently numerous cases of aplastic anemia were seen in the hematology departments at the University of Istanbul and Cerrahpasa Medical schools (Aksoy and Erdem, 1978). M. Aksoy, Director of Hematology, initiated a series of studies among the 28,500 shoe workers in the Istanbul area between 1967 and 1975 and his work was summarized in his book, *Benzene Carcinogenicity* (1988). In a study of 217 workers (Aksoy et al., 1971) engaged in shoe manufacturing using a benzene-based cement, in which exposure was estimated to range from 30 to 210 ppm, 23% displayed hematological abnormalities indicative of benzene poisoning, i.e., leukopenia, thrombocytopenia, leukopenia and thrombocytopenia in the same individuals, pancytopenia (decreases in all three circulating blood cell types), etc. He reported on 32 patients who had been exposed to benzene at 150–650 ppm for 4 months to 15 years and were found to be pancytopenic. The most common feature observed in these patients was arrested maturation of bone marrow cells. Thus, bone marrow samples demonstrated excessive immature cells in the myeloid and erythroid series. The picture of the bone marrow underlying pancytopenia ranged from complete aplasia to hyperplasia.

Yin et al. (1987) evaluated benzene toxicity among 508,818 workers in 19,969 factories in China. The airborne concentration of benzene in 50,255 workplaces within these factories was found to be less than 40 mg/m^3 (12.5 ppm). Overall the geometric mean concentration was 18.1 mg/m^3 with a range of 0.06–844.74 mg/m^3. In 1.3% of the workplaces benzene concentrations exceeded 1000 mg/m^3 (312.5 ppm). The overall prevalence of benzene poisoning, defined as leukopenia, was given as 0.5%, but industry-specific values included 0.54% in the painting industry and 1.25% in the shoemaking industry. In all, 24 cases of aplastic anemia were observed, primarily where benzene concentrations exceeded 93–1156 mg/m^3 (29–361 ppm).

28.4 HEMATOTOXICITY AND BONE MARROW FUNCTION

Both functional and mechanistic studies of benzene-induced hemopathies require a basic understanding of bone marrow structure and function. The term bone marrow refers to the hollow space within all bones where cells destined for the circulation can be made. In the neonatal period when most of the marrow is engaged in blood cell production, the marrow has a red appearance because the majority of developing cells belong to the erythroid line. With age less of the marrow is normally required to satisfy the body's need for blood cells and, except in certain disease states, the requirement can be met by the red marrow of the vertebrae, ribs, sternum, and pelvis, and to some degree the ends of the long bones. Thus, much of marrow contains fat and has a white to yellow appearance. Microscopic evaluation of both circulating blood and bone marrow has been used from the earliest studies to evaluate the effects of benzene. With the evolution of the field of cell biology newer techniques have been employed in studying benzene toxicity, some of which will be discussed here.

The idea that circulating blood cells arise from a common precursor in the marrow called the pluripotential stem cell, plus the concept that damage to the hematopoietic environment, or stroma, of the marrow could impair bone marrow function, helped to redefine the field of hematology and also permitted people studying benzene toxicity to evaluate loci within the marrow which might be targets at which benzene toxicity might be initiated. Thus, microscopic evaluation of circulating cells in the blood or in samples of bone marrow have been supplemented with (1) studies of bone marrow cell colony-forming units, and (2) advances in immunology have led to the identification of cells at various stages of differentiation leading to mature circulating cells on the basis of antibody responses to specific sites on cell membranes.

We now know that the process by which circulating blood cells develop is initiated when a pluripotential stem cell undergoes cell division to yield an identical stem cell and a cell that is now

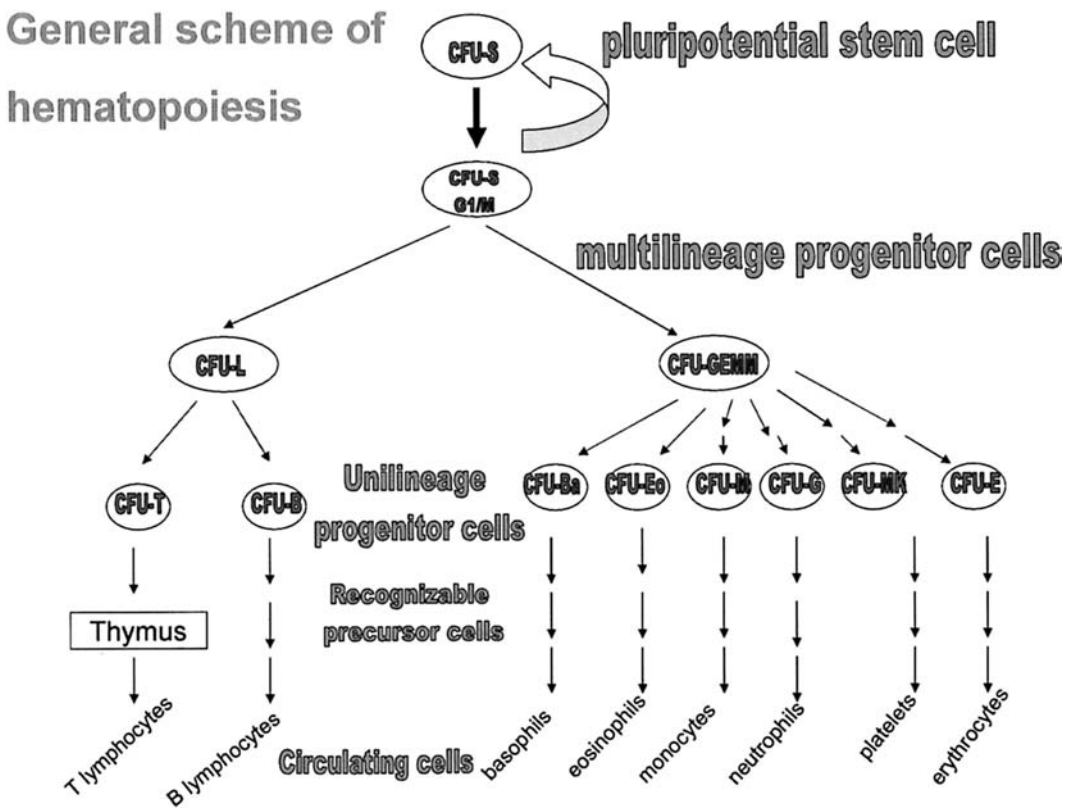


FIGURE 28.1 Scheme for differentiation of bone marrow cells from pluripotential stem cells to mature, circulating blood cells. Nomenclature is based on studies using the "colony forming unit" technique.

receptive to cytokines which drive differentiation through stages described as multilineage progenitor cells, unilineage progenitor cells, indentifiable precursor cells, and ultimately, maturation to circulating cells. Figure 28.1 shows a commonly accepted scheme for these events. The process involves differentiation and maturation to cells, which, in theory, should be identifiable either morphologically or via the use of appropriate biological markers. However, the other physiological function associated with the marrow is amplification. From a single stem cell it is necessary to derive a specified number of mature circulating cells, a task that is accomplished by mitosis of cells at various stages of development. As cells undergo morphological and physiological changes aimed at eventually defining specific cell types at the most mature stage, they also undergo mitoses that serve to increase their numbers. Each mitotic event, beyond that of the pluripotential stem cell, can yield two daughter cells. Thus, the amplification of cell numbers reflects a geometric progression controlled by the number of mitotic events at each stage. For example, among the erythroid cells there must be a sufficient number of mitotic events to provide 5 million mature red blood cells per microliter of blood; leukocytes of various types are present in much smaller numbers, but they have a shorter lifetime in the circulation. A great many mitotic events are required in each pathway from primitive to mature cells. Therefore, a bone marrow cell sample will have predominantly more differentiated and mature cells than early precursor cells, which serves to complicate the identification of earlier cells. The technological solution to the problem involved the development of a system in which each cell type serves as the original cell in a colony-forming unit (CFU). Whereas it may be difficult to observe a primitive unilineage red blood cell precursor, we can grow colonies of cells, which we call CFU-E, each of which derives from one erythroid cell and thereby permits us to determine the number of these

specific erythrocyte precursors in each preparation. By the same token colonies of each of the cell types indicated in Figure 28.1 can be grown and will indicate the number of those cells in any given bone marrow sample.

CFU-S was first demonstrated by Till and McCulloch (1961), who irradiated mice to render the bone marrow nonfunctional. Upon injecting bone marrow cells from a normal mouse into the irradiated mouse they found that the spleen of the recipient mouse could give rise to colonies of either erythroid or myeloid cells. The cells that gave rise to the spleen colonies are considered to be pluripotential stems cells and because they were first identified using the spleen colony-forming assay they are called CFU-S (colony-forming unit-spleen). The significance of this approach to the study of benzene-induced bone marrow damage was emphasized by Uyeki et al. (1977) and Gill et al. (1980), who reported on the reduction of CFU-S in bone marrow of mice exposed on several occasions to benzene at 4000–5000 ppm. Green et al. (1981), Harigaya et al. (1981), and Cronkite et al. (1982) also observed decreases in pluripotential stem cells during chronic benzene exposure, albeit at much lower benzene doses, i.e., 100–400 ppm. Sensitivity to benzene exposure has been observed in early erythroid cells, e.g., CFU-E, BFU-E, and in early myeloid cells, e.g., CFU-C, CFU-GM (Hildebrand and Murphy, 1983; Seidel et al., 1989). Indeed the addition to the hydroquinone, catechol, 1,2,4-benzenetriol, or muconaldehyde at appropriate doses may be toxic to each of the CFUs indicated in Figure 28.1.

The processes described in Figure 28.1 relate to those cells that undergo differentiation, maturation, and amplification in the bone marrow. However, these events cannot proceed in the absence of a viable hematopoietic microenvironment, otherwise termed the stroma. Weiss (1976) described the hematopoietic microenvironment as ". . . branching vascular sinuses lying in a fibroblastic stroma which supports hematopoiesis." Reticuloendothelial cells and monocytes/macrophages, fibroblasts, and fat cells, plus key structural features, i.e., the bone itself and the sinusoids, constitute the hematopoietic microenvironment. Dexter and his coworkers (1977, 1979) developed an *in vitro* system in which stroma was grown as an adherent layer of cells and served to support the growth of stem cells.

Thus, Garnett et al. (1983) demonstrated that bone marrow stromal cultures from benzene-treated mice were structurally altered and failed to support the growth and differentiation of stem cells from untreated mice. Longacre et al. (1981a) reported that DBA/2 mice were more susceptible to benzene toxicity than C57Bl/6 mice. Gaido and Wierda (1985) suggested that the differential sensitivity was based on effects of benzene metabolites on stromal cells. Addition of hydroquinone or *p*-benzoquinone to stromal cell cultures reduced their ability to support stem cell development (Gaido and Wierda, 1984). Evidence supporting the requirement for the oxidation of hydroquinone to *p*-benzoquinone in stromal cells for the production of benzene toxicity came from studies showing that the inhibition of the peroxidatic activity of prostaglandin synthase reduced hydroquinone oxidation and was protective of stromal cell function (Gaido and Wierda, 1987; Schlosser et al., 1990; Renz and Kalf, 1991). Ross et al. (1990) proposed that the " . . . activation and deactivation of quinones . . . " by DT-diaphorase (NQO1) in bone marrow stroma modulated the toxicity of hydroquinone in stromal cells. Thomas et al. (1990) narrowed the range of specific targets for hydroquinone within the stroma by demonstrating that the stromal macrophage was more sensitive to the toxic effects of hydroquinone than the fibroblastoid stromal cells. They suggested that the basis of the differential sensitivity was that the fibroblastoid cells were 4-fold higher in DT-diaphorase than the macrophages. The accumulated evidence suggests that damage to the stroma may be a significant cause of bone marrow depression and aplastic anemia.

28.5 IMMUNOTOXICITY

Many components of the immune system are represented in the contents of circulating blood. As a result it is expected that bone marrow depression would lead to impairment of the immune system. The increased susceptibility to infection as a result of bone marrow depression is of major significance

and may well be the leading cause of death related to chronic exposure to benzene (e.g., Young and Maciejewski, 2000; Greenberg, 2000).

Immunology as a separate discipline began in the period immediately preceding World War I and several early immunologists, in response to the reports of Selling, examined the impact of benzene on immunocompetence. Hirschfelder and Winternitz (1913) used a rabbit model for lobar pneumonia, found that treatment with benzene at levels which Selling found to produce bone marrow depression caused a marked reduction in the capacity to survive the infection. White and Gammon (1914) proposed that benzene-treated rabbits were more susceptible to tuberculosis than controls. Camp and Baumgartner (1915) in an early study on the inflammatory reaction used three test systems in rabbits made leukopenic by benzene treatment, i.e., rubbing croton oil into a needle scratch on the ear of a rabbit; application of heat to the rabbit ear; intramuscular injection of granular "carmine." Each of these normally produce signs of inflammation including infiltration of polymorphonuclear leukocytes. In benzene-treated animals there was no leukocyte infiltration and large numbers of bacteria were found at the site of injury. Simonds and Jones (1915) and Hektoen (1916) noted that blood from benzene-treated animals demonstrated reductions in red blood cell lysis, bacterial agglutinins, opsonin activity, and phagocytosis. More recently it was demonstrated that among the functions inhibited following benzene exposure is impairment of the immune response to *Listeria monocytogenes* (Rosenthal and Snyder, 1985) .

There has been general but not unanimous agreement in the literature that the first cells to be shown to decrease in numbers in response to benzene exposure are the leukocytes. However, which of these is most vulnerable has been debated. Earlier literature suggested that granulocytes decrease first, whereas recent evidence has focused on lymphocytes (Svirbely et al., 1944; Snyder, et al., 1978, 1980, 1982; Rozen et al., 1984; Ayoma, 1986; Farris et al., 1997). All reported decreases in circulating B and/or T lymphocytes following exposure to benzene. Irons and Moore (1980) examined the effects of injected benzene in rabbits and reported a selective decrease in circulating B lymphocytes. In a series of papers Irons and coworkers (Pfeifer and Irons, 1981, 1982; Wierda et al., 1981; Wierda and Irons, 1982) reported on the inhibition of lectin-stimulated proliferation of lymphocytes and suggested that the mechanism, in part, was related to the ability of benzene or hydroquinone to cause the cells to begin to cycle. Once in cycle, however, the cells failed to proliferate because hydroquinone caused the disaggregation of microtubule assembly during mitosis.

Recent studies on the interaction of benzene and its metabolites with lymphocytes have focused on the effects on growth factors. For example, the benzene metabolites hydroquinone or *p*-benzoquinone added to mouse spleen lymphocytes resulted in an inhibition of the formation of interleukin 2 (IL-2), a T-cell growth factor, probably as a result of inhibition of RNA synthesis (Post et al., 1985, 1986). *p*-Benzoquinone also inhibited the processing of preinterleukins IL-1α and IL-1β to active interleukins (Niculescu and Kalf, 1995; Kalf et al., 1996; Renz and Kalf, 1991). The lack of IL-1 may have profound effects on bone marrow function including immune suppression. Pyatt et al. (1998) showed that 1 μM hydroquinone reversibly inhibited tumor necrosis factor α (TNF-α)-induced activation of NF-κB in $CD34^+$ T lymphocytes and the production of IL-2.These authors suggest that NF-κB may be a significant mediator of benzene-induced immunotoxicity.

The mononuclear phagocyte system (MPS) is another target for benzene (Lipton, 1995). The MPS develops in the bone marrow (Figure 28.1) by way of the CFU-GM. Further differentiation separates the monocyte/macrophage line from the granulocytes. Sequential differentiation through the CFU-M, the monoblast, and the promonocyte lead to the formation of the monocyte. The monocytes leave the marrow and travel via the blood to various organs where they become the resident macrophages of each organ. In the liver they are called Kupfer cells, in the lung they are called pulmonary alveolar macrophages, in the brain they are called microglial cells, etc. Their roles are to consume dying cells and to provide defense against infections and other foreign challenges. Macrophages are unique in that they can exist in a given organ in a quiescent state but can be activated by antigens (North, 1978; Karnovsky and Lazdins, 1978; Cohn, 1978). Activation is demonstrated by increases

in the capacity of macrophages for chemotaxis, phagocytosis, cytotoxicity, and the production of a variety of mediators (Laskin and Pendino, 1995). Indeed, excessive increases in these activities have been associated with acetaminophen-induced hepatotoxicity (Laskin and Pilaro, 1986).

Treatment of mice with benzene display increased activation and/or maturation of bone marrow macrophages (Laskin et al., 1989; MacEachern and Laskin, 1992; MacEachern et al., 1992), coincidental with increases in the production of TNF-α, IL-1, and hydrogen peroxide. TNF-α and IL-1 have been shown to increase the levels of the inducible nitric oxide synthase resulting in an increase in nitric oxide production (Laskin and Laskin, 1999). Benzene treatment also resulted in increased expression of the mRNA for the inducible form of nitric oxide synthase and higher nitric oxide production (Punjabi et al., 1994). Nitric oxide had been shown to decrease bone marrow cell proliferation (Punjabi et al., 1992). When bone marrow leukocytes were exposed to benzene, hydroquinone, 1,2,4-benzenetriol, or *p*-benzoquinone at bone marrow-depressive doses, the cells were more sensitive to stimulants of NO production such as lipopolysaccharide or interferon-gamma than controls (Laskin et al., 1995). Thus, the impact of benzene on the immune system via activation of macrophages may provide an alternative mechanism by which benzene reduces bone marrow function.

Lewis and coworkers (Lewis et al., 1988; Klan et al., 1990; Manning et al., 1994) have focused on the effects of benzene and its metabolites on the MPS. Benzene and several of its metabolites were examined for their ability to inhibit a number of macrophage functions. These studies were performed using peritoneal macrophages from mice immunized with bacillus Calmette-Guerin to increase macrophage yield. The resulting cells appeared to be activated macrophages. The cells were subsequently cultured and the effects of benzene and several of its metabolites evaluated with respect to their effects on macrophage function. Whereas benzene itself was ineffective, *p*-benzoquinone inhibited the release of hydrogen peroxide, Fc receptor-mediated phagocytosis, interferon-gamma priming for tumor cell cytolysis, and bacterial lipopolysaccharide (LPS) triggering of cytolysis. Hydroquinone inhibited hydrogen peroxide release and priming for cytolysis, benzenetriol inhibited phagocytosis and priming for cytolysis, and catechol only inhibited peroxide release. Whereas the results quoted above may appear to be in conflict, the source of the macrophages and the mechanism of activation may account for the differences in response. Macrophages taken directly from the bone marrow may respond differently from peritoneal macrophages activated by bacterial antigens.

28.6 CARCINOGENESIS AND LEUKEMOGENESIS

Should we distinguish between carcinogenic activity and leukemogenic activity of chemicals? Maltoni and Scarnato (1979) administered benzene at doses of 50 or 250 mg/kg/d, orally, 4–5 d per week for 52 weeks to rats and observed 8 of 35 rats with tumors in the high-dose group and 2 of 30 in the low-dose group. The major form of tumor was carcinoma of the Zymbal gland. They later performed inhalation studies (Maltoni et al., 1982, 1983) and reported similar effects. Bioassays by the National Toxicology Program (Huff et al., 1989) confirmed the results of Maltoni and found Zymbal gland tumors in the mouse, as well as malignant tumors of the mouth and skin, plus malignant lymphoma and mammary carcinoma. The National Toxicology Program, while studying short-term carcinogenesis assays using the Tg.AC transgenic mouse, found that application of benzene to the skin resulted in pappilomas as well as myelogenous leukemia (Spaulding et al., 1999). Because only those cancers that are classified as leukemias have been related to benzene exposure in humans, as opposed to laboratory animals, this discussion will be restricted to the leukemogenic activity of benzene.

When Santesson announced that benzene was the cause of hematotoxicity and aplastic anemia in exposed workers the medical community accepted his conclusions because a significant percentage of workers in benzene-related industries displayed the signs of the disease after relatively brief exposure; the observations turned out to be consistent from facility to facility and Santesson was a credible authority. When the issue of benzene-induced leukemia arose, following the initial

case report by LeNoir and Claude (1897), acceptance of the cause-and-effect association was slower in developing because fewer authorities supported the concept and few cases were observed. Furthermore, studies of workplace populations exposed to benzene (e.g., Greenburg, 1939; Helmer, 1944; Hamilton-Patterson and Browning, 1944; Savilahti, 1956) emphasized descriptors of bone marrow depression. Nevertheless, Selling who had reproduced benzene toxicity in rabbits 20 years earlier, stated in 1935, "There is evidence in the literature that benzol may be a causal agent in the production of leukemia." He cited the reports of Delore and Borgomano (1928) and of Falconer (1933), who had observed cases of acute leukemia in people who worked with benzene. He also discussed the controversial paper of Lignac (1933) who treated mice with benzene and claimed to have observed the development of leukemia. For many years shadows were cast on this work because, despite a number of attempts, it was not possible to reproduce the results. The issue was raised again in a report by Mallory et al. (1939) who cited a case of leukemia among the benzene-exposed rotogravure workers described by Greenburg (1939).

On the basis of his clinical observations Vigliani, who was Direttore, Instituto di Medicina Industriale di Torino dell ′E.N.P.I., categorized benzene toxicity into four subtypes: (1) typical aplastic anemia, (2) typical cases of aplastic anemia except that the bone marrow appeared to be quite active in the formation of undifferentiated cells, (3) atypical aplastic anemia in which the marrow appeared to be either "hyperplastic" or "metaplastic," and (4) frank aleukemic leukemia (Penati and Vigliani, 1938). Unfortunately the journal in which his paper was published was obscure and unavailable to most investigators and his report was largely ignored. His attempts to further his arguments were cut short by World War II. Following the war he became Director of the Institute of Occupational Medicine at the University of Milan and proceeded to report on retrospective studies in France and Italy that involved 77 fatalities resulting from occupational exposure to benzene, in which it was demonstrated that approximately half died of aplastic anemia and half of leukemia (Vigliani and Saita, 1964; Vigliani and Forni, 1969). He subsequently reviewed data from Italian benzene-exposed workers between 1928 and 1938, where 60 cases of aplastic anemia and 10 of leukemia were observed (Vigliani and Forni, 1976).

The bulk of the cases of benzene-related illness in Italy were associated with the shoemaking industry where benzene was used as a solvent for rubber cement. A similar industry flourished in Turkey where shoemaking was to some extent a cottage industry with families engaged in making shoes in their poorly ventilated homes using materials collected from a central point of distribution. A petroleum-based solvent had been used as a base for the glue in Turkey up to about 1955–60, when a change was made to a benzene-based glue (Aksoy, 1988). Subsequently, Aksoy, who headed the Department of Hematology at the University of Istanbul, recognized that among the patients in his clinic were an unusual number of cases of aplastic anemia and leukemia. Because he was aware of the work of Vigliani, both from his publications and through personal contacts, he studied the shoemakers of Istanbul, a cohort which he estimated to number 28,500, and found widespread bone marrow depression as cited above. However, he also described several cases of "preleukemia," which today would be classified as myelodysplastic syndrome, and subsequently reported that of 51 cases of pancytopenia, 13 developed leukemia (Aksoy et al., 1972).

In the early 1970s benzene was one of the earliest problems investigated by the newly established Occupational Safety and Health Administration (OSHA) and its research arm, the National Institute for Occupational Safety and Health (NIOSH). Although the work of Vigliani and Aksoy strongly suggested that tighter controls on benzene exposure in the workplace were required, the database was flawed because the reported results were not based on well-founded epidemiological studies and there were no acceptable data on exposure assessment. As a result NIOSH and OSHA focused on a well-defined cohort of workers in Ohio who were engaged in the manufacture of a rubberized material termed Pliofilm (Infante et al., 1977) where benzene was used as a solvent for the rubber. The cohort was made up of white men who worked in the factory between 1940 and 1949. They reviewed death certificates for those who had died and accounted for all but 25% of the cohort. Seven cases of leukemia associated with benzene exposure in the Pliofilm operation were identified.

The standardized mortality ratio (SMR) for benzene-exposed Pliofilm workers was 560 for the group at large and 2100 for workers exposed for more than 5 years (Rinsky et al., 1981, 1987). They went on to develop an argument based on cumulative exposure using the metric "ppm-years of exposure" and suggested that, given an exposure range of 40 ppm-years to 400 ppm-years the projected SMR values for an excess of benzene-associated leukemia would range from 109 to 6637. It is notable that an internal study at the Dow Chemical Company (Ott et al., 1978) also reported an excess of leukemia among workers exposed to benzene.

The studies of Rinsky et al. (1981, 1987) and of Ott et al. (1978) demonstrated an association between benzene exposure and leukemia but theirs were relatively small-scale studies. An opportunity to examine a larger cohort arose as a result of efforts by S.-N. Yin of the Institute of Health of the Chinese Academy of Medical Sciences who initiated a study of Chinese workers exposed to benzene (Yin et al., 1987, 1996) or benzene-containing mixtures in 28 provinces of China between 1979 and 1981. Of the 528,729 workers in the cohort, 96.23% were examined. The report indicated an overall prevalence of so-called "benzene poisoning" of 0.51%. Twenty-four cases of aplastic anemia and nine of leukemia, most of which were of the acute nonlymphocytic variety, were reported. Studies in 55,255 workplaces demonstrated an average benzene concentration of 18.1 mg/m^3 (5.6 ppm); however, in those workplaces where most of the leukemia and aplastic anemia were observed the benzene air concentrations were estimated to range between 93 and 115 mg/m^3 (29–361 ppm) over varying periods. A follow-up study (Yin et al., 1989) focused on an exposed cohort of 28,460 workers that were compared with a group of controls of approximately the same size. An SMR value for leukemia (undifferentiated) of 5.74 was calculated for the group occupationally exposed to benzene.

Over the past 60-70 years benzene exposure has been associated with a wide range of different types of leukemia/lymphoma. Eventually it was realized that the predominant form was acute myelogenous leukemia (AML). The report of by Yin et al. (1996) summarized a study involving 73,000 benzene-exposed workers in China compared with 35,000 controls. Statistical examination of the results showed a significant association between benzene exposure and the development of leukemia in general, AML specifically, aplastic anemia, and myelodysplasia. The data did not show an increase in chronic myelogenous leukemia (CML) (a form of leukemia not observed among the Chinese population) or acute lymphocytic leukemia (AAL). They also reported, for the first time, increases in multiple myeloma and non-Hodgkin's lymphoma (NHL). This was the largest study ever performed to investigate benzene-induced leukemia. The Australian Health Watch (Consensus Documents, 2000, 2001), a university-based program supported by the Australian Institute of Petroleum which surveys leukemia in the petroleum industry in Australia, found an increased incidence of AML and CML among these workers. This was the first time that benzene had been implicated in CML. They found no evidence for either lymphatic cancer (NHL and multiple myeloma) or multiple myeloma alone.

The fact that CML is commonly seen in western countries (25–30% of all cases of leukemia in the United States are CML) suggests an ethnogenetic basis for the disease. Epidemiological studies in Europe or the United States have not been large enough to find an association with benzene although there is a great deal of NHL in this country unrelated to benzene (about 4% of all leukemias). The Chinese study detected NHL in a Chinese population but it was not observed in a large study in Australia (albeit smaller than the Chinese study). Furthermore, multiple myeloma was increased in the Chinese study but not in the Australian study. There are many other reports in the leukemia literature suggesting ethnogenetic links of susceptibility. Thus, it would not be surprising if there is a benzene-sensitive population in China susceptible to NHL that cannot be found in Western countries. More data are required, especially in the western countries, to determine whether benzene exposure is linked to NHL.

28.7 BENZENE METABOLISM

It has been well established that animals in which benzene metabolism has been impaired are protected against benzene-induced bone marrow damage (Snyder et al., 1982). It was concluded,

therefore, that one or more benzene metabolites must play a role in initiating benzene toxicity, and by extension, benzene-induced leukemogenesis. The pathway of benzene metabolism has been well explored (Snyder et al., 1993; Snyder and Hedli, 1996) and Figure 28.2 summarizes the major pathways of benzene metabolism. CYP 2E1 is the enzyme primarily responsible for the hydroxylation of benzene. The intermediate oxide-oxepin undergoes a spontaneous, nonenzymatic rearrangement to form phenol. Phenol is further hydroxylated to form hydroquinone, catehol, and 1,2,4-benzenetriol, the latter being largely derived from hydroquinone. Alternatively, epoxide

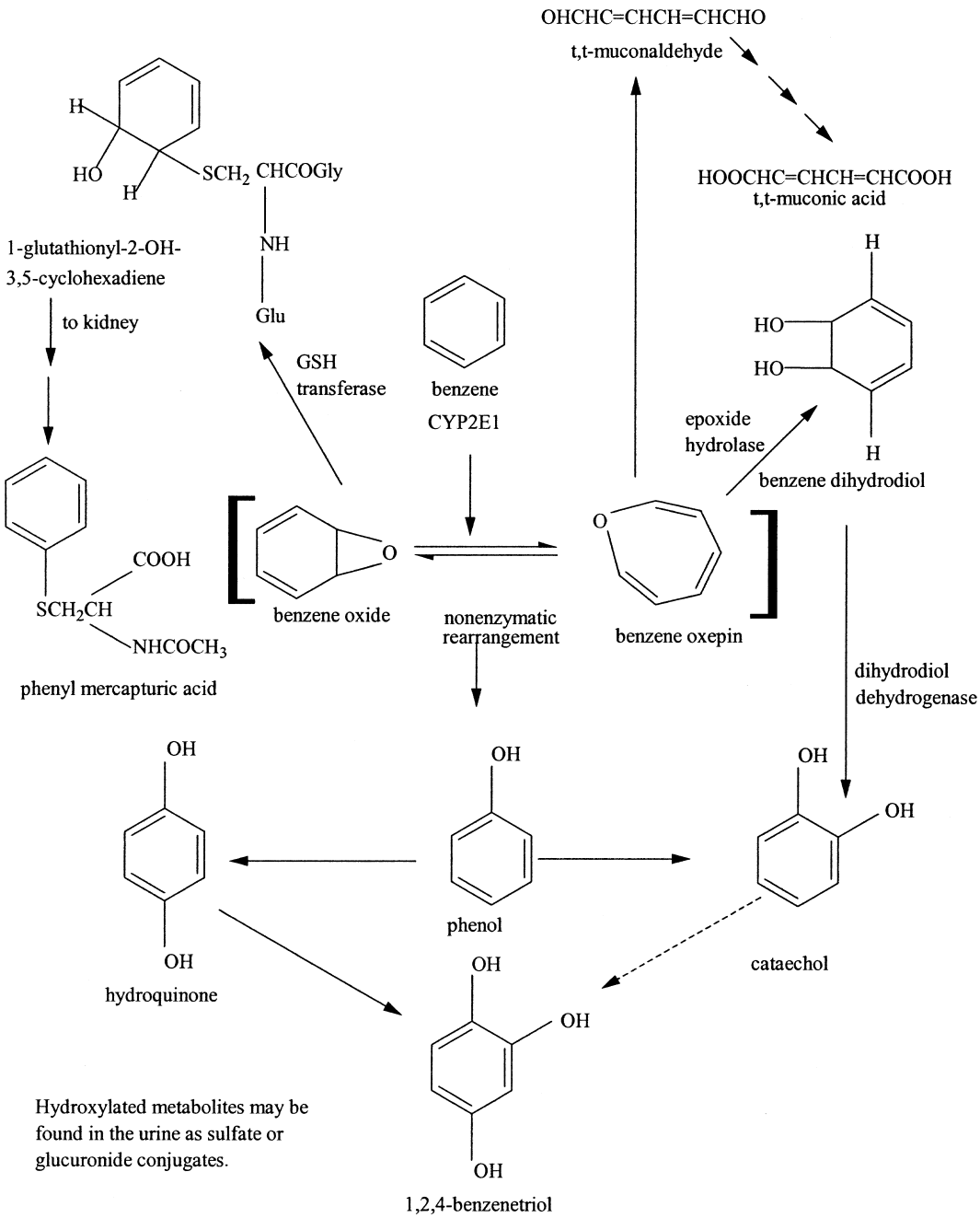


FIGURE 28.2 Pathways of benzene metabolism.

hydrolase may add water to benzene oxide to yield the dihydrodiol, which can be oxidized to catechol via the enzyme dihydrodiol dehydrogenase. The benzene ring may be opened to yield *t*, *t*-muconaldehyde and 6-OH-*t*, *t*-hexadienoic acid. The ring opening is postulated to be the result of an attack by an as-yet-unidentified reactive oxygen species (Zhang et al., 1995a). Further metabolism results in muconic acid, a well-described urinary metabolite. Glutathione can react with benzene oxide to yield 1-glutathionyl-1,2-OH-cyclohexadiene, which in the kidney is converted to the phenylmercapturic acid. The phenolic compounds appear in the urine as sulfates and glucuronides. Other metabolites that have been observed in urine include a *N*-acetyl-*S*-(2,5-dihydroxyphenyl)-L-cysteine, N7-phenylguanine (thought to be an end product of the breakdown of DNA containing a benzene-related adduct), and two protein-adduct-related end products, *S*-phenylcysteine and *N*-phenylvaline (Medieros et al., 1997).

Apparently, several reactive metabolites are formed during benzene metabolism. Hydroquinone, catechol, and 1,2,4-benzenetriol can be oxidized to highly reactive, bone-marrow-toxic quinines (probably via myeloperoxidase in bone marrow) which could covalently bind to DNA or proteins. *t, t*-Muconaldehyde, and 6-OH-*t*, *t*-2,4-hexadienal have been shown to be bone marrow depressants. Furthermore, there is evidence for synergistic effects of ring-opening products and hydroxylated metabolites of benzene (Eastmond et al., 1987; Guy et al., 1990, 1991; Robertson et al., 1991; Amin and Witz, 2001).

28.8 MECHANISM(S) OF BENZENE TOXICITY/LEUKEMOGENESIS

The mechanism of benzene toxicity/leukemogenesis has been the subject of many studies over the past 50 years. The first, and one of the most important, was the suggestion by Parke and Williams (1953), as a result of their studies of benzene metabolism, that it was likely that one or more of the benzene metabolites might be important in the mechanism of toxicity. The observation that inhibition of benzene metabolism by toluene (Andrews et al., 1977) or partial hepatectomy (Sammett et al., 1979), which both reduced the production of benzene metabolites and protected against benzene toxicity, supported the Parke and Williams (1953) hypothesis. It is clear that after inhalation benzene is carried to the liver where it undergoes a series of metabolic reactions (Hedli et al., 1997; Hoffmann et al., 1999). Metabolites leave the liver and travel to the bone marrow where secondary metabolites are formed which are probably the direct cause of impaired bone marrow function. Metabolites viewed as most likely to play a role in benzene-induced hematotoxicity are hydroquinone, catechol, and 1,2,4-benzene triol (each of which can be oxidized in the bone marrow to respective quinones) and *t*, *t*-muconaldehyde.

The concept that metabolites of xenobiotic chemicals can lead to toxicity was strengthened by the demonstration that metabolically generated, chemically reactive intermediary metabolites can covalently join with macromolecules, such as proteins and DNA, with the result that macromolecular functions are changed. (The demonstration of these events and discussions of their implications has been the subject of a series of international symposia, the proceedings of the most recent of which can be found in Dansette et al., 2001.) The metabolites, referred to as biological reactive intermediates (BRIs), react nonenzymatically and with less overall specificity than might be observed in enzymatically driven reactions. Thus, *in vitro* benzene oxide, a product of the epoxidation of benzene by CYP 2E1, and *p*-benzoquinone, an oxidation product of the benzene metabolite hydroquinone, can covalently bind to both proteins and DNA. However, *in vivo* greater specificity of bonding sites occurs than *in vitro* (Snyder, 2002) and binding to proteins is more reproducible and extensive that binding to DNA.

The proposed mechanism of carcinogenesis of so-called "genotoxic carcinogens" that has arisen over the past several decades is that BRIs react covalently with DNA with the result that the genetic code is misread during DNA replication or mRNA synthesis, or attempts to repair covalently bound DNA result in repair error. The resultant impairment of DNA function could result in mutation-based cell transformation to a cancer cell. It has been argued that a single covalently bound BRI could

initiate this series of events. It is more likely, however, that several mutations, reflective of genetic instability, are required to elicit a carcinogenic response (Fearon and Vogelstein, 1990; Finlay, 1993; Arends, 2000; Bronchud, 2002; Nowak et al., 2002).

The dogma suggests that, unlike other toxicological effects, there cannot be a threshold dose for a genotoxic carcinogen. Attempts to regulate exposure to benzene have employed risk assessment techniques directed to genotoxic carcinogens. If the key event, however, is a series of reactions involving binding to proteins, molecules that turn over quickly and can be readily replaced when damaged, it is necessary to reevaluate the "no threshold" concept. Consequently it is of both theoretical and regulatory interest to fully understand the mechanism of both benzene-induced aplastic anemia and leukemia.

Inhalation of benzene at a sufficiently high dose for a sufficiently long period of time leads to bone marrow depression and eventually marrow aplasia. The route of administration plays a significant role in determining the effectiveness that a given dose will display in producing toxicity. Benzene given to mice benzene (200 mg/kg) intradermally displayed significantly higher levels of benzene metabolites in bone marrow than animals given the same dose by gavage (Hoffmann et al., 2001). The oral route permitted loss of benzene in the expired air at a greater rate than when given in the skin. The pattern of metabolites was not affected by the route of administration. Inhalation of benzene permits absorption of a significant percentage of the inspired vapor. Recent studies in humans confirm the frequently stated suggestion that short peaks of high exposure during chronic lower-dose exposure seem to be more effective in damaging bone marrow leading to a leukemogenic response than long-term exposure at constant levels (Collins et al., 2003).

In the bone marrow benzene metabolites have a variety of effects depending on concentration. For example, in both human and mouse bone marrow stem/progenitor cells hydroquinone at concentrations below 5 μM causes proliferation of granulocytic precursors (Stillman et al., 2000; Hazel et al., 1995, 1996a, 1996b; Hazel and Kalf, 1996; Hoffmann et al., 2001). At higher concentration, e.g., 10 μM or higher, the cells commence apoptosis (Moran et al., 1996). At sufficient doses animals treated with benzene display decreased bone marrow cellularity and reductions in various bone marrow CFUs (Snyder, 2002). The growth of specific CFUs *in vitro* can also be inhibited by benzene metabolites. Benzene metabolites have been shown to interfere with the normal functioning of several bone marrow cytokines necessary for growth and differentiation of specific bone marrow cell types. Furthermore, benzene treatment appears to render the bone marrow stroma (hematopoietic microenvironment) inactive in the support of growth and differentiation of bone marrow cells.

Whereas the death of critical cells and/or the inhibition of their function can be accepted as a probable cause of benzene-induced aplastic anemia, leukemogenesis appears to be a somewhat more complex process. The prevailing opinion is that in cases where exposure to benzene does not lead to marrow aplasia, cells may survive but may be inherently damaged. The resulting bone marrow appears dysplastic, with aberrant cells and chromosome damage. Chronic exposure to benzene leads to chromosome aberrations, which have been related to leukemogenesis. Chromosome damage has not been excluded as a factor in the production of aplastic anemia.

It has been postulated that covalent binding of reactive metabolites of benzene to cellular macromolecules could be the basis of chromosome damage and subsequent leukemogenesis. However, covalent binding of benzene metabolites to DNA has been found to be of a low order and erratic *in vivo*. There is extensive binding to proteins but it appears to be nonspecific. Any number of proteins and specific enzymes may be shown to be bound by reactive metabolites of benzene but sorting out those specific to toxicity requires further work. A more recent hypothesis suggested that benzene metabolites alter the activity of topoisomerase II, an enzyme that can both assist in DNA repair, or when inhibited, contribute to DNA damage leading to chromosomal aberrations and cell transformation. The role of benzene metabolites in stimulating differentiation of myeloblasts to myelocytes, the inhibition of further maturation to neutrophils, coupled with inhibition of apoptosis at the myelocyte stage, could act as a mechanism for promoting the growth of the transformed cells (Hazel et al.,

1995, 1996a, 1996b, 1996c). Thus, the combined effects of benzene metabolites at various stages in cellular function could lead to eventual leukemogenesis.

Benzene metabolites have been shown to interfere with cellular processes, each of which may contribute to the ultimate toxic/leukemogenic effects. There is no well-developed road map that leads from benzene exposure, through benzene metabolism, to aplasia or leukemia. Part of the problem lies in the lack of an animal model that can reproduce the benzene-induced leukemia most frequently seen in humans. The nearest to a model has been the Tg.AC mouse, which when treated with benzene by skin application gives rise to both papillomas and myelogenous leukemia. The transgenic animal results from the insertion of v Harvey *ras* oncogene into the mouse genome. The insertion may render the animal more susceptible to leukemia than animals that do not carry the extra *ras* gene. Further studies with these and other models are needed before we fully understand the mechanism of benzene-induced leukemia.

28.9 CLOSING COMMENTS

The study of the mechanism of benzene toxicity and leukemogenesis is entering a new phase. Several laboratories are engaged in studying the effects of benzene inhalation on the genome in animal models by using a variety of array technologies. There is intensive work in the areas of the mechanism and the interpretation of the formation of chromosome aberrations. Examination of the effects of benzene in humans is continuing in several studies including those in China and Australia. It is already possible to suggest that susceptibility to benzene toxicity/leukemogenesis may be related to differences between individuals in the expression of CYP 2E1, NQ01, and glutathione transferases. Other factors in the bone marrow may also vary genetic susceptibility to benzene. Genomic information may, in the future, provide a method for identifying those people most susceptible to benzene-related diseases.

REFERENCES

Aksoy, M., Benzene hematotoxicity, in *Benzene Carcinogenicity,* Aksoy, M., Ed., CRC Press, Boca Raton, FL, 1988, pp. 59–112.

Aksoy, M., Dinçol, K., Akgün, T., Erdem, S., and Dinçol, G., Haematological effects of chronic benzene poisoning in 217 workers, *Br. J. Ind. Med.,* 28, 296–302, 1971.

Aksoy, M., Dinçol, K., Erdem, S., Akgün, T., and Dinçol, G., Details of blood changes in 32 patients with pancytopenia associated with long term exposure to benzene, *Br. J. Ind. Med.,* 29, 56–64, 1972.

Aksoy, M. and Erdem, S., Followup study on the mortality and development of leukemia in 44 pancytopenic patients with chronic benzene exposure, *Blood,* 52, 285–292, 1978.

Amin, R.P. and Witz, G., DNA-protein crosslink and DNA strand break formation in HL-60 cells treated with trans, trans-muconaldehyde, hydroquinone and their mixtures*, Int. J. Toxicol.,* 20, 69–80, 2001.

Andrews, L.S., Lee, E.W., Witmer, C.M., Kocsis, J.J., and Snyder, R., Effects of toluene on the metabolism, disposition and hematopoietic toxicity of benzene, *Biochem. Pharmacol.,* 26, 293–300, 1977.

Arends, J.W., Molecular interactions in the Vogelstein model of colorectal carcinoma, *J. Pathol.,* 190, 412–416, 2000.

ATSDR (Agency for Toxic Substances and Disease Registry), 7. Regulation and advisories, in *Toxicological Profile for Benzene (Update*), U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry, Atlanta, GA, 1997, pp. 325–348.

Avis, S. and Hutton, C.J., Acute benzene poisoning: a report of three fatalities, *J. Forensic Sci.,* 38, 599–602, 1993.

Ayoma, K., Effects of benzene inhalation on lymphocyte subpopulations and immune response in mice, *Toxicol. Appl. Pharmacol.,* 85, 92–101, 1986.

Bass, M., Sudden sniffing death, *J. Am. Med. Assoc.,* 212, 2075–2079, 1970.

Bonnet, P., Morele, Y., Raoult, G., Zissu, D., and Gradiski, D., Determination de la concentration lethale$_{50}$ des principaux hydrocarbures aromatiques chex le rat, *Arch. Mal. Rof.,* 43, 261–265, 1982.

Brewer, R.K. and Weiskotten, H.G., The action of benzol III. The urinary phenols with special reference to the diphasic leucopenia, *J. Med. Res.,* 35, 71–77, 1916.

Bronchud, M.H., Is cancer really a 'local' cellular clonal disease? *Med. Hypotheses,* 59, 560–565, 2002.

Camp, W.E. and Baumgartner, E.A., Inflammatory reactions in rabbits with a severe leucopenia, *J. Exp. Med.,* 22, 174–194, 1915.

Carpenter, C.P., Shaffer, C.B., Weil, C.S., and Smyth, H.F., Studies on the inhalation of 1:3-butadiene; with a comparison of its narcotic effect with benzol, toluol, and styrene, and a note on the elimination of styrene by the human, *J. Ind. Hyg. Toxicol.,* 26, 69–78, 1944.

Ciranni, R., Barale, R., and Adler, I.D., Dose-related clastogenic effects induced by benzene in bone marrow cells and in differentiating spermatogonia of Swiss CD1 mice, *Mutagen,* 6, 417–421, 1991.

Cohn, Z.A., The activation of mononuclear phagocytes: Fact, fancy, and future. *J. Immunol.* 121, 813–816, 1978.

Collins, J.J., Ireland, B., Buckley, C.F., and Shepperly, D., Lymphatohameatopoeitic cancer mortality among workers with benzene exposure, *Occup. Environ. Med.,* 60, 676–679, 2003.

Consensus Document, Health Watch, The Australian Institute of Petroleum Health Surveillence Program, Eleventh report, The University of Adelaide, Department of Public Health, Adelaide, Australia, 2000..

Consensus Document, Lympho-haematopoietic Cancer and Exposure to Benzene in the Australian Petroleum Industry, Technical report and appendices, Monash University, Department of Epidemiology and Preventive Medicine, and Deakin University, Unit of Occupational Hygiene and School of Health Sciences, Victoria, Australia, 2001.

Cronkite, E.P., Drew, R.T., Inoue, T., Hirabayashi, Y., and Bullis, J.E., Hematotoxicity and carcinogenicity of inhaled benzene. *Environ. Health Perspect.,* 82, 97–108, 1989.

Cronkite, P.E., Inoue, R., Carten, A., Miller, M., Bullis, J., and Drew, R.. Effects of benzene inhalation on murine pluripotent stem cells, *J. Toxicol. Environ. Health,* 9, 411–421, 1982.

Delore, P. and Borgomano, C., Leucemie aigue au cours d = intoxication benzenique, *J. Méd. Lyon.,* 9, 225, 1928. [As cited in: Hamilton, A., General Review: Benzene (benzol) poisoning, *Arch. Pathol.,* 11, 434–454, 601–637, 1931].

Dempster, A.M., Evans, H.L., and Snyder, C.A., The temporal relationship between behavioural and hematological effects of inhaled benzene, *Toxicol. Appl. Pharmacol.,* 76, 195–203, 1984.

Dexter, T.M., Wright, E.G., Krizsa, F., and Lajtha, L.G., Regulation of haematopoietic stem cell proliferation in long term bone marrow cultures, *Biomedicine.,* 27, 344–349, 1977.

Dexter, T.M., Haemopoiesis in long-term bone marrow cultures. A review, *Acta Haematol.,* 62, 299–305, 1979.

Drew, R.T. and Fouts, J.R., The lack of effects of pretreatment with phenobarbtial and chlorpromazine on the acute toxicity of benzene in rats, *Toxicol. Appl. Pharmacol.,* 27, 183–193, 1974.

Drozd, J. and Bockowski, E.J., Acute benzene poisoning, *J. Occup. Med.,* 9, 9–11, 1967.

Eastmond, D.A., Smith, M.T., and Irons, R.D., An interaction of benzene metabolites reproduces the myeotoxicity observed with benzene exposure, *Toxicol. Appl. Pharmacol.,* 91, 85–95, 1987.

Engelhardt, W.E. and Estler, W., Versuche uber die akut narkotische wirkung aliphatischer und aromatischer kohlenwasserstoffe. Die wirkung einmaliger einatmung vecsheidener konzentrationen benzin, benzol, toluol, xylol auf kannichen und katzen, *Arch. Hyg.,* 114, 249–260, 1935.

Erexson, G.L., Wilmer, J.L., Steinhagen, W.H., and Kligerman, A.D., Induction of cytogenetic damadge in rodents after short-term inhalation of benzene, *Environ. Mutagen.,* 8, 29–40, 1986.

Evans, H.L., Dempster, A.M., and Snyder, C.A., Behavioural changes in mice following benzene inhalation, *Neurobehav. Toxicol. Teratol.,* 3, 481–485, 1981.

Falconer, E.H., An instance of lymphatic leukemia following benzol poisoning, *Am. J. Med. Sci.,* 186, 353–361, 1933.

Farris, G.M., Robinson, S.N., Wong, B.A., Wong, V.A., Hahn, W.P., and Shah, R., Effects of benzene on splenic, thymic, and femoral lymphocytes in mice, *Toxicology,* 28, 137–148, 1997.

Fearon, E.R. and Vogelstein, B., A genetic model for colorectal cancer, *Cell,* 61, 759–767, 1990.

Finlay, G.J., Genetics, molecular Biology and colorectal cancer, *Mutat. Res.,* 290, 3–12, 1993.

Fishbein, L., An overview of environmental and toxicological aspects of aromatic hydrocarbons. I. Benzene, *Science Total Environ.,* 40, 189–218, 1984.

Flury, F., II. Moderne gewerbliche vergiftungen in pharmakologisch-toxicologischer hinsicht, *Arch. Exp. Pathol. Pharmakol.,* 138, 65–82, 1928.

Frantik, E., Hornychova, M., and Horvath, M., Relative acute neurotoxicity of solvents: Isoeffective air concentrations of 48 compounds evaluated in rats and mice, *Environ. Res.,* 66, 173–185, 1994.

Fujie, K., Ito, Y., and Maeda, S., Acute cytogenetic effect of benzene on rat bone marrow cells in vivo and the effect if inducers or inhibitors of drug-metabolizing enzymes, *Mutat. Res.,* 298, 81–90, 1992.

Gaido, K.W. and Wierda, D., *In vitro* effects of benzene metabolites on mouse bone marrow stromal cells, *Toxicol. Appl. Pharmacol.,* 76, 45–55, 1984.

Gaido, K.W. and Wierda, D., Modulation of stromal cell function in DBA 2/J and B6C3F1 mice exposed to benzene or phenol, *Toxicol. Appl. Pharmacol.,* 81, 469–475, 1985.

Gaido, K.W. and Wierda, D., Suppression of bone marrow stromal cell function by benzene and hydroquinone is ameliorated by indomethacin, *Toxicol. Appl. Pharmacol.,* 89, 378–390, 1987.

Garnett, H.M., Cronkite, P.E., and Drew, R., Effect of *in vivo* exposure to benzene on the characteristics of bone marrow adherent cells, *Leukoc. Res.,* 7, 803–810, 1983.

Gerarde, H.W., *Toxicology and Biochemistry of Aromatic Hydrocarbons,* Browning, E., Ed., Elsevier Publishing Company, New York, 1960.

Gill, D.P., Jenkins, V.K., Kempen, R.R., and Ellis, S., The importance of pluripotential stem cells in benzene toxicity, *Toxicology,* 16, 163–171, 1980.

Green, J., Snyder, C.A., Goldstein, B.D., and Albert, R., Acute and chronic dose/response effect of benzene inhalation on the peripheral blood, bone marrow, and spleen cells of CD-1 male mice, *Toxicol. Appl. Pharmacol.,* 59, 204–214, 1981.

Greenberg, P.L., Myelodysplastic syndrome, in *Hematology,* 3rd *ed.,* Hoffman, R., Benz, E.J., Jr., Shattil, S.J., Furie, B., Cohen, H.J., Silberstein, L.E., andMcGlace, P., Eds., Churchill Livingstone, New York, 1106–1129, 2000.

Greenburg, L., Benzol poisoning as an industrial hazrd. I. The chemistry and industrial uses of benzol, *Public Health Rep.,* 41, 1357–1375, 1926a.

Greenburg, L., Benzol poisoning as an industrial hazard. VI. Intensive study of selected industries with respect to factory conditions and pollution of the atmosphere by benzol, *Public Health Rep.,* 41, 1516–1539, 1926b.

Greenburg, L., Mayers, M.R., Goldwater, L., and Smith, A.R., Benzene (benzol) poisoning in the rotogravure printing industry in New York City, *J. Ind. Hyg. Toxicol.,* 21, 395–420, 1939.

Guy, R.L., Dimitriadis, E.A., Hy, P., Cooper, K.R., and Snyder, R., Interactive inhibition of erythroid ^{59}Fe utilization by benzene metabolites in female mice, *Chem. Biol. Intrtact.,* 74, 55–62, 1990.

Guy, R.L., Hu, P., Witz, G., Goldsatein, B.D., and Snyder, R., Depression of iron uptake into erythrocytes in mice by treatment with the combined benzene metbolites p-benzoquinone, muconaldehyde and hydroquinone*, J. Appl. Toxicol.,* 11, 443–446, 1991.

Hamilton, A., The growing menace of benzene (benzol) poisoning in American industry, *JAMA,* 78, 627-630, 1922.

Hamilton-Patterson, J.L. and Browning, E., Toxic effects in women exposed to industrial rubber solutions, *Br. Med. J.,* I, 349–352, 1944.

Harigaya, K., Miller, M.E., Cronkite, E.P., Drew, R.T., The detection of *in vivo* hematotoxicity of benzene by *in vitro* liquid bone marrow cultures, *Toxicol. Appl. Pharmacol.,* 60**,** 346–353, 1981.

Hazel, B.A., Baum, C., Kalf, G.F., Hydroquinone, a bioreactive metabolite of benzene, inhibits apoptosis in myeloblasts, *Stem Cells,* 14, 730–742, 1996a.

Hazel, B.A. and Kalf, G.F., Induction of granulocytic differentiation in myeloblasts by hydroquinone, a metabolite of benzene, involves the leukotriene D4 receptor, *Recept. Signal Transduct.,* 6, 1–12, 1996.

Hazel, B., O'Connor, A., Niculescu, R., and Kalf, G.F., Benzene and its metabolite, hydroquinone, induce granulocytic differentiation in myeloblasts by interacting with cellular signaling pathways activated by granulocyte colony-stimulating factor, *Stem Cells,* 13, 295–310, 1995.

Hazel, B.A., O'Connor, A., Niculescu, R., and Kalf, G.F., Induction of granulocytic differentiation in a mouse model by benzene and hydroquinone, *Environ. Health Perspect.*, 104 (Suppl. 6), 1257–1264, 1996b.

Hedli, C.C., Hoffmann, M.J., Ji, S., Thomas, P.E., and Snyder, R., Benzene metabolism in the isolated perfused mouse liver, *Toxicol. Appl. Pharmacol.,* 146, 60–68, 1997.

Hektoen, L., Effect of benzene on the production of antibodies, *J. Infec. Dis.,* 19, 69–84, 1916.

Helmer, K.J., Accumulated cases of benzene poisoning in the rubber industry, *Acta Med. Scand.,* 118, 354–375, 1944.

Hildebrand, R.L. and Murphy, M.J., Jr., The effects of benzene inhalation on murine hematopoietic precursor cells (CFU-e, BFU-e and CFU-gm), *Int. J. Cell Cloning,* 1, 240–253, 1983.

Hirschfelder, A.D. and Winternitz, M.C., Studies upon experimental pneumonia in rabbits, *J. Exp. Med.,* 17, 657–665, 1913.

Hoffmann, M.J., Hedli, C.C., and Snyder, R., Metabolism of [^{14}C]phenol in the isolated perfused mouse liver, *Toxicol. Sci.,* 49, 40–47, 1999.

Huff, J.E., Haeman, J.K., DeMarini, D.M., Eustis, S., Maronpot, R.R., Peters, C., Peprsing, R.L., Chrisp, C.E., and Jacobs, A.C., Multiple-site carcinogenicity of benzene in Fischer 344 rats and B6C3F1 mice, *Environ. Health Perspect.,* 82, 125–163, 1989.

Hunter, D., *The Diseases of Occupations,* Little Brown and Company, Boston, 1962.

Infante, P.F., Rinsky, R.A., Wagoner, J.K., and Young, R.J., Leukemia in benzene workers, *Lancet,* 2, 76–78, 1977.

Irons, R.D. and Moore, B.J., Effect of short term benzene administration on circulating lymphocyte subpopulations in the rabbit: evidence of a selective B-lymphocyte sensitivity, *Res. Commun. Chem. Pathol. Pharmacol.,* 27, 147–155, 1980.

Kalf, G.F., Renz, J.F., and Niculescu, R., p-Benzoquinone, a reactive metabolite of benzene, prevents the processing of pre-interleukins-1 alpha and -1 beta to active cytokines by inhibition of the processing enzymes, calpain, and interleukin-1 beta converting enzyme, *Environ. Health Perspect.,* 104 (Suppl. 6), 1251–1256, 1996.

Karnovsky, M.L. and Lazdins, J.K., Biochemical criteria for activated macrophages, *J. Immunol.,* 121, 809–813, 1978.

Kellerova, V., Electroencephalographic findings in workers exposed to benzene, *J. Hyg. Epidemiol. Microbiol. Immunol.,* 29, 337–346, 1985.

Klan, M.J., Adams, D.O., and Lewis, J.G., Effects of exposure to benzene in vivo on the murine mononuclear phagocyte system, *Toxicol. Appl. Pharmacol.,* 103, 198–205, 1990.

Kraut, A., Lilis, R., Marcus, M., Valciukas, J.A., Wolff, M.S., Landrigan, and P.J., Neurotoxic effects of solvent exposure on sewage treatment workers. *Arch. Environ. Health,* 43, 263–268, 1988.

Laskin, D.L., MacEachern, L., and Snyder, R., Activation of bone marrow phagocytes following benzene treatment of mice, *Environ. Health Perspect.,* 82, 75–79, 1989.

Laskin, D.L. and Pendino, K.J., Macrophages and inflammatory meddiators in tissue injury, *Annu. Rev. Pharmacol. Toxicol.,* 53, 655–677, 1995.

Laskin, J.D. and Laskin, D.L., *Cellular and Molecular Biology of Nitric Oxide,* Marcel Dekker, New York, 1999.

Laskin, D.L. and Pilaro, A.W., Potential role of activated macrophages in acetaminophenhepatotoxicity. I. Isolation and characterization of activated macrophages from rat liver, *Toxicol. Appl. Pharmacol.,* 86, 204–215, 1986.

Laskin, J.D., Rao, N.R., Punjabi, C.J., Laskin, D.L., and Snyder, R., Distinct actions of benzene and its metabolites on nitric oxide production by bone marrow leukocytes, *J. Leukoc. Biol.,* 57, 422–426, 1995.

Lazarew N.W. Uber die giftigkeit verschiedener kohlenwasserstoffdämpfe. *Arch. Exptl. Path. Pharmakol.,* 143, 223–233, 1929.

Lehmann, K.B., Quantitative untersuchungen über die aufnahme von benzol durch tier und mensch aus der luft, *Arch. Hyg*., 72, 307–326, 1910.

Le Noir, M.M. and Claude, H., [A case of purpura attributed to benzene poisoning.] *Bull. Méd. Hôp. Paris,* 14, 151–160, 1897. [As cited in Golding, B.T. and Watson, W.P., Possible mechanisms of carcinogenesis after exposure to benzene, in *Exocyclic DNA Adducts in Mutagenesis and Carcinogenesis,* Singer, B. and Bartsch, H., Eds., IARC Scientific Publications, Lyon, France, 1999.]

Lewis, J.G., Odom, B., and Adams, D.O., Toxic effects of benzene metabolites on mononuclear phagocytes, *Toxicol. Appl. Pharmacol.,* 92, 246–254, 1988.

Lignac, G.O.E., Benzene induced leukemia in humans and white mice, *Klin. Wochenschr.,* 12, 109–110, 1933. [The paper was originally written in German. It was helpful to have available in an English translation prepared by Leo Kanner Associates, Redwood City, CA in 1977].

Lipton, J.M., Histiocytic syndromes, in *Hematology,* 2nd ed., Hoffman, R., Benz, E.J., Shattil, S.J., Furie, B., Cohen, H.J., and Silberstein, L.E., Eds., Churchill Livingstone, New York, 1995, pp. 840–841.

Litovitz, T., Myocardial sensitization following inhalation abuse of hydrocarbons, *Occup. Med.,* 3, 567–568, 1988.

Longacre, S.L., Kocsis, J.J., and Snyder, R., Influence of strain differences in mice on the metabolism and toxicity of benzene, *Toxicol. Appl. Pharmacol.,* 60, 398–409, 1981a.

Longacre, S.L., Kocsis, J.J., Witmer, C.M., Lee, E.W., Sammett, D., and Snyder, R., Toxicological and biochemical effects of repeated administration of benzene to mice, *J. Toxicol. Environ. Health,* 7, 223–237, 1981b.

MacEachern, L. and Laskin, D.L., Increased production of tumor necrosis factor-alpha by bone marrow leukocytes following benzene treatment of mice, *Toxicol. Appl. Pharmacol.,* 113, 260–266, 1992.

MacEachern, L., Snyder, R., and Laskin, D.L., Alterations in the morphology and functional activity of bone marrow phagocytes following benzene treatment in mice, *Toxicol. Appl. Pharmacol.,* 117, 147–154, 1992.

Mallory, T.B., Gall, E.A., and Brickley, W.J., Chronic exposure to benzene (benzol). III. The pathologic results, *J. Ind. Hyg. Toxicol.,* 21, 355–393, 1939.

Maltoni, C., Conti, B., and Cotti, G., Benzene: multipotential carcinogen. Results of long term bioassay performed at the Bologna Institute of Oncology, *Am. J. Ind. Med.,* 4, 589–630, 1983.

Maltoni, C., Cotti, G., Valgimigli, L., and Mandrioli, A., Zymbal gland carcinomas in rats following exposure to benzene by inhalation, *Am. J. Ind. Med.,* 3, 11–16, 1982.

Maltoni, C. and Scarnato, C., First experimental demonstration of the carcinogenic effects of benzene, *Med. Lav.,* 5, 352–357, 1979.

Manning, B.W., Adams, D.O., and Lewis, J.G., Effects of benzene metabolites on receptor-mediated phagocytosis and cytoskeletal integrity in mouse peritonean macrophages, *Toxicol. Appl. Pharmacol.,* 126, 214–223, 1994.

Medieros, A.M., Bird, M.G., and Witz, G., Potential biomarkers of benzene exposure*, J. Toxicol. Environ. Health,* 29, 519–539, 1997.

Midzenski, M., McDiarmid, M.A., Rothman, N., and Kolodner, K., Acute high dose exposure to benzene in shipyard workers, *Am. J. Ind. Med.,* 22, 553–565, 1992.

Molnar, J., Paksy, K., and Naray, M., Changes in the rat = s motor behaviour during 4-hr inhalation exposure to prenarcotic concentrations of benzene and its derivates, *Acta Physiol. Hung.,* 67, 349–354, 1986.

Moran, J.L., Siegel, D., Sun, X.M., and Ross, D., Induction of apoptosis by benzene metabolites in HL-60 and CD34+ human bone marrow progenitor cells, *Mol. Pharmacol.,* 50, 610–615, 1996.

Niculescu, R. and Kalf, G.F., A morphological analysis of the short-term effects of benzene on the development of the hematological cells in the bone marrow of mice and the effects of interleukin-1α on the process, *Arch. Toxicol.,* 69, 141–148, 1995.

North, R.J., The concept of the activated macrophage*, J. Immunol.,* 121, 806–809, 1978.

Nowak, M.A., Komarova, N.L., Sengupta, A., Jallepalli, P.V., Shih, L.-M., Vogelstein, B., and Lengauer, C., The role of chromosomal instability in tumor initiation, *Proc. Natl. Acad. Sci. USA,* 99, 16226–16231, 2002.

NRC (National Research Council), *Spacecraft Maximum Allowable Concentrations for Selected Airborne Contaminants*, Vol. 2 (Appendix B2), National Academy of Sciences, Washington, DC, 1996, pp. 39–103.

Ott, M.G., Townsend, J.C., Fishbeck, W.A., and Langner, R.A., Mortality among individuals occupationally exposed to benzene, *Arch. Environ. Health,* 33, 3–10, 1978.

Parke, D.V. and Williams, R.T.**,** Studies in detoxication 49. The metabolism of benzene containing [$^{14}C_1$] benzene, *Biochem. J.,* 54, 231–238, 1953.

Penati, F. and Vigliani, E.C., Sul problema delle mielopatie aplastische pseudoaplastische e leucemiche d benzolo, *Ras. Med. Ind.,* 9, 345–361, 1938.

Pfeifer, D. and Irons, R.D., Inhibition of lectin-stimulated lymphocyte agglutination and mitogenesis by hydroquinone: reactivity with intracellular sulfhydryl groups, *Exp. Mol. Pathol.,* 35, 189–198, 1981.

Pfeifer, D. and Irons, R.D., Effect of benzene metabolites on phytohemagglutinin-stimulated lymphopoiesis in rat bone marrow, *J. Reticuloendothel. Soc.,* 31, 155–170, 1982.

Post, G., Snyder, R., and Kalf, G.F., Inhibition of RNA synthesis and interleukin-2 production in lymphocytes *in vitro* by benzene and its metabolites, hydroquinone and p-benzoquinone, *Toxicol. Lett.,* 29, 161–167, 1985.

Post, G., Snyder, R., and Kalf, G.F., Metabolism of benzene and phenol in macrophages *in vitro* and the inhibition of RNA synthesis by benzene metabolites, *Cell Biol. Toxicol.,* 2, 231–246, 1986.

Punjabi, C.J., Laskin, D.L., Heck, D.E., and Laskin, J.D., Production of nitric oxide by murine bone marrow cells, *J. Immunol.,* 149, 2179–2184, 1992.

Punjabi, C.T., Laskin, J.D., Hwang, S.M., MacEachern, L., and Laskin, D.L., Enhanced production of nitric oxide by bone marrow cells and increased sensitivity to macrophage colony-stimulating factor (CSF) and granulocyte-macrophage CSF after benzene treatment of mice, *Blood,* 83, 3255–3263, 1994.

Pyatt, D.W., Stillman, W.S., and Irons, R.D., Hydroquinone. A reactive metabolite of benzene, inhibits NF-κB in primary human CD4$^+$ T Lymphocytes, *Toxicol. Appl. Pharamcol.,* 149, 178–184, 1998.

Ranaldi, R., Bassani, B., Villani, P., Lombardi, C.C., Tanzarella, C., and Pacchierotti, F., Measurement and characterisation of micronuclei in cultured primary lung cells of mice following inhalation exposure to benzene, *Mutagenesis,* 13, 453–460, 1998.

Reinhardt, C.F., Azar, A., and Maxfield, M.E., Cardiac arrhythmias and aerosol sniffing, *Arch. Environ. Health,* 22, 265–279, 1971.

Renz, J.F. and Kalf, G.F., Role for interleukin-1 (IL-1) in benzene-induced hematotoxicity: uninhibition of conversion of pre-IL-1 α to mature cytokine in murine macrophages by hydroquinone and prevention of benzene-induced hematotoxicity in mice by IL-1 α, *Blood,* 78, 938–944, 1991.

Rinsky, R.A., Smith, A.B., Hornung, R., Filoon, T.G., Young, R.J., Okun, A.H, and Landrigan, P., Benzene and Leukemia. An epidemiological risk assessment, *N. Engl. J. Med.,* 316, 1044–1050, 1987.

Rinsky, R.A., Young, R.J., and Smith, A.B., Leukemia in benzene workers, *Am. J. Ind. Med.,* 2, 217–245, 1981.

Robertson, M.L., Eastmond, D.A., and Smith, M.T., Two benzene metabolites, catechol and hydroquinone, produce synergistic induction of micronuclei and toxicity in cultured human lymphocytes, *Mutat. Res.,* 249, 201–209, 1991.

Rosenthal, G.J. and Snyder, C.A., Modulation of the immune response to *Listeria monocytogenes* by benzene inhalation, *Toxicol. Appl. Pharmacol.,* 80, 502–510, 1985.

Ross, D., Siegal, D., Gibson, N.W., Pacheco, D., Thomas, D.J., Reasor, M., and Wierda, D., Activation and deactivation of quinones catalyzed by DT-diaphorase. Evidence for bioreductive activation of diaziquone (AZQ) in human tumor cells and detoxification of benzene metabolites in bone marrow stroma, *Free Radic. Res. Commun.,* 8, 373–381, 1990.

Rozen, M., Snyder, C.A., and Albert, R.E., Depression in B- snf T-lymphocyte mitogen-induced blastogenesis in mice exposed to low concentrations of benzene, *Toxicol. Lett.,* 20, 343–349, 1984.

Sammett, D., Lee, E.W., Kocsis, J.J., and Snyder, R.. Partial hepatectomy reduces both metabolism and toxicity of benzene, *J. Toxicol. Environ. Health,* 5, 785–792, 1979.

Santesson, C.G., Über chronischeV Toxicol. Appl. Pharmacol. ergiftungen mit Steinkohlenteerbenzin: vier Todesfälle, *Arch. Hyg. Berl.,* 31, 336–376, 1897.

Savilahti, M., More than 100 cases of benzene intoxication in a shoe factory. Observations on hematological symptoms and course of the process in one year, *Arch. Gewerbepath. Gerwerbehyg.,* 15, 147–157, 1956.

Schlosser, M.J., Shurina, R.D., and Kalf, G.F., Prostaglandin H synthesis catalyzed oxidation of hydroquinone to a sulfhydryl-binding and DNA-damaging metabolite, *Chem. Res. Toxicol.,* 3, 333–339, 1990.

Seidel, H.J., Barthel, E., and Zinser, D., The hematopoietic stem cell compartments in mice during and after long-term inhalation of three doses of benzene, *Exp. Hematol.,* 17, 300–303, 1989.

Selling, L., A preliminary report on some cases of purpura hæmorrhagica due to benzol poisoning, *Johns Hopkins Hosp. Bull.,* 21, 33–37, 1910.

Selling, L., Benzol as a leucotoxin. Studies on the degeneration of the blood and haematopoietic organs, *Johns Hopkins Hosp. Rep*., 17, 83–148, 1916.

Simonds, J.P. and Jones, H.M., The effect of injections of benzol upon the production of antibodies, *J. Med. Res.,* 33, 197–209, 1915.

Smyth, H.F., Carpenter, C.P., Weil, C.S., Pozzani, U.C., and Striegel, J.A., Range finding toxicity data: list VI, *Ind. Hyg. J.,* March-April, 95–107, 1962.

Snyder, C.A., Goldstein, B., Sellakumar, A., Bromberg, I., Laskin, S., and Albert, R., The inhalation toxicology of benzene incidence of hematopoietic neoplasms and hematotoxicity in AKR/J and C57Bl/6J mice, *Toxicol. Appl. Pharmacol.,* 54, 323–331, 1980.

Snyder, C.A., Goldstein, B., Sellakumar, A., Bromberg, I., Laskin, S., and Albert, R., Toxicity of chronic benzene inhalation: CD-1 mice exposed to 300 ppm, *Bull. Environ. Contam. Toxicol.,* 29, 385–391, 1982.

Snyder, C.A., Goldstein, B., Sellakumar, A., Nohnan, S., Bromberg, I., and Laskin, S., Hematotoxicity of inhaled benzene to Sprague-Dawley rats and AKR mice at 300 ppm, *J. Toxicol. Environ. Health,* 4, 605–618, 1978.

Snyder, R., Benzene and leukemia, *Crit. Rev. Toxicol.,* 32, 155–210, 2002.

Snyder, R. and Hedli, C.C., An Overview of Benzene Metabolism, *Environ. Health Perspect.,* 104, 1165–1171, 1996.

Snyder, R. and Kocsis, J.J, Current concepts of chronic benzene toxicity, *CRC Crit. Rev. Toxicol.,* 3, 265–288, 1975.

Snyder, R., Witz, G., and Goldstein, B.D., The toxicology of benzene, *Environ. Health Perspect.,* 100, 293–306, 1993.

Spaulding, J.W., French, J.E., Furedi-Machachek., M., Haseman, J.K., and Tennant, R.W., Development of a transgenic mouse model for carcinogenesis bioassays:evaluation of chemically induced skin tumors in Tg.AC mice, *Toxicol. Sci.,* 49, 241–254, 1999.

Srbova, J., Teisinger, J., and Skramovsky, S., Absorption and elimination of inhaled benzene in man, *Arch. Ind. Hyg. Occup. Med.,* 2, 1–8, 1950.

Stillman, W.S., Varela Garcia, M., and Irons, R.D., The benzene metabolite, hydroquinone, selectively induces 5q31- and -7 in human CD34+CD19- bone marrow cells, *Exp. Hematol.,* 28, 169–176, 2000.

Styles, J.A. and Richardson, C.R., Cytogenetic effects of benzene: dosimetric studies on rats exposed to benzene vapour, *Mutat. Res.,* 135, 203–209, 1984.

Svirbely, J.L., Dunn, R.C., and Von Oettingen, W.F., The acute toxicity of vapors of certain solvents containing appreciable amounts of benzene and toluene, *J. Ind. Hyg. Toxicol.,* 25, 366–373, 1943.

Svirbely, J.L., Dunn, R.C., and Von Oettingen, W.F., The chronic toxicity of moderate concentrations of benzene and of mixtures of benzene and its homologues for rats and dogs, *J. Ind. Hyg. Toxicol.,* 26, 37–46, 1944.

Tegeris, J.S. and Balster, R.L., A comparison of the acute behavioural effects of alkylbenzenes using a functional observation battery in mice, *Fundam. Appl. Toxicol.,* 22, 240–250, 1994.

ten Berge, W.F., Zwart, A., and Appleman, L.M., Concentration-time mortality relationship of irritiant and systemically acting vapours and gases*, J. Hazard. Mater.*, 13, 301–309, 1986.

Thomas, D.J., Sadler, A., Subrahmanyam, V.V., Siegel, D., Reasor, M.J., Weirda, D., and Ross, D., Bone marrow stromal cell bioactivation and detoxification of benzene the metabolite hydroquinone: comparison of macrophages and fibroblastoid cells, *Mol. Pharmacol.,* 37, 255–262, 1990.

Tice, R.R., Costa, D.L., Drew, R.T., Cytogenetic effects of inhaled benzene in murine bone marrow: induction of sister chromatid exchanges, chromosomal aberrations, and cellular proliferation inhibition in DBA/2 mice, *Proc. Natl. Acad. Sci.* USA*.,* 77, 2148–2152, 1980.

Till, J.E. and McCulloch, A.E., A direct measure of the radiation sensitivity of normal mouse bone marrow cells, *Radiat. Res.,* 14, 213–222, 1961.

Uyeki, E.M., Ashkar, A.E., Shoeman, D.W., and Bisel, T.U., Acute toxicity of benzene inhalation to hemopoietic precursor cells, *Toxicol. Appl. Pharmacol.,* 40, 49–57, 1977.

Vigliani, E.C. and Forni, A., Benzene, chromosome changes and leukemia. Letter to the Editor, *J. Occup. Med.,* 11, 148–149, 1969.

Vigliani, E.C. and Forni, A., Benzene and leukemia, *Environ. Res.,* 11, 122–127, 1976.

Vigliani, E.C. and Saita, G., Benzene and leukemia, *N. Engl. J. Med.,* 271, 872–876, 1964.

Von Oettingen, W.F., *Public Health Bulletin 255,* U.S. Public Health Service, Washington, DC, 1940.

Weiskotten, H.G., Schwartz, S.C., and Steensland, H.S., The action of benzol I. On the significance of myeloid metaplasia of the spleen, *J. Med. Res.,* 33, 127–140, 1915–16.

Weiskotten, H.G., Schwartz, S.C., and Steensland, H.S., The action of benzol II. The deuterophase of the diphasic leucopenia and antigen-antibody reaction, *J. Med. Res.,* 35, 63–69, 1916–17.

Weiskotten, H.G. and Steensland, H.S., The action of benzol IV. Spontaneous infections with special reference to the diphasic leucopenia (rabbit), *J. Med. Res.,* 37, 215–223, 1917.

Weiskotten, H.G. and Steensland, H.S., The action of benzol V. The diphasic leucopenia as a polynuclear amphophile phenomenon (rabbit), *J. Med. Res.,* 39, 485–494, 1918–19.

Weiskotten, H.G., Wyatt, T.C., and Gibbs, R.F.D., The action of benzol.VI. Benzol vapor leucopenia (rabbit), *J. Med. Res.,* 41, 425–438, 1920.

Weiskotten, H.G., Wyatt, T.C., and Gibbs, R.F.D., The action of benzol VII. Thrombocytopenia and thrombocytosis coincident with marrow necrosis and marrow regeneration (rabbit), J. *Med. Res.,* 44, 593–599, 1924.

Weiss, L., The hematopoietic microenvironment of the bone marrow: an ultrastructural study of the stroma in rats, *Anat. Rec.,* 186, 161–184, 1976.

White, W.C. and Gammon, A.M., The influence of benzol inhalations on experimental tuberculosis in rabbits, *Trans. Assoc. Am. Phys.,* 29, 332, 1914.

Wierda, D. and Irons, R., Hydroquinone and catechol reduce the frequency of progenitor B lymphocytes in mouse spleen and bone marrow, *Immunopharmacology,* 4, 41–54, 1982.

Wierda, D., Irons, R., and Greenlee, W., Immunotoxicity in C57Bl/6 mice exposed to benzene and arochlor 1254, *Toxicol. Appl. Pharmacol.,* 60, 410–417, 1981.

Wolf, M.A., Rowe, V.K., McCollister, D.D., Hollingsworth, R.L., and Oyen, F., Toxicological studies of certain alkylated benzenes and benzene, *A.M.A. Arch. Ind. Health,* 14, 387–398, 1956.

Yin, S., Li, G., Hu, Y., Zhang, X., Jin, C., Inoue, O., Seije, K., Kasahara, M., Nakatsuka, H., and Ikeda, M., Symptoms and signs of workers exposed to benzene, toluene or the combination, *Ind. Health.,* 25, 113–130, 1987.

Yin, S.-N., Hayes, R.B., Linet, M.S., Li, G.-L., Dosemeci, M., Travis, L.B., Zhang, Z.-N. Li, D.-G., Chow, W.-H., Wacholder, S., Blot, W.J., The Benzene Study Group, An expanded cohort study of cancer among benzene-exposed workers in China, *Environ. Health Perspect.,* 104 (Suppl. 6), 1339–1341, 1996.

Yin, S.-N., Li, Q., Liu, Y., Tian, F., Du, C., and Jin, C., Occupational exposure to benzene in China, *Br. J. Ind. Med.,* 44, 192–195, 1987.

Yin, S.-N., Li, G.-L., Tain, F.-D., Fu, Z.-I., Jin, C., Chen, Y.-J., Luo, S.-J., Ye, P.-Z., Wang, G.-C., Zhang, X.-C., Wu, H.-N., Zhong, Q.-C., A retrospective chort study of leukemia and other cancers in benzene workers, *Environ. Health Perspect.,* 82, 207–213, 1989.

Young, N.S. and Maciejewski, J.P., Aplastic anemia, in *Hematology*, 3rd ed., Hoffman, R., Benz, E.J., Jr., Shattil, S.J., Furie, B., Cohen, H.J., Silberstein, L.E., McGlace, P.. Eds., Churchill Livingstone, New York, 2000, pp. 314–315.

Zhang, L., Eastmond, D.A., and Smith, M.T., The nature of chromosomal aberrations detected in humans exposed to benzene, *Crit. Rev. Toxicol.,* 32, 1–42, 2002.

Zhang, J., Goldstein, B.D., and Witz, G., Iron-stimulated ring-opening of benzene in a mouse liver microsomal system. Mechanistic studies and formation of a new metabolite*, Biochem. Pharmacol.,* 50, 1607–1617, 1995a.

Zhang, J., Schafer, F., Schoenfeld, H., Cooper, K.R., Snyder, R., Goldstein, B.D. and Witz, G., The hematotoxic effects of 6-hydroxy-trans,trans-2,4-hexadienal, a reactive metabolite of trans,trans-muconaldehyde, in CD-1 mice, *Toxicol. Appl. Pharmacol.,* 132, 213–219, 1995b.

29 Carbon Monoxide

James J. McGrath

CONTENTS

29.1 INTRODUCTION

Carbon monoxide (CO), a gas produced by the incomplete combustion of carbon-based fuels including oil, gasoline, wood, charcoal, and coal, is the most ubiquitous pollutant in the lower atmosphere (Jaffee, 1970). Although CO was present in large quantities in the earth's primitive atmosphere (Hart, 1979) and produced and consumed by microorganisms long before early humans discovered fire, it was the use of fire and the concomitant production of CO in confined areas that made CO an environmental hazard. Although they could not, of course, identify CO, the ancient Greeks and Romans were aware of the toxic phenomenon associated with fire and lack of ventilation and used CO to both execute criminals and commit suicide.

Carbon monoxide poisoning is unique in its close association with the evolution of technology. Poisoning from CO increased drastically in the fifteenth century with the use of coal for domestic heating. The sharp increase resulted directly from inhalation of CO formed by incomplete combustion in home-heating appliances and explosions and fires in coal mines. Illuminating gas (a mixture of hydrogen, CO, and methane), introduced as a fuel for domestic heating, increased further the hazard of CO poisoning. In more recent years, the introduction of the internal combustion engine and the development of numerous technological processes that produce CO have contributed further to the hazards associated with this gas. Carbon monoxide poisoning is recognized as a major public health problem in as much as it may be responsible for more than one half of all fatal poisonings reported each year (Raub et al., 2000).

The effects of CO on health have been the subject of several recent reviews (Penney, 1996, 2000; U.S. Environmental Protection Agency [U.S. EPA], 2000). This chapter will consider (1) the sources of CO in the environment, (2) the transport of oxygen (O_2) in the blood and tissues, (3) the effects of CO on hemoglobin and myoglobin, (4) the uptake and elimination of CO, (5) other mechanisms of

CO toxicity, (6) the effects of CO at altitude, (7) the endogenous production of CO, (8) physiological aspects of CO, and (9) exposure limits for CO set by various agencies.

29.2 SOURCES OF CO IN THE ENVIRONMENT

Humans are exposed to CO produced and/or emitted by energy conversion processes, tobacco smoking, and normal physiological processes. Accordingly, exposure to CO may occur in both ambient air and during a variety of normal, occupational, recreational, or other human activities.

The U.S. EPA (2003) estimated that the total emissions of CO in the United States in 2001 was 120.8 million short tons (STN) (Table 29.1). Fuel combustion by on-road vehicles, including automobiles and trucks, the single largest emission category, contributed 74.8 million STN or 62% of the total. Nonroad engines, including lawn and garden, aircraft, railroads, and vessels, emitted 24.7 million STN or 20.4% of the total. Accordingly, the total emissions from internal combustion engines were 99.5 million STN per year or 82.4% of total CO emissions. The third major CO emission category was waste disposal and recycling. This grouping includes the combustion of wastes in municipal and other incinerators and from open burning of refuse. These sources emitted 3.2 million STN or 2.7% of total CO emissions.

Other categories comprise metals processing, electrical utilities, industrial, and other fuel combustion, other chemicals and allied products, petroleum and related industries, and storage and transport. These categories contributed 6.8 million metric tons or 5.4% of total CO emissions. The miscellaneous category includes emissions from forest wild fires, slash burning, agricultural fires, and structural fires. This grouping contributed 11.4 million STN or 9.4% of total CO emissions. Forest wildfires accounted for 7.1 million STN or 5.9% of total CO emissions. Residential wood burning produces approximately 2.5 million STN of CO or 2.1% of all CO emissions. Therefore, the internal combustion engine remains the principal anthropogenic source of CO.

Tobacco smoking was a major source of CO exposure for nonsmokers in the United States in the decades before the 1990s. Goldsmith (1970) estimated that 40–60% of the adults in any community were affected by cigarette smoking. However, increased awareness of the health risks posed by tobacco smoking to smokers and nonsmokers alike, and legislation restricting smoking on airplanes, public buildings, and in many restaurants has limited CO exposure.

Cigarette smoke contains up to 4% (40,000 ppm) CO by volume. Carbon monoxide inhaled during smoking is diluted with air so that the alveolar gas of smokers may contain 400–500 ppm CO. Smoke produced by a burning cigarette is divided into several smoke streams (Hoegg, 1972). Mainstream smoke, the smoke drawn directly into the lungs has received the most attention. All the other smoke

TABLE 29.1 Nationwide Carbon Monoxide Emission Estimated for 2001

Source category	Short tons (10^6)	% of total
On-road vehicles	74.8	62.0
Nonroad engines and vehicles	24.7	20.4
Waste disposal and recycling	3.2	2.7
Metals processing	1.4	1.1
Fuel combustion electrical Utility	0.5	0.4
Fuel combustion industrial	1.2	1.0
Fuel combustion other	2.9	2.4
Chemical and allied products	0.4	0.3
Petroleum and related industries	0.2	0.1
Storage and transport	0.2	0.1
Miscellaneous	11.4	9.4

Source: U.S. EPA (2003).

streams are emitted into the environment and are responsible for the health concerns associated with "passive smoking," i.e., the inhalation of environmental tobacco smoke (ETS) by nonsmokers in the vicinity of a smoker. Side-stream smoke generated by the burning end of the cigarette during the puff interval contributes approximately 95% of the total smoke, whereas the smolder stream, also generated during the puff interval, contributes 4%. The remaining smoke is emitted from the glowing end of the cigarette during the puff (the glow stream), from the sides of the cigarette during the puff (the effusion stream), and from the sides of the cigarette between puffs (the diffusion stream). Although the amount of CO produced by cigarette smoking constitutes a minor percentage of the total atmospheric burden, smoking in closed spaces can significantly elevate CO levels (U.S. Department of Health, 1979).

The major determinants of CO levels caused by smoking are the size of the space in which smoking occurs, the number and type of tobacco products smoked, and the amount and effectiveness of ventilation. Hoegg (1972) reported side stream and mainstream values for CO produced by a machine smoking cigarettes in a 25-m^3 nonventilated chamber. There was a linear relationship between the number of cigarettes smoked and the chamber CO concentration. Smoking 24 cigarettes produced a chamber concentration of 69.8 ppm CO. Side stream smoke and mainstream smoke from a single cigarette contained 75.5 and 16.0 ml of CO, respectively. The side stream-mainstream ratio for CO was 4.7. This study separated mainstream smoke from side stream smoke and demonstrated that side stream smoke is an important source of CO in a closed environment.

Coburn et al. (1964) reported on the effects of smoking on the buildup of CO in confined areas. In hospital wards where smoking was prohibited, CO concentrations averaged 2.2 ppm (but reached 5 ppm). Air taken from smoke-filled conference rooms contained from 4.3 to 9.0 ppm CO. Ten cigarettes burned in a small room produced an atmospheric concentration of 20 ppm CO.

Lawther and Commins (1970) demonstrated the potential for exposure to CO from "passive smoking" in a nonsmoker wearing a CO sensor seated next to a smoker in a chamber. The chamber CO concentration rose to 20 ppm in 1 h after the smoking of seven cigarettes. Transient peaks of up to 90 ppm CO were measured in the breathing zone of the nonsmoker. Russell et al. (1973) reported an increase in mean COHb levels of 1.6–2.6% in 12 nonsmokers sitting in a smoke-filled room with six smokers. Furthermore, they suggested that the amount of CO absorbed by a nonsmoker in these experiments was approximately equal to that absorbed by a person who had smoked and inhaled one cigarette.

A variety of personnel are exposed to elevated CO levels during the course of a workday. Workers in industries such as casting, welding, drying or preheating, boiler cleaning, blast furnace operations, or transport of hot coke or slag are exposed to high levels of CO. Occupational groups involved with vehicles, metal processing, chemical processing, stone and glass processing, printing, welding, electrical assembly and repair work, and certain types of graphic artwork have been found to have average COHb levels in excess of 2% (Wright and Shephard, 1979). Lawther and Commins (1970) reported elevated COHb levels in policemen, garage workers, tollbooth operators, greenhouse workers, and firemen. However, they concluded that in all groups, with the possible exception of greenhouse workers, COHb levels were elevated more by cigarette smoking than by occupational exposure.

Davies (1975) reported on CO concentrations in a submarine. In a nuclear submarine, 75–90% of the total CO load is from tobacco smoking. In a Polaris submarine with a 150-man crew, 100 smokers produce approximately 200 l of CO in a breathable air volume of 4000 m^3. In current nuclear submarine operations, the mean CO level for more than 90% of diving time varies between 5 and 15 ppm. Bondi et al. (1978) reported ambient concentrations aboard a submarine of 7 ppm and COHb levels of 2.1, 1.7, and 1.7% in nonsmokers at the start, middle, and end of a 40-day patrol. Seufert and Kiser (1996) reported that, after a 62-h submergence, expired CO levels in nonsmokers approached the presubmergence levels of smokers who smoked 21 cigarettes per day. The mean CO concentration increased from 2.6 before submergence to 9.2 ppm at the end of the 62-h dive.

Significant amounts of CO can be generated in enclosed areas by ice-resurfacing machines. Johnson et al. (1965) reported that operation of a propane-powered ice-resurfacing machine produced average values of 157–304 ppm CO in an ice-skating arena. Anderson (1971) measured CO concentration up to 250 ppm in an ice-skating rink after an episode of illness among skaters marked by headache and nausea.

Under certain conditions, closed-circuit anesthetic machines using volatile anesthetics can become a source of CO exposure. When the CO_2 absorbent (soda lime) is dry, significant production of CO can result from the degradation of the anesthetic. Exposure of a patient to CO can result in COHb levels of up to 7% (Woehlck et al., 1997a, 1997b).

The possibility of risking elevated COHb levels without being exposed to CO also exists. Stewart et al. (1972) reported on a coworker who had breathed varnish remover vapors while "stripping" furniture. Further study revealed that the methylene chloride in the varnish metabolized in the body to CO, producing elevated COHb levels. This observation was confirmed by Ratney et al. (1974), who reported COHb levels of 9% in factory workers exposed to methylene chloride levels of 180–200 ppm. On the basis of their study, Ratney recommended that the allowable limits of exposure to methylene chloride be decreased to avoid body burdens of CO greater than those allowed persons exposed to exogenous CO.

29.3 OXYGEN TRANSPORT

29.3.1 In the Blood

An understanding of the transport of O_2 within the body is essential to understand the mechanisms of CO toxicity. Oxygen is transported from the lungs to the tissues in reversible combination with hemoglobin (Hb), a conjugated protein molecule in which the protein, globin, is joined with heme, an iron-porphyrin moiety. The Hb molecule is remarkable; it reversibly binds O_2 in the lungs, where the concentration of O_2 is high and releases it in the tissues where O_2 is low. The amount of O_2 transported from the lungs to the tissues as oxyhemoglobin (O_2Hb) depends on the Po_2 in the inhaled air, the amount of functional Hb, and the resulting percentage of Hb saturated with O_2.

The relationship between Po_2 and Hb saturation with O_2 is depicted by the sigmoidal-shaped O_2 Hb dissociation curve (Figure 29.1). Two regions of the normal curve are of particular interest. In the flatter region between O_2 tensions of 70–100 mmHg, there is little change in O_2Hb saturation or arterial blood O_2 content with changes in Po_2; a decrease in Po_2 from 100 to 70 decreases Hb saturation to only about 93%. This property of Hb enables a subject to experience a moderate reduction in Po_2 without much of a reduction in the amount of O_2 transported by the blood to the tissue.

The steeper region between O_2 tensions of 10 and 40 mmHg, is the Po_2 range found in metabolically active tissues. Because the amount of O_2 that Hb can bind depends on the local Po_2, O_2Hb dissociates in this region and releases O_2 to the metabolizing tissue. At a Po_2 of 40 mmHg, Hb saturation is only 75%. If the Po_2 is decreased further to 10 mmHg, Hb saturation decreases to about 13%. In this way, the flat, upper part of the curve protects the body by enabling blood to load O_2 in the lungs over a broad range of Po_2 values, whereas the steep middle and lower parts protect the metabolizing tissues by causing the blood to unload large amounts of O_2 in the tissues with relatively small decreases in Po_2. In healthy subjects at sea level only the upper portion of the O_2Hb dissociation curve is used in unloading O_2; the lower part may be viewed as a reserve that is drawn on only during exercise or in pathological conditions.

29.3.2 In the Tissues

Oxygen dissociates from Hb in the tissues and diffuses through the plasma, capillary endothelium, and interstitium and enters the myocyte, where it is transported to the mitochondria (McGrath, 2000b). The plasma, capillary wall, interstitial fluid, and sarcolemma of the cell do not contain a heme protein for transporting O_2; the cardiac and muscle myocytes do. Oxygen is transported through the cell cytoplasm of the myocyte to the mitochondria via two simultaneous pathways: direct diffusion and myoglobin (Mb)-facilitated diffusion (Wittenberg and Wittenberg, 1987). Within the mitochondria, the O_2 enters into a series of reactions whereby ATP is produced. ATP, in turn, diffuses out of the mitochondria and powers the numerous energy-requiring activities of the cell, among which is contraction of the myocyte. During myocyte contraction, chemical energy, released by the breakdown of ATP, is converted to mechanical work. The continued production of ATP depends on the uninterrupted

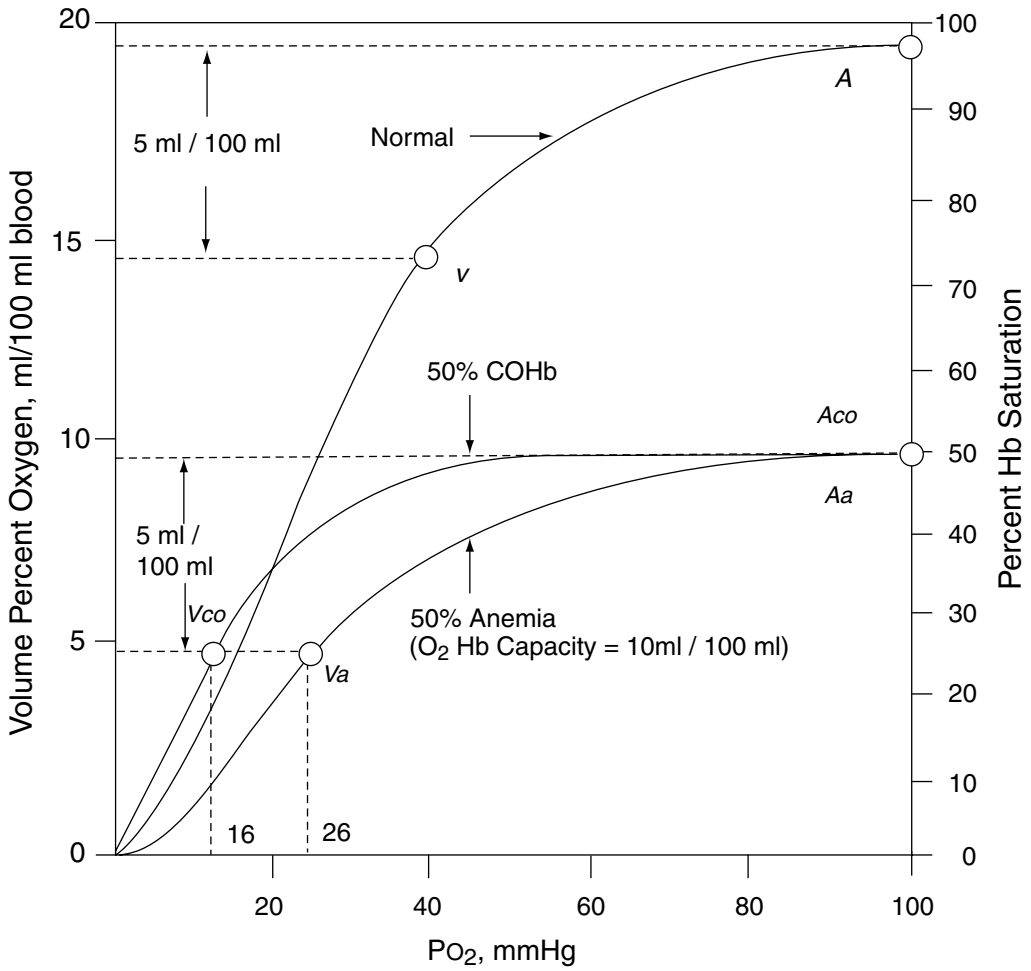


FIGURE 29.1 Oxyhemoglobin dissociation curve of normal human blood, blood with 50% Hb due to anemia, and of blood containing 50% COHb (U.S. EPA 2000).

delivery of O_2 to the mitochondria. Hence, Mb serves as a critical link between the capillary supply of O_2 provided by circulating Hb and the production of ATP by the O_2-consuming cytochromes in the mitochondrial membrane.

Myoglobin is an intracellular iron-containing protein, which binds O_2 reversibly (Wittenberg and Wittenberg, 1989) with a greater affinity than Hb (Roughton, 1964), as a result Mb takes up O_2 from Hb in the blood. The dissociation curve for Mb is a rectangular hyperbola that lies to the left of the sigmoidal-shaped curve for Hb. Myoglobin is a small molecule compared with Hb (17,000 vs. 68,000 Da) with sufficient intracellular mobility to diffuse rapidly and contribute substantially to the total intracellular O_2 flux. Although Mb diffuses at 1/20th the rate of free O_2, the fluxes of free and Mb-bound O_2 are of the same order (Wittenberg and Wittenberg, 1990) because the Mb concentration in the myocyte exceeds the free O_2 concentration.

The main functions of Mb are to temporarily "store" and facilitate the diffusion of O_2 between Hb and the tissues (Peters et al., 1994). Myoglobin has a CO affinity constant approximately eight times lower than Hb ($M = 20$ to 40 versus 218, respectively) (Coburn and Mayers, 1971; Haab, 1990). As with Hb, the combination velocity constant between CO and Mb is only slightly lower than for O_2, but the dissociation velocity constant is much lower. The combination of greater affinity (Mb is 90% saturated at P_{O_2} of 20 mmHg) and lower dissociation velocity constant for CO favors retention of CO

in the muscular tissue. Thus, a considerable amount of CO can be potentially stored in the skeletal muscle (Luomanmaki and Coburn, 1969).

29.4 MECHANISM(S) OF CARBON MONOXIDE TOXICITY

29.4.1 Effects on Hemoglobin

Claude Bernard described the classic mechanism of CO toxicity in 1857 (Bernard, 1857) when he determined that CO produces hypoxia through its effect on O_2 transport. Carbon monoxide combines with Hb to form COHb; this reaction is rapid and the affinity of CO for Hb is many times greater than the affinity of O_2 for Hb (Root, 1965). However, binding of O_2 to Hb is complex; the greater the number of heme molecules bound to CO, the greater is the affinity of the unbound hemes for O_2. In this way, CO reduces the amount of available O_2 not only by occupying O_2-binding sites, but also by altering the characteristic S-shaped relationship between P_{O_2} and O_2Hb in normal blood. The affinity of CO for Hb, represented by the Haldane coefficient, *M*, is about 218 (210–250) times greater than the affinity of O_2 for Hb (U.S. EPA, 2000). However, the rates of Hb binding and dissociation are slower for CO than for O_2. Accordingly, under steady-state conditions, one part of CO and 218 parts of O_2 would form equal parts of O_2Hb and COHb. The Haldane relationship (Guttierez, 1982) is:

$$COHb / O_2Hb = M (P_{CO} / P_{O_2})$$

Where:

COHb = carboxyhemoglobin
O_2Hb = oxyhemoglobin
P_{CO} = partial pressure of CO
P_{O_2} = partial pressure of O_2

The classic mechanism of CO toxicity is illustrated in Figure 29.1. Points A and V represent the arterial and venous P_{O_2} values, respectively, in a healthy individual with a normal O_2Hb dissociation curve. At a mixed venous P_{O_2} of 40 mmHg (Point V), 5 ml of O_2 per 100 ml of blood is delivered to the metabolizing tissue. In an anemic subject, where the O_2-carrying capacity of the blood is reduced to 10 ml/100 ml (Aa), venous P_{O_2} must drop to 26 mmHg (Point Va) to release 5 ml of O_2 per 100 ml of blood to the tissues. In contrast, in a person poisoned by CO (50% COHb), where the O_2-carrying capacity of the blood is reduced to 10 ml/100 ml (Aco), the venous P_{O_2} will have to drop to 16 mmHg (Vco, severe hypoxia) to release the same 5 vol% O_2. As the dissociation curve shifts to the left, its shape is transformed into a near-rectangular hyperbola.

Moreover, because the shift occurs over a critical saturation range for releasing O_2 to the tissues, a reduction in O_2Hb by CO binding will have more severe effects on O_2 release than an equivalent reduction in O_2Hb caused by anemia. Any further demand for O_2 under conditions such as exercise might result in hypoxia and loss of consciousness in the CO-poisoned individual. Thus, even though the content of O_2 in blood may be reduced to the same extent by anemia and CO exposure, the symptoms produced by CO poisoning are more severe because of the increased tenacity, induced by CO, with which the remaining O_2 is bound to the Hb molecule.

29.4.2 Effects on Myoglobin

Carbon monoxide is distributed throughout the body primarily bound to Hb and Mb. In the intravascular compartment (blood) CO binds with Hb to form COHb, and in the extravascular compartment (principally heart and skeletal muscle) CO binds with Mb to form carboxymyoglobin (COMb). Nearly all CO found in the body outside the vascular compartment is bound to Mb; there is little binding of CO to the cytochromes. At near-normal arterial PO_2, skeletal and cardiac muscle tissue

COMb and blood COHb were found to be similar (~1:1) (Coburn and Mayers, 1971; Coburn et al., 1973). However, this distribution of CO is altered markedly in certain conditions such as hypoxemia and hemorrhage (Coburn et al., 1973). Under these circumstances, CO leaves the intravascular compartment and enters the heart and skeletal muscle where it increases the concentration of COMb.

The observation that during hypoxia CO leaves the intravascular compartment and enters the extravascular compartment has been made repeatedly in diverse experimental models (McGrath, 2000a). In experiments using ^{14}CO in anesthetized dogs, Luomanmaki and Coburn (1969) observed that when arterial P_{O_2} was increased from 50 to 500 mmHg radioactivity in the blood remained unchanged; however, as arterial P_{O_2} was decreased to less than 40 mmHg, radioactivity decreased to levels as low as 50% of control. When arterial P_{O_2} was returned to normal, the ^{14}CO reentered the blood.

These workers also studied the shift of CO out of the blood during hypoxia by measuring the rate of increase of blood COHb when CO was administered, at a constant rate, into the rebreathing system. They found that COHb increased at a constant rate up to a saturation of 50%. With additional CO, however, COHb increased at a slower rate, suggesting that proportionally greater quantities of CO were going in to extravascular stores. The rate of COHb buildup became nonlinear at a COHb level of 50%, corresponding to an arterial P_{O_2} of 80 mm Hg (Coburn, 1970).

In a study designed to estimate intracellular P_{O_2} during exercise, Clark and Coburn (1975) measured the effects of hypoxia on compartmental shifts of CO between blood and muscle. In human subjects breathing 21% O_2 while exercising on a bicycle ergometer COMb/COHb increased 163% above baseline. When the subjects breathed 13–14% O_2 while exercising, COMb/COHb increased 210% above baseline. Carboxyhemoglobin levels decreased about 6% and 11%, respectively, in subjects breathing 21% and 13–14% O_2 during maximum exercise.

The shift of CO out of the blood during bouts of hypoxia has been confirmed in studies conducted on both men and women undergoing maximal aerobic capacity tests while inhaling CO (Horvath et al., 1988). Carbon monoxide shifted into extravascular spaces during maximum work but returned to the vascular space within 5 min after exercise stopped. Agostoni et al, (980) presented a theoretical model supporting these observations; they developed a series of equations predicting that with decreased venous P_{O_2}, CO moves out of the vascular compartment and into skeletal and heart muscle, increasing the formation of COMb in the tissues.

Binding of CO to Mb to form COMb in heart and skeletal muscle in vivo has been demonstrated at levels of COHb below 2% in heart and 1% in skeletal muscle (Coburn and Mayers, 1971; Coburn et al., 1973). At rest, the COMb/COHb ratio (0.4 to 1.2) does not increase with an increase in COHb up to 50% saturation and appears to be independent of the duration of exposure (Sokal et al., 1984). During exercise, however, the relative rate of CO binding increases more for Mb than for Hb, and CO diffuses from blood to skeletal muscle (Werner and Lindahl, 1980); consequently, the COMb/COHb increases for both skeletal and cardiac muscles (Sokal et al., 1986). During heavy smoking (COHb levels of 10%), as much as 30% of the cardiac Mb may be saturated with CO (Coburn, 1970). A similar shift in CO has been observed during hypoxia because a fall in myocyte intracellular P_{O_2} below a critical level increases the relative affinity of Mb for CO (Coburn and Mayers, 1971). The consequent reduction in of Mb function (O_2 storage and facilitated O_2 diffusion) may have a profound effect on O_2 transport within the tissue.

Thus, CO can interrupt O_2 delivery to the mitochondria by combining with two distinct transport proteins located at two different sites in the O_2 delivery pathway. In the intravascular compartment, CO reduces the O_2 carrying capacity of the blood and O_2 delivery by combining with Hb to form COHb. In the intravascular compartment CO reduces the intracellular transport of O_2 to the mitochondria by combing with Mb to form COMb.

29.5 UPTAKE AND ELIMINATION OF CARBON MONOXIDE

Carbon monoxide is transported from the airways to the Hb in the red blood cell (RBC) entirely by physical processes; transport from the airway to the alveoli is in the gas phase while transport across

the air–blood interface is in the liquid phase. In the gas phase, the key mechanisms of transport are convective flow created by the mechanical action of the respiratory muscles and diffusion in the alveolar region of the lung. In the liquid phase, CO diffuses along a pressure gradient from the alveolus across the alveolocapillary membrane through the plasma, across the RBC membrane, and, finally, into the RBC stroma where reaction with Hb can take place (Figure 29.2).

Carbon monoxide is rapidly exchanged between the air and blood compartments because of the enormous surface area and short diffusion distances of the lung, and the steep CO concentration gradient between air and blood. The rapid binding of CO to Hb keeps the partial pressure of CO within the RBC low and maintains a pressure gradient between air and blood.

Inhalation of CO-free air reverses the gradient (higher CO pressure in the blood than in alveolar air) and CO diffuses back into the alveolus. During CO inhalation the air–blood gradient for CO is much higher than the blood–air gradient; therefore, CO uptake will be a proportionately faster process than CO elimination. The rate of formation and elimination of COHb and its concentration in blood is controlled by numerous physical factors and physiological mechanisms. The relative

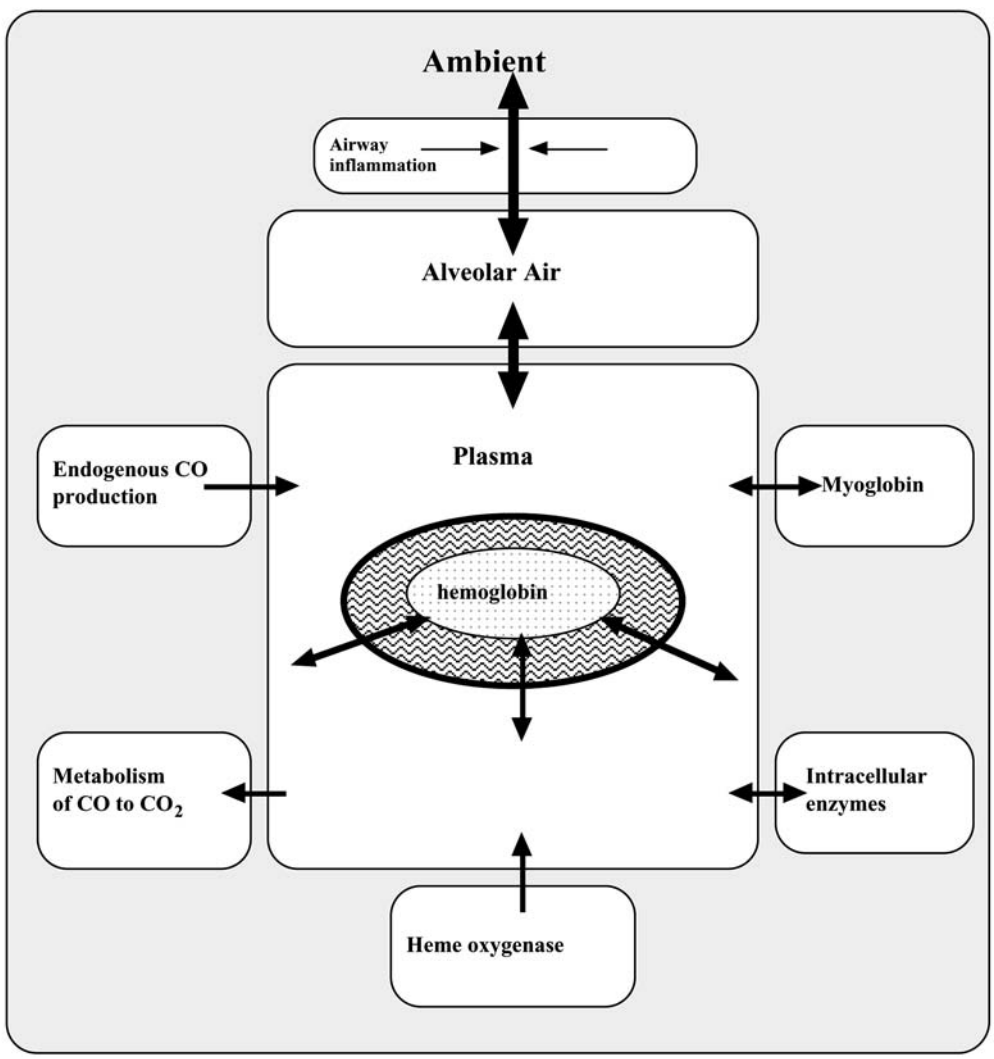


FIGURE 29.2 Principle transport pathways and body stores of carbon monoxide (U.S. EPA 2000).

contribution of these mechanisms to overall COHb kinetics is determined by environmental conditions, the physical activity of an individual, and other complex physiological processes. During exercise, the two most important physiological variables are the alveolar ventilation and cardiac output. Both of these mechanisms increase CO uptake by maintaining a high CO gradient. Consequently, the rates of CO uptake and COHb formation will be proportional to the intensity of exercise.

Although the lung is the major route by which CO enters the body, little CO actually diffuses into the lung tissue parenchyma. The epithelium of the airways presents a significant barrier to CO diffusion, and even at high CO concentrations diffusion and uptake of CO by the lung parenchyma is low. The rate at which CO dissolved in the mucosa of the airways will diffuse into the submucosa and interstitium depends on the concentration and duration of CO exposure and on the surface area of the respiratory tract. Exposures of the oronasal cavity in monkeys to high concentrations of CO ($>$400 ppm) for a short period (5 s) increased the blood COHb level to $<$3.5%. Exposures of the whole lung, however, increased COHb to almost 60% (Schoenfisch et al., 1980). Thus, diffusion of CO across the airway mucosa contributes little to overall COHb concentration.

Although there is extensive information on the rate of CO uptake and COHb formation, the information on the elimination of CO from the blood and body stores is more limited. Elimination of CO from blood is a much slower process (Pace et al., 1950). The half-time for CO loss from the blood among individuals breathing air varies considerably. For COHb concentrations of 2–10%, the reported half-times range from 2 to 6.5 h (Peterson and Stewart, 1970; Landaw, 1973). The half-time for CO elimination in nonsmokers is longer in men (4.5 h) than in women (3.2 h) (Deller et al., 1992). During sleep the elimination rate decreases to 8 h in men and 4.3 h in women. The day-to-night differences have been attributed to lower ventilation rates during sleep and the lower muscle mass and, therefore, lower Mb levels in women.

Normobaric O_2 administered to fire victims and CO-poisoned individuals resulted in about the same CO elimination half-time, 91 and 87 min, respectively (Levasseur et al., 1996). Inhaling higher O_2 concentrations increased the elimination of CO; breathing 100% O_2, shortened the half-time for elimination by almost 75% (Peterson and Stewart, 1970). The average half-time for COHb elimination in individuals with COHb levels (1.16%) breathing hyperbaric O_2 was 26 min, compared with 71 min when breathing normobaric O_2 (Jay and McKindley, 1997). Increasing Po_2 to 3 atm reduced the half-time to about 20 min, a 14-fold decrease over that seen when breathing room air (Landaw, 1973; Jay and McKindley, 1997). Although the washout of CO can be somewhat accelerated by an admixture of 5% CO_2 in O_2, hyperbaric O_2 treatment is more effective in facilitating displacement of CO. Therefore, hyperbaric O_2 is used as a treatment of choice in CO poisoning.

29.5.1 Other Mechanisms of Carbon Monoxide Toxicity

Symptoms of CO poisoning have been described from observations made on humans poisoned by CO, studies conducted on human volunteers in low-level exposure studies, and blood gas measurements made on acutely ill patients brought to the emergency room (Guttierez, 1982). They range from visual disturbances, headache, and decreased manual dexterity at COHb levels of 10–20% to stupor, convulsions and ultimately death at concentrations of 40% or greater. However, symptoms relating to CO exposure are often vague and some studies estimate that the diagnosis of CO poisoning is missed in 30% of cases presenting to the emergency room (Barret et al., 1985). Moreover, COHb levels are not always correlated with CO toxicity nor do they predict the risk for the development of long-term effects (Sokal, 1975; Sokal and Kralkowska, 1985; Lasater, 1986) leading some investigators to speculate that the Hb-mediated effect may not be the only mechanism of CO toxicity.

Although the classic mechanism of CO toxicity described thus far is accepted throughout the medical and scientific communities and provides the basis for treatment of CO intoxication, many observations suggest that other potential mechanisms may be important. Moreover, there is a large and growing database on the potential physiological role of CO as a gaseous messenger molecule.

The early studies suggesting other potential effects of CO (as opposed to a Hb-mediated effect) have been reviewed by McGrath (1982). These studies generally fall into four categories: dosimetry studies, tissue CO tension, isolated tissue studies, and altered lethality studies. In the dosimetry studies, CO is administered by different routes or rates to achieve the same COHb levels. In one such study, Chen and McGrath (1979) measured blood glucose and lactate levels in rats challenged with CO at two different rates to achieve the same COHb levels. One group inhaled 5000 ppm CO for 10 min (INH-I), a second group inhaled 2000 ppm for 40 min (INH-II), a third group received 100% CO i.p. Blood glucose and lactate concentrations were determined when COHb concentrations reached the same level (64–67%). Both groups of animals receiving CO by inhalation became unconscious at COHb levels of 60%, whereas rats receiving CO i.p. remained conscious throughout the experiment. Blood glucose levels were higher in the INH-I and INH-II (239 and 360 mg/dl, respectively) than the IP rats (196 mg/dl). Blood lactate levels increased in both groups of rats inhaling CO, but, remained virtually unchanged in the rats receiving CO i.p. These studies demonstrate that, despite similar COHb levels, CO delivered by inhalation is more stressful and elicits more powerful hyperglycemic and heart rate responses than CO delivered by injection.

In the CO tissue tension studies the partial pressure of CO is measured in the tissues of animals inhaling CO. A bubble of CO-free nitrogen is injected i.p. and the concentration of CO in the bubble is measured at various times. Using these techniques Göthert et al. (1970) found, in rabbits breathing 1000 ppm CO, concentrations of 154 ppm in the gas bubble after 2 h and 460 ppm after 25 h. These studies indicate that the partial pressure of CO in the tissues is significant and that unbound CO is available to exert other toxic effects.

In the isolated tissue studies, the effects of CO are assessed on a tissue suspended in or perfused with a Hb-free salt solution equilibrated with CO. Using these techniques, McFaul and McGrath (1987) demonstrated the vasodilatory effects of CO on the coronary circulation of isolated rat hearts. Moreover, vasodilation was not the result of decreased O_2 content in the perfusate and was not mediated by adrenergic influences, adenosine, or prostaglandins. In a later study they demonstrated the effects to be due to the stimulating effects of CO on cyclic guanine monophosphate (GMP) levels (Ramos et al., 1989).

In the lethality studies, the lethality of CO was altered by factors that did not affect COHb levels. In one such study, Winston et al. (1974) described the effects of pretreatment with several drugs on the lethality of inhaled CO. Mice were pretreated with ethanol, chlorpromazine, or pentobarbital and then exposed for 4 h to 1900 ppm CO or hypoxia. Pretreatment with either chlorpromazine or ethanol increased both CO and hypoxia lethality, whereas pretreatment with phenobarbital had no effect on CO lethality but increased hypoxia lethality.

29.5.2 Reactive Oxygen Species

A rapidly growing database (U.S. EPA, 2000) indicates that injury from nitrogen- and O_2-based free radicals may be yet another mechanism of CO toxicity, during both CO exposure and the reoxygenation following exposure. Thus, Thom (1990) demonstrated that CO could cause brain lipid peroxidation in rats. Ninety minutes after CO exposure products of lipid peroxidation were increased in rats 75% over the baseline values. Unconsciousness was associated with a brief period of hypotension, but the hypotension per se caused no apparent injury. Lipid peroxidation was not correlated with COHb levels and occurred only after the animals were returned to CO-free air.

Exposure of rats to CO at concentrations of 20 ppm or more for 1 h caused platelets to release the nitric oxide free radical (CNO) (Thom et al., 1994). Similarly, cultured bovine pulmonary endothelial cells exposed to CO also release CNO, which can cause death by a CNO-related process occurring 18 to 24 h after the exposure (Thom and Ischiropoulos, 1997). The mechanism appears to be based on elevations in steady-state CNO concentrations and production of peroxynitrite (Thom et al., 1994, 1997). Peroxynitrite is a relatively long-lived, strong oxidant, produced by the reaction between NO and superoxide radical (Huie and Padmaja, 1993). Thus, exposure to environmentally

relevant concentrations of CO can cause platelets and endothelial cells to release NO and NO-derived oxidants, which can increase the production of substances that adversely affect cell physiology.

Carbon monoxide may cause vascular injury, the extent of which is determined by the concentration of CO and the duration of exposure. Leakage of albumin and leukocyte sequestration have been shown following exposures of rats to 50 ppm or more for 1 h, by a process mediated by CNO-derived oxidants (Thom, 1993; Ischiropoulos et al., 1996; Thom et al., 1999a, 1999b). Rats exposed to 1000–3000 ppm CO for 1 h demonstrate brain oxidative stress associated with this mechanism (Thom, 1993; Ischiropoulos et al., 1996).

The mechanism by which CO elevates steady-state CNO concentrations appears to be based on altering the intracellular "routing" of CNO in endothelial cells and platelets. It is known that the association and dissociation rate constants of CNO with hemoproteins exceed the rate constants for O_2 or CO. Moreover, CO incubated with CNO-Mb or CNO-Hb slowly displaced the CNO (Moore and Gibson, 1976). Carbon monoxide replacement occurred even with excess CNO-heme protein. Replacement rates were enhanced by increasing the CO concentration or by carrying out the reaction in the presence of thiols, which react with the liberated CNO. These conditions, including the presence of thiols, exist in cells exposed to CO. Exposures to up to 1070 nmol of CO did not alter the rate of production of CNO by platelets and endothelial cells, but did enhance liberation of CNO (Thom et al., 1994, 1997; Thom and Ischiropoulos, 1997). Studies with rats exposed to 50 ppm CO or more show that CO can increase the concentration of CNO available to react with cells in both lung and brain.

Carbon monoxide can elevate the steady-state concentration of nitric oxide (*NO)-derived oxidants in the lungs of rats (Thom et al., 1999b). Consequences from this change include increases in the concentration of reactive oxygen species, production of *NO-derived oxidants including peroxynitrite, and physiological evidence of lung injury. Lung capillary leakage increased significantly 18 h after rats had been exposed to 50 ppm CO or more for 1 h. Both *NO and H_2O_2 were elevated by CO.

Exposure to extremely high concentrations of CO also causes cellular changes that appear not to be related to hypoxia. Rats exposed to 10,000 ppm CO exhibit mitochondrial dysfunction characterized by impaired high-energy phosphate synthesis and production of hydroxyl-free radicals (Brown and Piantadosi, 1992; Piantadosi et al., 1995). Exposure to 2500 ppm CO causes hydroxyl radicals to be produced by the mitochondria, by a process that evidently could not be related to hypoxic stress (Piantadosi et al., 1997). Evidence for mitochondrial dysfunction has not been observed at lower CO concentrations *in vivo*. However, under conditions of high metabolic demand, exposure to even 1000 ppm CO in the absence of an overt hypoxic stress can result in impaired energy production in the brain (Meilin et al., 1996). Xanthine oxidase is an additional source of partially reduced O_2 species found in animals exposed to CO. Conversion of xanthine dehydrogenase, the enzyme normally involved with uric acid metabolism, to xanthine oxidase, the radical-producing form of the enzyme, occurred in the brains of rats exposed to approximately 3000 ppm CO (Thom, 1992). Lower CO concentrations did not trigger this change. Therefore, xanthine oxidase is unlikely to be a free radical source following exposures to CO at concentrations found in ambient air.

Carbon monoxide dilates blood vessels. Koehler et al. (1982) studied the effects of hypoxic hypoxia and CO hypoxia on cerebral blood flow in newborn lambs. Cerebral blood flow increased 47% more with CO hypoxia than hypoxic hypoxia. Portions of the observed increases in cerebral blood flow were independent of perturbations in O_2 supply. In a setting where cellular oxidative metabolism was not impaired by CO, elevations in cerebral blood flow appeared to be mediated by NO (Meilin et al., 1996).

Although cerebral vasodilation mediated by NO was reported with exposures to 1000 ppm CO, that level of exposure did not alter pulmonary vasoconstriction in isolated, perfused rat lungs (Cantrell and Tucker, 1996). Exposure to 150,000 ppm CO did not change pulmonary artery pressure in an *in situ*, blood-perfused preparation, although CO did inhibit hypoxic pulmonary vasoconstriction (Tamayo et al., 1997). In humans, exposure to CO for sufficient time to achieve COHb levels of approximately 8% did not alter forearm blood flow, blood pressure, or heart rate (Hausberg and Somers, 1997).

Exposure to 2000 ppm CO increased cerebral blood flow in the brain of awake rats (Mendelman et al., 2002). The stability of mitochondrial NADH redox level during the exposure indicated that tissue hypoxia did not develop. The elevation in blood flow was inhibited by a NOS inhibitor, indicating that NO was responsible for the CO-induced elevation in blood flow. The CO exposure also decreased pH and increased extracellular potassium ion, possibly due to ion-pump inhibition. The amplitude of the electrocorticogram wave activity decreased, indicative of compromised physiological activity. These changes were not observed in rats anesthetized with pentobarbital during the exposure, although the anesthesia did not effect the CO-induced elevation in blood flow nor change the mitochondrial NADH redox level. The authors concluded that CO acts by separate mechanisms to alter cerebral vasoactivity and neuronal metabolic responses and that both processes are independent of hypoxic stress.

Thus, CO, at levels found in the environment, has the ability to generate free radicals and these agents have the potential to cause pathophysiological changes or mediate physiological processes.

29.5.3 The Effects of Carbon Monoxide at Altitude

Carbon monoxide poisoning, commonly encountered in a variety of indoor and outdoor settings, poses a special danger at altitude (McGrath, 2000b). Emissions of CO from light-duty vehicles and trucks are increased at altitude. Although the CO emissions of newer automobiles have decreased dramatically in the United States, factors such as tampering with emission control devices, numerous "cold" starts, the age of the vehicle fleet, and the increased numbers of automobiles and sports vehicles being driven at altitude all contribute CO to the ambient air. Moreover in the underdeveloped countries the preponderance of vehicles driven emit high CO concentrations. Other common sources of CO exposure in mountain recreational communities include hibachi and charcoal grills, heating devices (space heaters, wood stoves, and fireplaces), snowmobiles, chainsaws, motorized lawn and garden equipment and other small engines, motorboats, and camping equipment.

Foutch and Henrichs (1988) reported on the fatal CO exposure of two young, healthy mountain climbers who succumbed to fumes generated by a small cook stove in the enclosed space of their tent at 4300 m on Denali in Alaska. Turner et al. (1988) measured CO levels in various shelters (tents, igloos, and snow caves) produced by heaters used for cooking and melting snow on a climb of Denali. Measurements were made during the climb at between 2000 and 5200 m. Mean CO concentrations in the shelters usually exceeded the 35 ppm 1 h limit set by EPA, and a mean value of 165 ppm, with a maximum of 190 ppm, was measured in one snow cave.

Fernandez-Bremauntz and Ashmore (1995) compared measurements of CO made concurrently inside vehicles and at fixed-site monitoring stations in Mexico City. During the study ambient CO concentrations were very high, in excess of the United States (9 ppm) and the Mexican (13 ppm) 8 h standards for CO. In-vehicle concentrations of CO for all modes of transportation were always higher than the concurrent ambient concentrations measured at the fixed-site monitors. Average, in-vehicle/ambient CO ratios for each mode of transportation were: automobile, 5.2; minivan, 5.2; minibus, 4.3; bus, 3.1; trolleybus, 3.0; and metro, 2.2. Ambient CO concentrations at selected stations varied from 3 to 28 ppm during peak commuting hours and, depending on the locations, CO levels were increased above ambient in automobiles by 37–47 ppm and in minivans by 29–52 ppm.

Snowmobiling can be a significant source of CO exposure. The typical snowmobile, powered by a two-stroke engine, is not equipped with pollution control equipment. Carbon monoxide emissions from snowmobiles are extraordinary; CO emissions from a typical snowmobile ranged from 9.9 g/mile at 10 mph to 19.9 g/mile at 40 mph. This contrasts with comparable figures for CO emissions from a modern automobile of 0.01 to 0.04 g/mile. During the winter of 1993–1994, over 87,000 tourists traveled by snowmobile in Yellowstone National Park (Snook and Davis, 1997) where altitudes range from 1,600 to 3,442 m (5282–11,358 ft). Near the West Yellowstone entrance, a point where over 1000 snowmobiles a day enter the park, one-hour air samples exceeded 35 ppm

CO in the winter of 1994–95. Snowmobilers typically travel for several hours in large groups along narrow trails such as the Continental Divide Snowmobile Trail (Wilkinson, 1995), a 250-mile trail that crosses the Continental Divide at 2,909 m (9,600 ft). Snook and Davis (1997) studied the CO exposure of a snowmobiler while traveling in the wake of a lead snowmobile on a 2 to 3 mile straight trail in Grand Teton National Park, WY. Average CO measurements 25 and 125 feet behind the lead snowmobile were as high as 23 ppm with individual measurements as high as 45 ppm.

Altitude has a significant influence on the physiology of humans and consequently on the toxicokinetics of CO, especially on the sojourner (U.S. EPA, 1978). At sea level, at a body temperature of 37°C, barometric pressure (PB) of 760 torr, and air saturated with water vapor (BTPS conditions) the pressure of O_2 in the inspired air (P_{IO_2}) is 149 torr. At an altitude of 3000 m (9840 ft; PB = 526 torr), the P_{IO_2} is only 100 torr, resulting in acute hypoxic hypoxia. Direct measurements of blood gases on over 1000 nonacclimatized individuals at this altitude found the partial pressure of O_2 in alveolar air to be only 61 torr (Boothby, 1954). The hypoxic stimulus triggers physiological responses that endeavor to maintain O_2 transport. The magnitude and character of these responses depend on the elevation, exercise intensity, and the length of stay at the altitude. During the first several days, pulmonary ventilation increases progressively until a new steady-state level is achieved (Burki, 1984; Bender et al., 1987). The physiological responses to altitude will also affect both CO uptake and CO elimination.

However, COHb levels are increased in the newcomer to altitude not only because of the physiological changes that increase CO uptake (i.e., hyperventilation) but also because CO competes with O_2 for Hb-binding sites. Hence, the biological effects depend on the partial pressures of both CO and O_2. This means that, at identical CO levels, COHb levels will be higher at altitude (McGrath, 2000b). Benignus (1995) developed a model to predict COHb levels following inhalation of 200 ppm CO at two different barometric pressures and two inhaled O_2 concentrations. In general, the model provides support for COHb being elevated when PB is reduced at a constant inhaled O_2 concentration and when O_2 is reduced at constant PB. Carboxyhemoglobin increased from 7.59 to 8.60%, respectively, when PB was reduced from 760 to 456 mmHg and increased from 7.59 to 10.43% when O_2 was reduced from 20.93 to 12%. Carbon monoxide uptake is also enhanced at altitude in humans breathing CO (McGrath and Schreck, 1993). In subjects breathing 9 ppm CO for 1 h, COHb increased from 0.78 to 1.464% at 100 m and from 0.959 to 1.982% at 3497 m.

Carbon monoxide appears to have a greater effect on coronary disease at altitude. Leaf and Kleinman (1996) investigated the effect of exposure to CO (COHb = 3.9%) at sea level and a simulated high altitude of 2100 m (6930 ft) on the incidence of cardiac ectopy in subjects with coronary artery disease. Seventeen men with documented coronary artery disease and stable angina pectoris performed cardiopulmonary exercise stress tests after random exposure to either CO or clean air at sea level or at a simulated altitude of 2100 m. The percentage of O_2 saturation in each subject's arterial blood was reduced from a baseline level of 98% to approximately 94% after CO or simulated altitude and to approximately 90% after CO at simulated altitude. The average incidence of exercise-induced ventricular ectopy was approximately doubled after all exposures (CO, altitude, CO and altitude), and a significant trend of increased ectopy with decreased O_2 saturation in arterial blood was observed. These workers concluded that exposure to increased levels of hypoxemia, resulting from hypoxic and/or CO exposures, increased the susceptibility to ventricular ectopy during exercise in individuals with stable angina pectoris.

Kleinman et al. (1998) reported on the effects of combined exposure to altitude and CO in patients with stable angina who resided at or near sea level. The subjects performed cardiopulmonary exercise stress tests at sea level or at a simulated altitude of 2100 m (6930 ft) while being exposed to either clean air or CO (3.9% COHb). Compared with sea level, the time to onset of angina was reduced by simulated altitude (11%), by breathing CO at sea level (9%) and by breathing CO at simulated altitude (18%). Other cardiopulmonary parameters were also adversely affected by concomitant CO-altitude exposure. The authors concluded that high altitude exacerbates the effects of exposure to CO in unacclimatized individuals with coronary artery disease.

29.5.4 Endogenous Production of Carbon Monoxide

Body stores of CO are increased both by endogenous production and by inhalation of CO from the ambient environment.

The principal processes that influence the body stores of CO have been described by Coburn (1970) and are depicted in Figure 29.2. The model assumes that approximately one-half of the body stores of CO arise from endogenous production and the other half from inhaling ambient air containing 2 ppm CO. Carbon monoxide is exchanged between alveolar air and blood and is distributed by blood to other tissues. Studies on dogs (Coburn et al., 1967; Luomanmaki and Coburn, 1969) found that, over the range of 2 to 35% COHb, an average of 77% of total body CO remains in the vascular compartment. The rest of CO diffuses to extravascular tissues, primarily skeletal muscle where it is bound to Mb. Compared with dogs, the extravascular CO stores in humans are smaller and account for 10–15% of total body CO, and less than 1% of the body CO stores appears to be physically dissolved in body fluids (Coburn, 1970). No shift between blood and extravascular compartments in humans or animals was observed at low (<4%) COHb concentrations.

Body stores tend to be decreased by excretion via the lungs and metabolism of CO to CO_2. In normal humans, the conversion of CO to CO_2 is slow and has little effect on body stores. Although metabolism may be an insignificant mechanism of CO elimination compared with excretion by the lungs, Luomanmaki and Coburn (1969) have shown that absolute rates of CO metabolism may be directly proportional to blood COHb and have suggested that metabolism may become more important at higher blood COHb levels.

Carbon monoxide produced endogenously is derived from the normal degradation of the α methane moieties of RBC Hb. When Hb is metabolized to bile pigments, a carbon atom is separated from the porphyrin nucleus and, subsequently, catabolized by heme oxygenase (HO) into CO. One molecule of CO is formed for every heme decomposed to bile pigment. The rate-limiting enzyme for heme metabolism is HO, which converts heme to biliverdin, free iron, and CO. Three isoforms of HO have been characterized. The HO-1 is an inducible enzyme found in vascular endothelial cells, smooth muscle cells, bronchoalveolar epithelium, and pulmonary macrophages. The HO-1 is induced by its substrate, heme, as well as CNO, H_2O_2, several cytokines, and lipopolysaccharide (Arias-Diaz et al., 1995; Morita et al., 1995; Motterlini et al., 1996; Durante et al., 1997). The HO-2 is a constitutive enzyme found in certain neurons within the central nervous system, testicular cells, and vascular smooth muscle cells (Marks, 1994). Little is known about HO-3, which was identified in homogenates from a number of organs (McCoubrey et al., 1997).

A small fraction of endogenously produced CO arises from the metabolism of nonhemoglobin heme-containing compounds within the liver. Endogenous production of CO accounts for 0.42 ml/h in healthy males and leads normally to blood COHb levels of less than 1%; however, considerably higher levels are observed in patients with certain diseases (Coburn et al., 1963). In pathological conditions such as anemia, thalassemia, and other hematological diseases, erythrocytic Hb is degraded and CO production is amplified (Berk et al., 1974; Solanki et al., 1988). Luomanmaki and Coburn (1969) reported COHb values up to 2.74% produced by an endogenous production rate elevated to 3.4 ml of CO per h in hemolytic anemia patients. These studies demonstrate that normal COHb levels of 0.5–0.9% can be increased to 2.74% in various disease states. Coburn (1970) suggests that very high COHb levels (12%) are sufficient to inhibit oxidase systems involved in the degradation of Hb to CO.

Endogenous CO production varies considerably in healthy subjects (Longo, 1977). Healthy men and menstruating women (during the estrogen phase of the menstrual cycle) produce approximately 6.1 and 5.3 ml/h/kg body weight of CO, respectively. During the progestational or secretory phase of the menstrual cycle, however, endogenous CO production doubles (10.2 ml/h/kg in healthy women). During pregnancy, CO production increases further to 13.7 l/h/kg, and, immediately after pregnancy, CO production rates may exceed 25 ml/h/kg. Neonates also have a significant increase in endogenous CO production related to increased breakdown of erythrocytes.

Not all endogenous CO comes from Hb degradation, however. Other hemoproteins, such as Mb, cytochromes, peroxidases, and catalase, contribute approximately 20–25% to the total (Berk et al.,

1976). Approximately 0.4 ml/h of CO is formed by Hb catabolism, and about 0.1 ml/h originates from non-Hb sources (Coburn et al., 1964). This results in normal blood COHb concentrations between 0.4 and 0.7% (Coburn et al., 1965).

Carbon monoxide is also produced during inflammation and appears to be a marker of inflammation in individuals with upper respiratory tract infection (URTI) (Yamaya et al., 1998, 1999), bronchiectasis (Horvath et al., 1998b), and asthma (Zayasu et al., 1997; Horvath et al., 1998a). Although exhaled CO concentration averaged 5.7 ppm in patients with asthma who did not use corticosteroids, CO concentrations in patients with asthma using corticosteroids were about the same as in healthy individuals (1.7 and 1.5 ppm, respectively). The authors speculate that one of the anti-inflammatory effects of corticosteroids is the down-regulation of HO.

Exhaled CO concentrations are also related to the severity of asthma (Yamaya et al., 2001). The mean values of exhaled CO in severe asthma over 1 year were 6.7 ppm compared with 1.2 ppm in nonsmoking control subjects. Exhaled CO concentrations in patients with unstable severe asthma were significantly higher than in patients with stable severe asthma.

Patients with bronchiectasis may produce a substantial amount of CO (e.g., 11.8 ppm). As with asthma, induction of HO appears to be the primary mechanism involved in the production of CO (Horvath et al., 1998a, 1998b). Critical illness also seems to be associated with elevated production of CO (Meyer et al., 1998). When compared with controls, ill patients (not characterized) had higher COHb in both arterial and central venous blood. Moreover, the authors speculate that up-regulation of the inducible isoform of heme HO in the lung and subsequent production of CO caused the positive arteriovenous COHb difference. Andersson et al. (2002) sampled air separately from the upper and lower airways of healthy subjects, patients with a history of allergic rhinitis, and patients with URTI. They demonstrated that nasal CO levels were increased in subjects with allergic rhinitis and in patients with URTI. The authors concluded that upper airway CO levels increase in parallel with different inflammatory stimuli, such as allergy and infection, and that CO may be useful as a marker of nasal inflammation.

Carboxyhemoglobin concentrations are also increased in inflammatory pulmonary diseases including bronchial asthma, pneumonia, and idiopathic pulmonary fibrosis (Yasuda et al., 2002). In patients with bronchial asthma, changes in COHb concentrations and forced expiratory volume in 1 s were significantly correlated. Carboxyhemoglobin concentrations were also correlated with exhaled CO concentrations. Numerous drugs affect endogenous CO production. In general, any drug that increases bilirubin production, primarily through the catabolism of Hb, will promote endogenous CO production. Nicotinic acid, allyl-containing compounds (acetamides and barbiturates), diphenylhydantoin, progesterone, and contraceptives all increase bilirubin and, consequently, CO production. Another mechanism that increases CO production is stimulation of HO and subsequent degradation of cytochrome P-450-dependent, mixed-function oxidases. Several types of compounds, such as a carbon disulfide and sulfur-containing chemicals (parathion and phenylthiourea), act on different moieties of the P-450 system leading to an increase in endogenous CO production (Landaw et al., 1970).

Other sources of CO involving HO activity include autooxidation of phenols, photooxidation of organic compounds, and 5–8 lipid peroxidation of cell membrane lipids (Rodgers et al., 1994). The P-450 system also is involved in oxidative dehalogenation of dihalomethanes, solvents widely used in homes and industry (Kim and Kim, 1996). Metabolic degradation of these solvents and other xenobiotics results in the formation of CO that can lead to very high (>10%) COHb levels (Manno et al., 1992; Pankow, 1996).

Inhaled CO exerts effects *in vivo* similar to those of exerted by endogenously produced CO, because the nanomolar tissue concentrations resulting from inhaled CO are comparable to or greater than concentrations produced by cells with HO. Liver parenchyma has been estimated to generate approximately 0.45 nmol CO/g liver/min (Goda et al., 1998). Carbon monoxide synthesis by smooth muscle cells is approximately 8 pmol/mg protein/min for human aorta and 23–37 pmol/mg protein/min for rat aorta (Cook et al., 1995; Grundemar et al., 1995).

Carbon monoxide production by unstimulated pulmonary macrophages is 3.6 pmol/mg protein/min, and, after stimulation with lipopolysaccharide, increases to about 5.1 pmol/mg protein/min (Arias-Diaz et al., 1995). The rate of synthesis of CO varies widely for nerve cells. Cerebellar granule cells generate approximately 3 fmol/mg protein/min, olfactory nerve cells produce 4.7 pmol/mg protein/min, and rat cerebellar homogenates can generate as much as 56.6 pmol/mg protein/min (Maines, 1988; Ingi and Ronnett, 1995; Nathanson et al., 1995; Ingi et al., 1996b).

29.5.5 Physiological Aspects of Carbon Monoxide

The physiological aspects of endogenously-produced CO is being increasingly appreciated, especially its role as a gaseous messenger molecule involved with smooth muscle relaxation, inhibition of platelet aggregation vasodilation, and neurotransmission.

In recent years, CO has been shown to play a physiological role in vasomotor control and neuronal signal transduction (Morita and Kourembanas, 1995; Ingi et al., 1996a). A main physiological role for CO appears to be regulation of guanylate cyclase (GC) activity. Both CO and CNO can activate GC, although activation by CO is approximately 30-fold less (Stone and Marletta, 1994). In neuronal cells possessing both HO and NO synthase, regulation of cyclic guanosine monophosphate (cGMP) synthesis is mediated in a reciprocal fashion by producing either CO or CNO (Maines, 1993; Ingi et al., 1996a). Endogenous CO also appears to have a role as a pyrogenic mediator in CNS (Jang et al., 2002).

The vasorelaxing effect of CO is independent of the presence of the intact endothelium and is partially inhibited by the blockade of either the cGMP pathway or big conductance calcium-activated (KCa) channels (Wang et al., 1997a). These results suggest that CO may affect the vascular contractile responses by activating both a cGMP signaling pathway and KCa channels in the same vascular tissues.

Carbon monoxide appears to be involved in the regulation of basal tone in resistance vessels. Kozma et al. (1999) examined the role of endogenous CO in the microcirculation by manipulating HO in large and small arteries and in isolated first-order gracilis muscle arterioles of rat. Strong heme oxygenase inhibitors decreased the diameter of pressurized gracilis muscle arterioles, whereas a weak HO inhibitor did not. A strong inhibitor also elicited development of isometric tension in the muscular branch of the femoral artery but not in the aorta or femoral artery. The arteriolar constrictor responses to a strong inhibitor varied in relation to the intravascular pressure, were blunted in preparations exposed to exogenous CO, and were unaffected by an endothelin receptor antagonist. Importantly, a strong inhibitor amplified the constrictor response to increases of pressure in gracilis arterioles. Accordingly, the constrictor effect of HO inhibitors is caused by magnification of myogenic tone due to withdrawal of a vasodilatory mechanism mediated by endogenous CO.

Carbon monoxide causes smooth muscle relaxation by stimulating sGC (Utz and Ullrich, 1991; Wang et al., 1997a). Smooth muscle relaxation also may occur because of activation of calcium-dependent potassium channels, although this effect may be linked to GC activity (Trischmann et al., 1991; Wang et al., 1997b). Carbon monoxide-mediated smooth muscle relaxation is involved with control of microvascular hepatic portal blood flow (Goda et al., 1998; Pannen and Bauer, 1998) and suppression of contractions in the gravid uterus (Acevedo and Ahmed, 1998). It also may play a role in gastrointestinal motility (Acevedo and Ahmed, 1998).

In macrophages, cGMP synthesis promotes chemotaxis, and cGMP-mediated synthesis and secretion of tumor necrosis factor have been linked to both CO and CNO (Belenky et al., 1993; Arias-Diaz et al., 1995).

29.6 CARBON MONOXIDE EXPOSURE LIMITS

A number of agencies have established exposure limits for CO for a variety of situations (Table 29.2). The National Ambient Air Quality Standard for CO, set by EPA and reviewed every 5 years, is 9 ppm for 1 h and 35 ppm averaged over 8 h.

TABLE 29.2 Carbon Monoxide Exposure Limits Set by Various Agencies for Different Situations

Situation	Concentration, ppm
NAAQS[a]	
1 h	35
8 h	9
OSHA TLV-TWA,[b] 8 h	25
NIOSH PEL, 8 h TWA	35
Spacecraft SMACs[c]	
1 h	55
24 h	20
180 d	10
NRC EEGL[d], 10 min	1500
NRC EEGL, 1 h	400
NRC EEGL, 24 h	50
NRC EEGL, 90 h	20
Indoor air (most common triggering action by local authorities)[e]	9

[a] National Ambient Air Quality Standard set by EPA.
[b] Threshold limit value, time-weighted average.
[c] Spacecraft maximum allowable concentrations.
[d] Emergency exposure guidance levels.
[e] Penney (2003).

The threshold limit value for CO set by the American Conference of Governmental and Industrial Hygienists (ACGIH) is 25 ppm. NIOSH has set a permissible exposure limit (PEL) of 35 ppm 8 h time-weighted average (TWA).

The continuous exposure limit for CO during 180-d flights in U.S. spacecraft is 10 ppm or a target COHb concentration of 1.6%, because higher CO levels might compromise the high levels of judgment and performance required of pilots and other occupants of space vehicles. The emergency exposure guidance level (EEGL) for CO recommended to military and space agencies by the Committee on Toxicology-National Research Council are 1500 ppm for 10 min, 400 ppm for 60 min, 50 ppm for 24 h, and 20 ppm for 90 h. The indoor air CO level most commonly triggering action by local authorities is 9 ppm (Penney, 2003).

REFERENCES

Acevedo, C.H. and Ahmed, A., Hemeoxygenase-1 inhibits human myometrial contractility via carbon monoxide and is upregulated by progesterone during pregnancy, *J. Clin. Invest.*, 101, 949–955, 1998.

Agostoni, A., Stabilini, R., Viggiano, G., Luzzana, M., and Samaja, M., Influence of capillary and tissue PO2 on carbon monoxide binding to myoglobin: a theoretical evaluation, *Microvasc. Res.*, 20, 81–87, 1980.

Anderson, D.E., Problems created for ice arenas by engine exhaust, *Am. Ind. Hyg. Assoc. J.*, 32, 790–801, 1971.

Andersson, J.A., Uddman, R., and Cardell, L.O., Increased carbon monoxide levels in the nasal airways of subjects with a history of seasonal allergic rhinitis and in patients with upper respiratory tract infection, *Clin. Exp. Allergy*, 32, 224–227, 2002.

Arias-Diaz, J., Vara, E., Garcia, C., Villa, N., and Balibrea, J.L., Evidence for a cyclic guanosine monophosphate-dependent, carbon monoxide-mediated, signalling system in the regulation of TNF-α production by human pulmonary macrophages, *Arch. Surg.*, 130, 1287–1293, 1995.

Barret, L., Danel, V., and Faure, J., Carbon monoxide poisoning, a diagnosis frequently overlooked, *J. Toxicol. Clin. Toxicol.*, 23, 309–313, 1985.

Belenky, S.N., Robbins, R.A., and Rubinstein, I., Nitric oxide synthase inhibitors attenuate human monocyte chemotaxis in vitro, *J. Leukoc. Biol.*, 53, 498–503, 1993.

Bender, P.R., Weil, J.V., Reeves, J.T., and Moore, L.G., Breathing pattern in hypoxic exposures of varying duration, *J. Appl. Physiol.*, 62, 640–645, 1987.

Benignus, V.A., A model to predict carboxyhemoglobin and pulmonary parameters after exposure to O2, CO2, and CO, *Aviat. Space Environ. Med.*, 66, 369–374, 1995.

Berk, P.D., Rodkey, F.L., Blaschke, T.F., Collison, H.A., and Waggoner, J.G., Comparison of plasma bilirubin turnover and carbon monoxide production in man, *J. Lab. Clin. Med.*, 83, 29–37, 1974.

Bernard, C., *Lecons sur les effets des sustances toxiques et medicamenteuses*, Bailliere, Paris, 1857.

Bondi, K.R., Very, K.R., and Schaefer, K.E., Carboxyhemoglobin levels during a submarine patrol, *Aviat. Space Environ. Med.*, 49, 851–854, 1978.

Boothby, W.M., Volume and partial pressures of respiratory gases at altitude, in *Respiratory Physiology in Aviation*, Boothby, W.M., Ed., U.S. Air Force School of Aviation Medicine, Randolph Field, TX, 1954, pp. 39–49.

Brown, S.D. and Piantadosi, C.A., Recovery of energy metabolism in rat brain after carbon monoxide hypoxia, *J. Clin. Invest.*, 89, 666–672, 1992.

Burki, N.K., Effects of acute exposure to high altitude on ventilatory drive and respiratory pattern, *J. Appl. Physiol. Respir. Environ. Exerc. Physiol.*, 56, 1027–1031, 1984.

Cantrell, J.M. and Tucker, A., Low-dose carbon monoxide does not reduce vasoconstriction in isolated rat lungs, *Exp. Lung Res.*, 22, 21–32, 1996.

Chen, K.C. and McGrath, J.J., Effects of intraperitoneal injection of carbon monoxide on circulating blood glucose and lactate, *Fed. Proc.*, 38, 1313, 1979.

Clark, B.J. and Coburn, R.F., Mean myoglobin oxygen tension during exercise at maximal oxygen uptake, *J. Appl. Physiol.*, 39, 135–144, 1975.

Coburn, R.F., The carbon monoxide body stores, *Ann. N. Y. Acad. Sci.*, 174, 11–22, 1970.

Coburn, R.F., Blakemore, W.S., and Forester, R.E., Endogenous carbon monoxide production in man, *J. Clin. Invest.*, 42, 1172–1178, 1963.

Coburn, R.F., Forster, R.E., and Kane, P.B., Considerations of the physiological variables that determine the blood carboxyhemoglobin concentration in man, *J. Clin. Investig.*, 44, 1899–1910, 1964.

Coburn, R.F., Forster, R.E., and Kane, P.B., Considerations of the physiological variables that determine the blood carboxyhemoglobin concentration in man, *J Clin Investig.*, 44, 1899–1910, 1965.

Coburn, R.F. and Mayers, L.B., Myoglobin O2 tension determined from measurement of carboxymyoglobin in skeletal muscle, *Am. J. Physiol.*, 220, 66–74, 1971.

Coburn, R.F., Ploegmakers, F., Gondrie, P., and Abboud, R., Myocardial myoglobin oxygen tension, *Am. J. Physiol.*, 224, 870–876, 1973.

Coburn, R.F., Williams, W.J., White, P., and Kahn, S.B., The production of carbon monoxide from hemoglobin in vivo, *J. Clin. Invest.*, 46, 346–356, 1967.

Cook, M.N., Nakatsu, K., Marks, G.S., McLaughlin, B.E., Vreman, H.J., Stevenson, D.K., and Brien, J.F., Heme oxygenase activity in the adult rat aorta and liver as measured by carbon monoxide formation, *Can. J. Physiol. Pharmacol.*, 73, 515–518, 1995.

Davies, D.M., The application of threshold limit values for carbon monoxide under conditions of continuous exposure, *Ann. Occup. Hyg.*, 18, 21–28, 1975.

Deller, A., Stenz, R., Forstner, K., and Konrad, F., [The elimination of carboxyhemoglobin—gender-specific and circadian effects], *Infusionsther Transfusionsmed.*, 19, 121–126, 1992.

Durante, W., Kroll, M.H., Christodoulides, N., Peyton, K.J., and Schafer, A.I., Nitric oxide induces heme oxygenase-1 gene expression and carbon monoxide production in vascular smooth muscle cells, *Circ Res.*, 80, 557–564, 1997.

Fernandez-Bremauntz, A.A. and Ashmore, M.R., Exposure of commuters to carbon monoxide in Mexico City, II. Comparison of in-vehicle and fixed-site concentrations, *J. Expo. Anal. Environ. Epidemiol.*, 5, 497–510, 1995.

Foutch, R.G. and Henrichs, W., Carbon monoxide poisoning at high altitudes, *Am. J. Emerg. Med.*, 6, 596–598, 1988.

Goda, N., Suzuki, K., Naito, M., Takeoka, S., Tsuchida, E., Ishimura, Y., Tamatani, T., and Suematsu, M., Distribution of heme oxygenase isoforms in rat liver. Topographic basis for carbon monoxide-mediated microvascular relaxation, *J. Clin. Invest.*, 101, 604–612, 1998.

Goldsmith, J.R., Contribution of motor vehicle exhaust, industry, and cigarette smoking to community carbon monoxide exposures, *Ann. N. Y. Acad. Sci.*, 174, 122–134, 1970.

Gothert, M., Lutz, F., and Malorny, G., Carbon monoxide partial pressure in tissue of different animals, *Environ. Res.*, 3, 303–309, 1970.

Grundemar, L., Johansson, M.B., Ekelund, M., and Hogestatt, E.D., Haem oxygenase activity in blood vessel homogenates as measured by carbon monoxide production, *Acta. Physiol., Scand.*, 153, 203–204, 1995.

Guttierez, G., Carbon monoxide toxicity, in *Air pollution-Physiological Effects*, McGrath, J.J. and Barnes, C.D., Eds., Academic Press, New York, 1982, pp. 127–147.

Haab, P., The effect of carbon monoxide on respiration, *Experientia*, 46, 1202–1206, 1990.

Hart, M.H., Was the pre-biotic atmosphere of the earth heavily reducing? *Origins Life*, 9, 261–266, 1979.

Hausberg, M. and Somers, V.K., Neural circulatory responses to carbon monoxide in healthy humans, *Hypertension*, 29, 1114–1118, 1997.

Hoegg, U.R., Cigarette smoke in closed spaces, *Environ. Health Perspect.*, 2, 117–128, 1972.

Horvath, I., Donnelly, L.E., Kiss, A., Paredi, P., Kharitonov, S.A., and Barnes, P.J., Raised levels of exhaled carbon monoxide are associated with an increased expression of heme oxygenase-1 in airway macrophages in asthma: a new marker of oxidative stress, *Thorax*, 53, 668–672, 1998a.

Horvath, I., Loukides, S., Wodehouse, T., Kharitonov, S.A., Cole, P.J., and Barnes, P.J., Increased levels of exhaled carbon monoxide in bronchiectasis: a new marker of oxidative stress, *Thorax*, 53, 867–870, 1998b.

Horvath, S.M., Agnew, J.W., Wagner, J.A., and Bedi, J.F., Maximal aerobic capacity at several ambient concentrations of carbon monoxide at several altitudes, *Res. Rep. Health Eff Inst.*, 1–21, 1988.

Huie, R.E. and Padmaja, S., The reaction of no with superoxide, *Free Radic. Res. Commun.*, 18, 195–199, 1993.

Ingi, T., Cheng, J., and Ronnett, G.V., Carbon monoxide: an endogenous modulator of the nitric oxide-cyclic GMP signaling system, *Neuron*, 16, 835–842, 1996a.

Ingi, T., Chiang, G., and Ronnett, G.V., The regulation of heme turnover and carbon monoxide biosynthesis in cultured primary rat olfactory receptor neurons, *J. Neurosci.*, 16, 5621–5628, 1996b.

Ingi, T. and Ronnett, G.V., Direct demonstration of a physiological role for carbon monoxide in olfactory receptor neurons, *J. Neurosci.*, 15, 8214–8222, 1995.

Ischiropoulos, H., Beers, M.F., Ohnishi, S.T., Fisher, D., Garner, S.E., and Thom, S.R., Nitric oxide production and perivascular nitration in brain after carbon monoxide poisoning in the rat, *J. Clin. Invest.*, 97, 2260–2267, 1996.

Jaffee, L.S., Sources, characteristics and fate of atmospheric carbon monoxide, *Ann. N. Y. Acad. Sci.*, 174, 76–88, 1970.

Jang, C.G., Lee, S.J., Yang, S.I., Kim, J.H., Sohn, U.D., and Lee, S.Y., Carbon monoxide as a novel central pyrogenic mediator, *Arch. Pharm. Res.*, 25, 343–348, 2002.

Jay, G.D. and McKindley, D.S., Alterations in pharmacokinetics of carboxyhemoglobin produced by oxygen under pressure, *Undersea Hyperb. Med.*, 24, 165–173, 1997.

Johnson, C.J., Moran, J.C., Paine, S.C., Anderson, H.W., and Siegel, J., Abatement of toxic levels of carbon monoxide in Seattle ice-skating rinks, *Am. J. Public Health*, 65, 1087–1090, 1965.

Kim, S.K. and Kim, Y.C., Effect of a single administration of benzene, toluene or m-xylene on carboxyhaemoglobin elevation and metabolism of dichloromethane in rats, *J. Appl. Toxicol.*, 16, 437–444, 1996.

Kleinman, M.T., Leaf, D.A., Kelly, E., Caiozzo, V., Osann, K., and O'Niell, T., Urban angina in the mountains: effects of carbon monoxide and mild hypoxemia on subjects with chronic stable angina, *Arch. Environ. Health*, 53, 388–397, 1998.

Koehler, R.C., Jones, M.D., Jr., and Traystman, R.J., Cerebral circulatory response to carbon monoxide and hypoxic hypoxia in the lamb, *Am. J. Physiol.*, 243, H27–H32, 1982.

Kozma, F., Johnson, R.A., Zhang, F., Yu, C., Tong, X., and Nasjletti, A., Contribution of endogenous carbon monoxide to regulation of diameter in resistance vessels, *Am. J. Physiol.*, 276, R1087–R1094, 1999.

Landaw, S.A,. The effects of cigarette smoking on total body burden and excretion rates of carbon monoxide, *J. Occup. Med.*, 15, 231–235, 1973.

Landaw, S.A., Callahan, E.W.J., and Schmid, R., Catabolism of heme in vivo: comparison of the simultaneous production of bilirubin and carbon monoxide, *J. Clin. Investig.*, 49, 914–925, 1970.

Lasater, S.R., Carbon monoxide poisoning, *CMAJ*, 134, 991–992, 1986.

Lawther, P.J. and Commins, B.T., Cigarette smoking and exposure to carbon monoxide, *Ann. N. Y. Acad. Sci.*, 174, 135–147, 1970.

Leaf, D.A. and Kleinman, M.T., Urban ectopy in the mountains: carbon monoxide exposure at high altitude, *Arch. Environ. Health*, 51, 283–290, 1996.

Levasseur, L., Galliot-Guilley, M., Richter, F., Scherrmann, J.M., and Baud, F.J., Effects of mode of inhalation of carbon monoxide and of normobaric oxygen administration on carbon monoxide elimination from the blood, *Hum. Exp. Toxicol.*, 15, 898–903, 1996.

Longo, L.D., The biological effects of carbon monoxide on the pregnant woman, fetus, and newborn infant, *Am. J. Obstet. Gynecol.*, 129, 69–103, 1977.

Luomanmaki, K. and Coburn, R.F., Effects of metabolism and distribution of carbon monoxide on blood and body stores, *Am. J. Physiol.*, 217, 354–363, 1969.

Maines, M.D., Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications, *FASEB J.*, 2, 2557–2568, 1988.

Maines, M.D., Heme oxygenase, a likely regulator of cGMP production in the brain: onduction in vivo of HO-1 compensates for depression in NO synthase activity, *Mol. Cell. Neurosci.*, 4, 398–405, 1993.

Manno, M., Rugge, M., and Cocheo, V., Double fatal inhalation of dichloromethane, *Hum. Exp. Toxicol.*, 11, 540–545, 1992.

Marks, G.S., Heme oxygenase: the physiological role of one of its metabolites, carbon monoxide and interactions with zinc protoporphyrin, cobalt protoporphyrin and other metalloporphyrins, *Cell Mol. Biol. (Noisy-le-grand)*, 40, 863–870, 1994.

McCoubrey, W.K., Jr., Huang, T.J., and Maines, M.D., Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3, *Eur. J. Biochem.*, 247, 725–732, 1997.

McFaul, S.J. and McGrath, J.J., Studies on the mechanism of carbon monoxide-induced vasodilation in the isolated perfused rat heart, *Toxicol. Appl. Pharmacol.*, 87, 464–473, 1987.

McGrath, J.J., Physiological effects of carbon monoxide, in *Air Pollution-Physiological Effects*, McGrath, J.J. and Barnes, C.D., Eds., Academic Press, New York, 1982, pp. 148–182.

McGrath, J.J., Biologic plausibility for carbon mooxide as a copollutant in PM epidemiology studies, *Inhalation Toxicol.*, 12, 91–407, 2000a.

McGrath, J.J., The interacting effects of altitude and carbon monoxide, in *Carbon Monoxide Toxicity*, Penney, D.G., Ed., CRC Press, Boca Raton, FL, 2000b, pp. 135–156.

McGrath, J.J. and Schreck, R.M., Carboxyhemoglobin levels in humans: effects of altitude, *Inhalation Toxicol.*, 5, 241–249,1993.

Meilin, S., Rogatsky, G.G., Thom, S.R., Zarchin, N., Guggenheimer-Furman, E., and Mayevsky, A., Effects of carbon monoxide on the brain may be mediated by nitric oxide, *J. Appl. Physiol.*, 81, 1078–1083, 1996.

Mendelman, A., Zarchin, N., Meilin, S., Guggenheimer-Furman, E., Thom, S.R., and Mayevsky, A., Blood flow and ionic responses in the awake brain due to carbon monoxide, *Neurol. Res.*, 24, 765–772, 2002.

Meyer, J., Prien, T., Van Aken, H., Bone, H.G., Waurick, R., Theilmeier, G., and Booke, M., Arterio-venous carboxyhemoglobin difference suggests carbon monoxide production by human lungs, *Biochem. Biophys. Res. Commun.*, 244, 230–232, 1998.

Moore, E.G. and Gibson, Q.H., Cooperativity in the dissociation of nitric oxide from hemoglobin, *J. Biol. Chem.*, 251, 2788–2794, 1976.

Morita, T. and Kourembanas, S., Endothelial cell expression of vasoconstrictors and growth factors is regulated by smooth muscle cell-derived carbon monoxide, *J. Clin. Investig.*, 96, 2676–2682, 1995.

Morita, T., Perrella, M.A., Lee, M.E., and Kourembanas, S., Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP, *Proc. Natl. Acad. Sci. USA*, 92, 1475–1479, 1995.

Motterlini, R., Hidalgo, A., Sammut, I., Shah, K.A., Mohammed, S., Srai, K., and Green, C.J., A precursor of the nitric oxide donor SIN-1 modulates the stress protein heme oxygenase-1 in rat liver, *Biochem. Biophys. Res. Commun.*, 225, 167–172, 1996.

Nathanson, J.A,. Scavone, C., Scanlon, C., and McKee, M., The cellular Na+ pump as a site of action for carbon monoxide and glutamate: a mechanism for long-term modulation of cellular activity, *Neuron*, 14, 781–794, 1995.

Pace, N., Strajman, E., and Walker, E.L., Acceleration of carbon monoxide elimination in man by high pressure oxygen, *Science*, 111, 652–654, 1950.

Pankow, D., *Carbon Monoxide due to Metabolism of Xenobiotics*, Penney, D.G., Ed., CRC Press, Boca Raton, FL, 1996, pp. 25–43.

Pannen, B.H. and Bauer, M., Differential regulation of hepatic arterial and portal venous vascular resistance by nitric oxide and carbon monoxide in rats, *Life Sci.*, 62, 2025–2033, 1998.

Penney, D.G., *Carbon Monoxide*, CRC Press, Boca Raton, FL, 1996.

Penney, D.G., *Carbon Monoxide Toxicity*, CRC Press, Boca Raton, FL, 2000.

Penney, D.G., Carbon Monoxide Headquarters, 2003. Available at http://www.coheadguarters.com/2003.

Peters, T., Jurgens, K.D., Gunther-Jurgens, G., and Gros, G., Determination of myoglobin-diffusivity in intact skeletal muscle fibers. An improved microscope-photometrical approach, *Adv. Exp. Med. Biol.*, 345, 677–683, 1994.

Peterson, J.E. and Stewart, R.D., Absorption and elimination of carbon monoxide by inactive young men, *Arch. Environ. Health*, 21, 165–171, 1970.

Piantadosi, C.A., Tatro, L., and Zhang, J., Hydroxyl radical production in the brain after CO hypoxia in rats, *Free Radic. Biol. Med.*, 18, 603–609, 1995.

Piantadosi, C.A., Zhang, J., and Demchenko, I.T., Production of hydroxyl radical in the hippocampus after CO hypoxia or hypoxic hypoxia in the rat, *Free Radic. Biol. Med.*, 22, 725–732, 1997.

Ramos, K.S., Lin, H., and McGrath, J.J., Modulation of cyclic guanosine monophosphate levels in cultured aortic smooth muscle cells by carbon monoxide, *Biochem. Pharmacol.*, 38, 1368–1370, 1989.

Ratney, R.S., Wegman, D.H., and Elkins, H.B., In vivo conversion of methylene chloride to carbon monoxide, *Arch. Environ. Health*, 28, 223–226, 1974.

Raub, J.A., Mathiev-Nolf, M., Hampson, N.B., and Thom, S.R., Carbon monoxide poising—a public health perspective, *Toxicology,* 145, 1–14, 2000.

Rodgers, P.A., Vreman, H.J., Dennery, P.A., and Stevenson, D.K., Sources of carbon monoxide (CO) in biological systems and applications of CO detection technologies, *Semin. Perinatol.*, 18, 2–10, 1994.

Root, W.S., Carbon monoxide, in *Handbook of Physiology*, Fenn, W.O. and Rahn, H., Eds., American Physiological Society, Washington, DC, 1965, pp. 1078–1098.

Roughton, F.J.W., Transport of oxygen and carbon dioxide, in *Handbook of Physiology*, Sect. 3, Vol. I, Chap. 31, Fenn, W.O. and Rahn, H., Eds., American Physiological Society, Washington, DC, 1964.

Russell, M.A., Cole, P/V., and Brown, E., Absorption by non-smokers of carbon monoxide from room air polluted by tobacco smoke, *Lancet*, 1, 576–579, 1973.

Schoenfisch, W.H., Hoop, K.A., and Struelens, B.S., Carbon monoxide absorption through the oral and nasal mucosae of cynomolgus monkeys, *Arch. Environ. Health*, 35, 152–154, 1980.

Seufert, K.T. and Kiser, W.R., End-expiratory carbon monoxide levels as an estimate of passive smoking exposure aboard a nuclear-powered submarine, *South Med. J.*, 89, 1181–1183, 1996.

Snook, L.M. and Davis, W.T., An investigation of driver exposure to carbon monoxide while traveling in the wake of a snowmobile, presented at 90th Annual Meeting and Exhibition of the Air and Waste Management Association, 1997.

Sokal, J., Majka, J., and Palus, J., Effect of work load on the content of carboxymyoglobin in the heart and skeletal muscles of rats exposed to carbon monoxide, *J. Hyg. Epidemiol. Microbiol. Immunol.*, 30, 57–62, 1986.

Sokal, J.A., Lack of the correlation between biochemical effects on rats and blood carboxyhemoglobin concentrations in various conditions of single acute exposure to carbon monoxide, *Arch. Toxicol.* 34, 331–336, 1975.

Sokal, J.A. and Kralkowska, E., The relationship between exposure duration, carboxyhemoglobin, blood glucose, pyruvate and lactate and the severity of intoxication in 39 cases of acute carbon monoxide poisoning in man, *Arch. Toxicol.*, **57**, 196–199, 1985.

Sokal, J.A., Majka, J., and Palus, J., The content of carbon monoxide in the tissues of rats intoxicated with carbon monoxide in various conditions of acute exposure, *Arch. Toxicol.*, 56, 106–108, 1984.

Solanki, D.L., McCurdy, P.R., Cuttitta, F.F., and Schechter, G.P., Hemolysis in sickle cell disease as measured by endogenous carbon monoxide production. A preliminary report, *Am. J. Clin. Pathol.*, 89, 221–225, 1988.

Stewart, R.D., Fisher, T.N., Hosko, M.J., Peterson, J.E., Baretta, E.D., and Dodd, H.C., Experimental human exposure to methylene chloride, *Arch. Environ. Health*, 25, 342–348, 1972.

Stone, J.R. and Marletta, M.A., Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states, *Biochemistry*, 33, 5636–5640, 1994.

Tamayo, L., Lopez-Lopez, J.R., Castaneda, J., and Gonzalez, C., Carbon monoxide inhibits hypoxic pulmonary vasoconstriction in rats by a cGMP-independent mechanism, *Pflugers Arch*., 434, 698–704, 1997.

Thom, S.R., Carbon monoxide-mediated brain lipid peroxidation in the rat, *J. Appl. Physiol.*, 68, 997–1003, 1990.

Thom, S.R., Dehydrogenase conversion to oxidase and lipid peroxidation in brain after carbon monoxide poisoning, *J. Appl. Physiol.*, 73, 1584–1589, 1992.

Thom, S.R,. Leukocytes in carbon monoxide-mediated brain oxidative injury, *Toxicol. Appl. Pharmacol.*, 123, 234–247, 1993.

Thom, S.R., Fisher, D., Xu, Y.A., Garner, S., and Ischiropoulos, H., Role of nitric oxide-derived oxidants in vascular injury from carbon monoxide in the rat, *Am. J. Physiol.*, 276, H984–H992, 1999a.

Thom, S.R. and Ischiropoulos, H., Mechanism of oxidative stress from low levels of carbon monoxide, *Res. Rep. Health Eff. Inst.*, 1–19, discussion 21–17, 1997.

Thom, S.R., Ohnishi, S.T., Fisher, D., Xu, Y.A., and Ischiropoulos, H., Pulmonary vascular stress from carbon monoxide, *Toxicol. Appl. Pharmacol.*, 154, 12–19, 1999b.

Thom, S.R., Ohnishi, S.T., and Ischiropoulos, H., Nitric oxide released by platelets inhibits neutrophil B2 integrin function following acute carbon monoxide poisoning, *Toxicol. Appl. Pharmacol.*, 128, 105–110, 1994.

Thom, S.R., Xu, Y.A., and Ischiropoulos, H., Vascular endothelial cells generate peroxynitrite in response to carbon monoxide exposure, *Chem. Res. Toxicol.*, 10, 1023–1031, 1997.

Trischmann, U., Klockner, U., Isenberg, G., Utz, J., and Ullrich, V., Carbon monoxide inhibits depolarization-induced Ca rise and increases cyclic GMP in visceral smooth muscle cells, *Biochem. Pharmacol.*, 41, 237–241, 1991.

Turner, W.A., Cohen, M.A., Moore, S., Spengler, J.D., and Hackett, P.H., Carbon monoxide exposure in mountaineers on Denali, *Alaska Med.*, 30, 85–90, 1988.

U.S. Department of Health, Education and Welfare, *Smoking and Health. A Report of the Surgeon General*, DHEW Publication No. 79-50066, U.S.DHEW, Washington, DC, 1979.

U.S. EPA (U.S. Environmental Protection Agency), Altitude as a Factor in Risk Assessment, U.S. Environmental Protection Agency, Washington, DC, 1978.

U.S. EPA (U.S. Environmental Protection Agency), Air Quality Criteria for Carbon Monoxide, U.S. Environmental Protection Agency, Washington, DC, 2000.

U.S. EPA (U.S. Environmental Protection Agency), National Air Quality and Emission Trends Report, U.S. Environmental Protection Agency, Washington, DC, 2003.

Utz, J. and Ullrich, V., Carbon monoxide relaxes ileal smooth muscle through activation of guanylate cyclase, *Biochem. Pharmacol.*, 41, 1195–1201, 1991.

Wang, R., Wang, Z., and Wu, L., Carbon monoxide-induced vasorelaxation and the underlying mechanisms, *Br. J. Pharmacol.*, 121, 927–934, 1997a.

Wang, R., Wu, L., and Wang, Z., The direct effect of carbon monoxide on KCa channels in vascular smooth muscle cells, *Pflugers Arch.*, 434, 285–291, 1997b.

Werner, B. and Lindahl, J., Endogenous carbon monoxide production after bicycle exercise in healthy subjects and in patients with hereditary spherocytosis, *Scand. J. Clin. Lab. Invest.*, 40, 319–324, 1980.

Wilkinson, T., Snowed under, *Natl. Parks*, 69, 32(1995)..

Winston, J.M., Creighton, J.M., and Roberts, R.J., Alteration of carbon monoxide and hypoxic hypoxia-induced lethality following phenobarbital, chlorpromazine, or alcohol pretreatment, *Toxicol. Appl. Pharmacol.*, 30, 458–465, 1974.

Wittenberg, B.A. and Wittenberg, J.B., (1987). Myoglobin-mediated oxygen delivery to mitochondria of isolated cardiac myocytes, *Proc. Natl. Acad. Sci. USA*, 84, 7503–7507.

Wittenberg, B.A. and Wittenberg, J.B., Transport of oxygen in muscle, *Annu. Rev. Physiol.*, 51, 857–878, 1989.

Wittenberg, J.B. and Wittenberg, B.A., Mechanisms of cytoplasmic hemoglobin and myoglobin function. *Annu. Rev, Biophys. Biophys. Chem.*, 19, 217–241, 1990.

Woehlck, H.J., Dunning, M., III, and Connolly, L.A., Reduction in the incidence of carbon monoxide exposures in humans undergoing general anesthesia, *Anesthesiology*, 87, 228–234, 1997a.

Woehlck, H.J., Dunning, M.B., III, Kulier, A.H., Sasse, F.J., Nithipataikom, K., and Henry, D.W., The response of anesthetic agent monitors to trifluoromethane warns of the presence of carbon monoxide from anesthetic breakdown. *J. Clin. Monit.* 13, 149–155, 1997b.

Wright, G.T. and Shephard, R.J., Physiological effects of carbon monoxide, *Int. Rev. Physiol.*, 20, 311–368, 1979.

Yamaya, M., Hosoda, M., Ishizuka, S., Monma, M., Matsui, T., Suzuki, T., Sekizawa, K., and Sasaki, H., Relation between exhaled carbon monoxide levels and clinical severity of asthma, *Clin. Exp. Allergy*, 31, 417–422, 2001.

Yamaya, M., Sekizawa, K., Ishizuka, S., Monma, M., Mizuta, K., and Sasaki, H., Increased carbon monoxide in exhaled air of subjects with upper respiratory tract infections, *Am. J. Respir. Crit. Care Med.*, 158, 311–314, 1998.

Yamaya, M., Sekizawa, K., Ishizuka, S., Monma, M., Sasaki, H., and Yamara, M., Exhaled carbon monoxide levels during treatment of acute asthma, *Eur. Respir. J.*, 13, 757–760, 1999.

Yasuda, H., Yamaya, M., Yanai, M., Ohrui, T., and Sasaki, H., Increased blood carboxyhaemoglobin concentrations in inflammatory pulmonary diseases, *Thorax*, 57, 779–783, 2002.

Zayasu, K., Sekizawa, K., Okinaga, S., Yamaya, M., Ohrui, T., and Sasaki, H., Increased carbon monoxide in exhaled air of asthmatic patients, *Am. J. Respir. Crit. Care Med.*, 156, 1140–1143, 1997.

30 Experimental, Clinical, Occupational Toxicology, and Forensic Aspects of Hydrogen Cyanide with Particular Reference to Vapor Exposure

Bryan Ballantyne and Harry Salem

CONTENTS

30.1 INTRODUCTION

Hydrogen cyanide was probably one of the essential reactive molecules in the prebiotic age that was responsible for the development of living organisms. Thus, in stellar atmospheres carbon and nitrogen atoms, derived from stellar core thermonuclear reactions, combine to produce the thermostable cyanide radical. Transfer of stellar matter results in a wide distribution of hydrogen cyanide (HCN) in interstellar clouds. Related to this and HCN liberation from comets entering the primordial atmosphere, the reactivity of HCN, and its condensation with other compounds, was possibly responsible for the prebiotic formation of most of the organic molecules required for the ultimate formation of living matter and organisms (Oró and Lazcano-Araujo, 1981). Thus, although the origin of terrestrial

life probably depended, at least in part, on the presence and reactivity of HCN and its chemical derivatives, paradoxically the molecule is lethally toxic to the majority of living organisms (Marrs and Ballantyne, 1987).

The Swedish chemist Karle Scheele isolated hydrocyanic acid from Prussian blue in 1782. He also distinguished himself in 1786 when he accidentally broke a vial of the material in a laboratory and died from vapor poisoning (Annotation, 1978). Berthellot, in 1787, showed that hydrogen cyanide contained H, C, and N, but not O. In 1802, Schrader demonstrated that cyanide could be isolated from bitter almonds. In 1815, Gay-Lussac prepared HCN in semipure form and named it hydrocyanic acid (Ballantyne, 1987a).

HCN and other free cyanides have widely differing use patterns, some of which are to the advantage of mankind and others to the detriment of the human race. Thus, HCN has found use in several industrial applications, synthesis of certain organic molecules, fumigation, and pest control. Additionally, HCN has been used in several deliberate antisocial and distasteful activities such as murder, suicide, chemical warfare, and judicial execution, and there are currently suggestions that it could be in the armamentarium of terrorists. Also, accidental and uncontrolled release of HCN into the environment may result in adverse ecotoxicological and human health effects.

This summary of HCN will cover the physicochemical properties, applications, mechanisms of toxic action, metabolism and detoxification, experimental and human acute and repeated exposure, general and organ-specific toxicity, clinical toxicology, management of human poisoning, forensic and occupational toxicology, and the contribution of HCN to morbidity and mortality in fires.

30.2 CHEMICAL IDENTIFICATION AND PHYSICOCHEMICAL PROPERTIES OF HCN

HCN is chemically identified as follows:

Synonyms:	formonitrile
	prussic acid (as aqueous solution)
	hydrocyanic acid (as aqueous solution)
CAS no:	74–90–8
RTECS no:	MW6825000

The physicochemical properties of HCN are important determinants of its ready diffusibility, ease of penetration through biological membranes, and ubiquitous distribution in biological fluids and tissues. The most relevant properties are as follows (Sharpe, 1976; Jenks, 1983a; EPA, 1984; Homan, 1987; Marrs and Ballantyne, 1987; Lewis, 1993; Hathaway et al., 1996; ATSDR, 1997):

Physical state:	water-white liquid at $<26.5°C$
Molecular weight:	27.04
Density (liquid):	0.7510 g ml^{-1} (0°C)
	0.7017 g ml^{-1} (10°C)
	0.6884 g ml^{-1} (20°C)
Surface tension:	19.68 dyn cm^{-1} (20°C)
Viscosity:	0.2014 cP (20.2°C)
Melting point:	−13.3°C
Boiling point:	26.5°C
Vapor pressure:	6.697 kPa (−29.5°C)=50.2 mmHg

	35.24 kPa (0°C)=265.3 mmHg
	107.6 kPa (27.2°C)=807 mmHg
Vapor density:	0.947 (air=1; 31°C)
Henry's Law constant:	5.1×10^{-2} atm-m^3 mol^{-1}
Heat of combustion:	667 kJ mol^{-1}
Heat of vaporization:	25.2 kJ mol^{-1}
Flash point (closed cup):	−17.8°C
Autoignition temperature:	538°C
Explosive limits:	61–41 vol% in air (100 kPa; 20°C)
Odor:	see section 30.16.
pK_a:	9.21 (25°C)
Log K_{ow}:	0.66
Solubility:	water—highly soluble
Stability:	in water HCN is in equilibrium with small concentrations of CN^-. At 10°C and pH 9.2 >90% is present as HCN. At 10°C and pH 8.5, 92% is unionized HCN (Rodkey and Robertson, 1979).
Conversion factors:	kPa × 7.5 = mmHg
	1 mg m^{-3} = 0.89 ppm (air; 20°C)
	ppm (w/v) = mg l^{-1} = μg ml^{-1} (in water)

Because HCN has a pK_a of 9.21 (Smith and Martell, 1989), solutions of CN compounds in water (NaCN and KCN) can form hydrogen cyanide at acid and neutral pH. Significant out-gassing of HCN is prevented with alkaline solutions of pH > 12. HCN normally adds across C=C double bonds only if they are adjacent to a powerful electron-withdrawing group; carbonyl or cyano groups. In these cases a Michael addition proceeds readily under basic catalysis, as with acrylonitrile to yield succinonitrile (Jenks, 1983a). HCN adds across the carbonyl group of aldehydes and ketones and opens the oxirane ring of epoxides. HCN reacts with formaldehyde to form glycolic nitrile, and this may be relative to the fact that with formaldehyde-embalmed tissues there is a rapid decrease in analytically recoverable CN.

Detailed reviews of the methods available for the chemical and biochemical quantitative analysis of HCN can be found in Troup and Ballantyne (1987).

30.3 SYNTHESIS AND USES

Various methods are available for the industrial synthesis of HCN. The Andrussaw process involves the high-temperature reaction of ammonia, methane, and air over a platinum catalyst, and the Shawinagan process involves the high-temperature reaction of ammonia with propane (Homan, 1987). Also, HCN is a principal by-product of the Sohio process for acrylonitrile synthesis and from coke oven gas by reaction with sodium carbonate (Jenks, 1983a). U.S. production of HCN has increased steadily; for example, it rose from 128×10^6 lb $year^{-1}$ in 1955 to 570×10^6 lb $year^{-1}$ in 1977 (Jenks, 1983a).

HCN is used in the manufacture of various dyes, pigments, chelating agents, nitriles, monomers, resins, and fibers, including the following:

1. Sodium cyanide (NaCN) is produced by the neutralization of HCN with sodium hydroxide. Water is removed by filtration, and NaCN recovered by crystallization (Jenks, 1983b).

2. Cyanogen chloride can be produced by the oxidation of HCN with chlorine in the presence of a metal catalyst.
3. Methyl methacrylate synthesis is based on the reaction of acetone with HCN to produce acetone cyanohydrin, which is stabilized with sulfuric acid to prevent alkaline decomposition and CN release, and then reacted with methanol to yield methyl methacrylate (Smiley, 1983).
4. Melamine synthesis can be accomplished by using urea and HCN as starting materials.
5. Adiponitrile (hexanedinitrile), an intermediate in the manufacture of nylon, can be manufactured by the reaction of HCN with butadiene to produce pentenenitrile and subsequently adiponitrile.
6. Azobis*iso*butyronitrile, a catalyst for vinyl and acrylate polymerization, and a blowing agent for foam rubber, is synthesized on the basis of a reaction between HCN, acetone, and hydrazine.
7. Synthetic glycine and alanine are produced commercially by reaction of formaldehyde and acetaldehyde, respectively, with HCN and ammonia (Yamamoto, 1983).
8. Synthetic lactic acid production involves the reaction of HCN with acetaldehyde.
9. *t*-Butylamine is made from HCN and *iso*butylene.

Cyanides are used in a variety of industrial processes including electroplating, metal case hardening, and extraction of gold and silver (Hartung, 1982; Klaasen, 1980). HCN, released from cylinders or from moistened NaCN, has been extensively used for fumigation and pest control because of its ease of penetration and effectiveness (Ballantyne, 1986, 1988; Green and Li, 1983; Parmagianni, 1983). It has been used particularly for elimination of insects and rodents from orchards, glasshouses, buildings, warehouses, ships, and certain storage facilities. However, this use has recently been significantly reduced because of the hazards, licensing requirements, and the availability of safer synthetic alternatives. Less desirable documented applications of HCN have included judicial execution, chemical warfare, and, more recently, a suggested potential for use in terrorist activities (Wexler, 1947; Borowitz et al., 1992, 2001; De Lorenzo, 1999; NRC, 1999).

30.4 BIODETOXIFICATION AND METABOLISM

Cyanide is rapidly detoxified in mammals; for example, detoxification rates have been estimated at 0.076 mg CN kg^{-1} min^{-1} in guinea pigs (Lendle, 1964) and at 0.017 mg CN kg^{-1} min^{-1} in humans (McNamara, 1976). The major detoxification pathway for cyanide is by enzymatic transformation to the significantly less acutely toxic thiocyanate (SCN), which is then renally excreted. SCN production varies between species, but this pathway may contribute to detoxification by converting up to 80% of a given CN dose (Okoh and Pitt, 1982; Silver et al., 1982). Rat LD_{50} values for NaSCN are 764 mg kg^{-1} peroral (p.o.) and 540 mg kg^{-1} intraperitoneal (i.p.) (RETECS, 1981–1982). The corresponding values for NaCN are 5.7 mg kg^{-1} and 4.72 mg kg^{-1} (Ballantyne, 1984a), indicating a reduction in the acute lethal toxicity of CN by 120-fold from the transulfuration process. The mean elimination $t_{1/2}$ for SCN has been estimated at 2.7 days in healthy subjects and 9 days for those with renal insufficiency. Elimination of SCN was essentially wholly renal, and the elimination constants were inversely proportional to the creatinine clearances (Schulz et al., 1979). There are two enzyme systems responsible for the transulfuration process (Ballantyne, 1987a; Sorbo, 1975): thiosulfate-cyanide transulferase (EC 2.8.1.1; rhodanese) and β-mercaptopyruvate-cyanide transulferase (EC 2.8.1).

Rhodanese, first described by Lang (1933), is located in mitochondria and catalyzes the transfer of a sulfane sulfur atom from sulfur donors to sulfur acceptors.

$$CN^- + S_2O_3^{2-} \rightarrow SCN^- + SO_3^{2-}$$

The basic reaction involves transfer of sulfane sulfur from the donor (thiosulfate) to the enzyme, forming a persulfide intermediate. The persulfide sulfur is transferred from the enzyme to the nucleophilic receptor (cyanide) to yield SCN. Rhodanese does not have high substrate specificity, and thus other acceptors can interact with the enzyme. However, with CN, the conversion to SCN is essentially irreversible, which may not be the case with other nucleophilic acceptors. Enzyme activity is variable between tissues and species, but is high in liver, kidney, and olfactory tissues for most species. Thus, Himwich and Saunders (1948) demonstrated that monkeys, rabbits, and rats have the highest rhodanese activity in liver and kidney, with the hepatic activity being approximately 10–20 times greater in the dog liver. In the dog, highest rhodanese activity was in the adrenal gland, being about 2.5 times that of the liver. Rhodanese is present in nasal tissues, particularly in the olfactory region (Dahl, 1989). The olfactory region of the rat had nearly seven times more rhodanese on a per mg mitochondrial protein basis than did the liver. Thus the nasal metabolism of cyanide may have an important influence on the toxicity of inhaled CN and cyanogens.

β-Mercaptopyruvate-cyanide transulfurases are present in blood, liver, and kidney and catalyze the reaction:

$$HS{\cdot}CH_2{\cdot}CO{\cdot}CO{\cdot}O^- + CN \rightarrow SCN^- + CH_3{\cdot}CO{\cdot}CO{\cdot}O^-$$

There is some evidence that the thiosulfate sulfurtransferase system may not necessarily be the primary simplistic detoxification mechanism for CN outlined above, principally because little thiosulfate penetrates the inner mitochondrial membrane to access the transferase enzyme. A more general view of the role of sulfur in the detoxification process, converting CN to SCN, is that the supply of sulfane sulfur is from a rapidly equilibrating pool of potential sulfane sulfur donors and that these include per- and polysulfides, thiosulfanates, polythionates, inorganic SCN, and protein-associated elemental sulfur. The sulfurtransferases catalyze the formation, interconversions, and reactions of compounds containing sulfane sulfur atoms (Westley, 1981; Westley et al., 1983). Overall it is possible that sulfane sulfur is derived from mercaptopyruvate via β-mercaptopyruate sulfurtransferase, and the various forms of sulfane sulfur are interconverted by rhodanese. The sulfane carrier transporting the sulfur formed is plasma albumin; the sulfane sulfur-albumin complex then reacts with CN. Binding is adequate enough that the mechanism could operate *in vivo*. Pharmacokinetic studies indicate that the conversion of CN to SCN is predominantly in the central compartment, with a volume of distribution approximating to the blood volume (Way, 1984). It is possible that the plasma albumin-sulfane complex is a primary CN detoxification buffer in normal metabolism (Westley, 1981; Vennesland et al., 1982).

Other detoxification pathways, of a more minor nature, include the following:

1. Exhalation as HCN, and as CO_2 resulting from oxidative metabolism. Thus, guinea pigs eliminated 1–2% of intravenously (i.v.) infused HCN as unchanged material in expired air (Friedberg and Schwarzkopf, 1969). Rats given $Na^{14}CN$ i.v. excreted 1.7% of the dose in expired air; 90% as CO_2 and 10% as CN (Boxer and Rickards, 1952). At 24 h after the subcutaneous (s.c.) injection of $Na^{14}CN$, 4.53% was excreted in expired air as 91% CO_2 and 9% as CN (Okoh, 1983). In mice given KCN (4.6 mg kg^{-1}, s.c.) containing 4.5 μCi of [^{14}C]KCN, approximately 1% KCN was exhaled as HCN and 2% as CO_2 (Johnson and Isom, 1985). Trace amounts of hydrogen cyanide may be detected in the breath of normal subjects, but show no correlation with blood CN concentrations. Most of the HCN in normal breath originates from oxidation of SCN by salivary peroxidase in the oropharyx, and as such measurements can only be used to monitor heavy exposure (Lundquist et al., 1988).
2. Reaction with cystine, producing β-thiocyanoalanine, followed by ring closure to 2-aminothiazoline-4-carboxylic acid (ATC) or its tautomer, 2-iminothiazoline-4-carboxylic acid (Wood and Cooley, 1956). ATC is metabolically inert. A rat injected i.v. with ^{35}S-cystine and given NaCN s.c., excreted 23% of the ^{35}S as 2-iminothiazolidine carboxylic acid in the

urine over 3 days. After i.p. injection and p.o. dosing of rats, Ruzo et al. (1978) found SCN was the main metabolite (>95%) and ATC was a minor metabolite (<5%). Using high-performance liquid chromatography (HPLC), Lundquist et al. (1995) found a detection limit for ATC of 0.3 μ*M* in urine, and was stable for at least 3 months when stored at –196 to +20ºC. In the urine of healthy nonsmokers, ATC was below the level of detection, but in five cigarette-smoking subjects the urinary concentrations ranged from <0.3 to 1.1 μ*M*. In rats given acetonitrile in drinking water (40 and 80 m*M*), the mean urinary ATC concentration was 4.0 μ*M* (SD 2.4 μ*M*); control rats had no detectable ATC in urine.

3. Combination with hydroxocobalamin to form cyanocobalamin, which is then excreted in urine and bile (Brink et al., 1950; Boxer and Rickards, 1951; Herbert, 1975).

Erythrocytes have a high affinity for cyanide, and they rapidly take up cyanide from plasma (Barr, 1966; Vesey et al., 1976; Schulz et al., 1983; Schulz, 1984). This sequestration of cyanide by erythrocytes has been suggested as being a protective function in the detoxification of CN (Vesey and Wilson, 1978). The rapid assimilation of cyanide by erythrocytes was confirmed by McMillan and Svoboda (1982), who also determined that 4,4-di*iso*thiocyano-2,2-disulfonic acid, an inhibitor of ion transport function, only marginally slowed the entry, suggesting that CN passes through the erythrocyte membrane as HCN. They were able to confirm that SCN is oxidized to CN and cyanate by erythrocytes, as proposed by other workers (Goldstien and Rieders, 1951, 1953; Pines and Crimble, 1952) and that hemoglobin (Hb) catalyzes SCN oxidation (Chung and Wood, 1971).

Most studies on the metabolism of CN have been conducted using acute dosages. However, Okoh (1983) found that with rats exposed to KCN daily in the diet (77 mol rat^{-1} day^{-1}) for 6 weeks the mode of CN elimination in urine and expired air was not significantly altered from that which resulted from controls without KCN in the diet.

30.4.1 Metabolic Sequelae of Cyanide Toxicity

A major consequence of cytochrome *c* oxidase inhibition, and the resultant disturbance of electron transfer, is a reduction in mitochondrial O_2 utilization with decreased ATP levels (Olsen and Klein, 1947). Anaerobic metabolism continues resulting in lactic acid accumulation and lactate acidosis. The combination of cytotoxic hypoxia and lactate acidosis both result in severe metabolic consequences, in particular, in the CNS, causing disturbances of perception and of consciousness. The endogenous buffering of lactate leads to a progressive fall in plasma HCO_3^-. In brains from CN-poisoned mice it has been demonstrated that lactate, inorganic phosphate, and ADP increase, whereas ATP, phosphocreatine, glycogen, and glucose decrease (Estler, 1965; Isom et al., 1975). Rats receiving an i.v. infusion of CN (4 mg kg^{-1} h^{-1}) had an increase in the tricarboxylic acid intermediates succinate, fumarate, and malate (Hoyer, 1984), indicating disturbance of NAD^+- and FAD^+-dependent redox reactions, including pyruvate oxidation. Experimentally, Yamamoto and Yamamoto (1977) compared right heart blood acid–base changes in rats resulting from p.o. NaCN intoxication with those changes produced by exposure to a N_2 atmosphere that caused acute anoxic anoxia. The p.o. NaCN doses were 7, 10, 15, and 20 mg kg^{-1}, and survival times ranged from 2.4 to 14.0 min and increased with decreasing dose. There was a clear correlation between dose and time to death ($r = -0.90$). For the NaCN groups there was no correlation between survival time and P_{O_2} (range, 45–70 mmHg; $r = 0.34$). As the NaCN dose was decreased and the survival time increased, there was a significant fall in both P_{CO_2} ($r = -0.68$) and plasma HCO_3^- ($r = -0.92$), with increase in H^+ and lactate. Thus, as time to death increased the metabolic disturbance was more marked at the lower doses. Comparison with the N_2 anoxic anoxia results showed a much higher P_{O_2} and greater inhibition of aerobic metabolism for the CN group, being clearly related to the differences in the mechanism of hypoxia; i.e., cytotoxic with CN, and arterial hypoxemia with deficiency of O_2 supply to tissues having the N_2 exposure. Increased H^+ concentration, with deceased pH, resulted from lactate accumulation due to buffering, leading to the lactate acidosis.

Lactate acidosis with hyperglycemia has been noted in dogs given 4 mg KCN kg^{-1} i.v. (Klimmek et al., 1979) and rats given NaCN solution i.v. (Salkowski and Penney, 1995). In addition, Katsuma et al. (1980) found that in rats the plasma lactate, glucose, and oxypurines increased along with blood CN following i.p. KCN. Plasma lactate and oxypurines increased biquadratically, with respective half-maximum effects at 1.63 and 2.09 μg CN ml^{-1}. Plasma glucose increased quadratically as a function of blood CN. Plasma allantoin was not significantly altered, and therefore the increased plasma oxypurine was ascribed to degradation of tissue ATP during anoxia. Although the hyperglycemia could be partly explained by a catecholamine response, the fact that a marked increase in plasma glucose was detected when the plasma lactate became near-maximal suggests that the hyperglycemia may be due to stimulation of gluconeogenesis by the increased plasma lactate. Jakob and Diem (1974) found that CN produced an increase in phosphorylase activity and increased glucose production in the isolated and perfused liver.

Isom et al. (1975) investigated the influence of sublethal doses of CN (5 mg KCN g^{-1}) on glucose catabolism in the mouse using ^{14}C-glucose, sodium glucuronate-6-^{14}C and sodium gluconate-1-^{14}C. They confirmed in the mouse that glucose is metabolized through three pathways: the Embden–Meyerhof–Parnas pathway and tricarboxylic acid cycle, the pentose phosphate shunt, and the glucuronate pathway. CN was found to increase the catabolism of carbohydrate by the pentose phosphate shunt and decrease utilization of the Embden–Meyerhof–Parnas pathway, tricarboxylic acid cycle, and glucuronate pathway. They suggested that increased catabolism of carbohydrate by the pentose phosphate shunt may produce a source of NADPN which can reduce NAD by means of a transhydrogenase enzyme, and in this way compensate for the aberrant redox state produced by CN intoxication. CN also blocks basal and glucagon-induced lipolysis (Camu, 1969). These findings indicate that CN can alter carbohydrate metabolism resulting in increased glycogenolysis and a shunting of glucose to the pentose phosphate pathway by decreasing the rate of glycolysis and inhibition of the tricarboxylic acid cycle.

30.4.2 Functional Implications of the Detoxification Mechanisms

Because of the rapid absorption and biodistribution of CN and its mechanism of toxic action by inhibition of intramitochondrial cytochrome *c* oxidase, cyanides are rapidly acting compounds. A major determinant to the severity and latency to onset of toxicity is that the balance between the quantitative rate of absorption versus the rate of the endogenous detoxification will be such as to prevent the accumulation of significantly toxic amounts of CN. As the dose and the quantitative rate of absorption increase, the rate of availability of sulfur substrate is a determining factor for detoxification, and a relative reduction in sulfurtransferase detoxification may occur with an accumulation of free CN. When the rate of accumulation of free (toxicologically effective) CN is slow there will be a delay to both latency of onset and progression of toxic effects. Within limits, this effect on the detoxification process produces a clear relationship between to a given end point and the dose of cyanide (Ballantyne, 1987a). If acute overexposure to large doses of CN occurs, then, because of the high quantitative absorption rate, there may be a swamping of endogenous detoxification, resulting in the prompt onset of severe signs with rapid time to death. The efficient detoxification prevents long-term bioaccumulation of CN. Thus, acute exposure to a sublethal dose of CN results in the development of signs of toxicity, but as detoxification proceeds the signs ameliorate and disappear as CN is excreted (mainly as SCN) without any bioaccumulation.

30.5 MECHANISM OF ACUTE TOXIC ACTION

Following absorption and biodistribution, CN is capable of inhibiting the biological activity of a large number of enzymes, some of which are listed in Table 30.1. Because of this, and because other biological systems may be adversely affected, the mechanism and presentation of CN toxicity may be complex. The mechanisms of inhibitory action on these enzymes include combination with

functionally essential metal ions, formation of cyanohydrins with carbonyl compounds required for enzyme activity (i.e., pyridoxal phosphate-dependent enzymes), slow irreversible inhibition due to scission of essential disulfide links, elimination of S as SCN, CN addition to Schiff base aldimine with formation of an aminonitrile. In a few cases, enzyme activity may be increased; e.g., acetylcholinesterase (Owasoyo and Iramain, 1980). However, the major mechanism for the acute lethal toxicity of CN is by inhibition of cytochrome *c* oxidase activity, which is the terminal oxidase of the respiratory chain. This results in a cytotoxic hypoxia.

In 1925, Warburg demonstrated that cyanide inhibits oxidations specifically by iron salts, and Keilin in 1929 showed that cyanide combines with the Fe^{3+} atom in cytochrome oxidase (Sykes, 1981). Spectrophotometric evidence indicates that HCN binds with both the reduced and oxidized forms of the cytochrome a_3 component of cytochrome *c* oxidase (Chance, 1952; Lemberg, 1996; Antonini et al., 1971; Nicholls et al., 1972; Van Buuren et al., 1972). Because the rate of interaction of CN with the oxidized form of the enzyme is about two orders of magnitude less then that for the reduced form, it has been suggested that the kinetically disruptive effect of CN on mitochondrial electron transport is at the reduced cytochrome a_3 level (Yonetani and Ray, 1965). Jones et al. (1984) examined the CN-binding properties of resting and pulsed cytochrome *c* oxidase in both their stable and transient turnover states. A model was developed which accounted for CN inhibition of the enzyme, the essential feature of which was the rapid and tight binding of CN to transient, partially reduced, forms of the enzyme populated during turnover. Experimental data computer-fitted to kinetic predictions from the model indicated that the CN-sensitive form of the enzyme binds the ligand with combination constants in excess of $10^6\ M^{-1}\ s^{-1}$ and K_D values of 50 n*M* or less. Kinetic difference spectra indicate that CN binds to oxidized cytochrome a_3, and that this occurs only when cytochrome a and Cu_A are reduced. It thus appears probable that cyanide reacts with the reduced form of cytochrome *c* oxidase, which may subsequently be converted to an oxidized enzyme-cyanide complex (Way, 1984). The oxidized form is relatively stable, but in the presence of reducing equivalents CN can dissociate from the enzyme-inhibitor complex to reactivate the enzyme

TABLE 30.1 Examples of Enzymes, Other Than Cytochrome *c* Oxidase, That Are Inhibited by Cyanide

Enzyme	Reference
Acetoacetate decarboxylase	Autor and Fridovich (1970)
D-Amino oxidase	Porter et al. (1972)
Carbonic anhydrase	Fenney and Brugen (1973)
Catalase	Kremer (1970)
Glutamate decarboxylase	Tursky and Sajter (1962)
2-Keto-4-hydroxyglutarate aldolase	Hansen and Dekker (1976)
Lipoxygenase	Aharony et al. (1982)
Nitrite reductase	Lafferty and Garrett (1974)
Ribulose diphosphate carboxylase	Marsho and Kung (1976)
Succinic dehydrogenase	Zanetti et al. (1973)
Superoxide dismutase	Fenney and Brugen (1973)
	Borders and Fredivish (1985)
Tyrosine aminotransferase	Yamamoto (1992)
Xanthine dehydrogenase	Coughlan et al. (1980)
Xanthine oxidase	Massey and Edmondron (1970)
	Coughlan et al. (1969)

(Ballantyne, 1987a). The potentially reversible nature of the inhibition is the basis for the use of certain antidotal treatments that reactivate the enzyme by depleting intracellular CN; e.g., shifting the equilibrium to the extracellular (plasma) compartment by cyanmethemoglobin (CNmetHb) formation, by chelation, or by conversion to SCN. Although inhibition of cytochrome *c* oxidase appears to be a major feature in the overall toxicity (lethal and nonlethal) produced by CN, other mechanisms may well be operative. For example, Pettersen and Cohen (1985) found an equivalent degree of inhibition of cytochrome *c* oxidase in brain and heart from mice following s.c. dosing with KCN at 4 or 20 mg kg^{-1}; respective mortalities were 0 and 100%. At 4 mg kg^{-1} sequential sacrifices showed that maximum inhibition of cerebral (40%) and myocardial (60%) cytochrome oxidase activity occurred at 10–20 min postdosing. Mice died within 5 min of 20 mg kg^{-1} KCN and had a 35% inhibition of brain and 60% inhibition of myocardial cytochrome oxidase activity. In further studies Pettersen and Cohen (1986) compared the *in vitro* effects of cyanide on mouse brain mitochondrial respiratory and cytochrome oxidase activities. Enzyme activity was inhibited in a linear fashion, with an IC_{50} of 4×10^{-4} *M*. Respiratory rates were slightly inhibited up to 10^{-4} *M* and 80% inhibited at 10^{-3} *M*. Thus, *in vitro,* large effects on respiration required >50% inhibition of cytochrome *c* oxidase activity. They suggested that 50% of cytochrome oxidase activity might be functional reserve. Further details of other mechanisms for cyanide toxicity are presented below in section 30.8 on neurotoxicity.

Because CN toxicity is by an intracellular (mitochrondrial) mechanism, resulting in a cytotoxic hypoxia, and because cyanide is sequestered by the erythrocyte (Vesey et al., 1976; Vesey and Wilson, 1978), it is the plasma CN concentration that is the prime determinant of cytotoxicity (Vesey, 1979; Ballantyne, 1979, 1987a). The inhibition of cytochrome *c* oxidase, consequent decrease in mitochondrial electron transport, and resultant cytotoxic hypoxia initially occurs in the presence of normal hemoglobin oxygenation. The reduction in oxidative phosphorylation leads to lactate acidosis. A major cause of lethal toxicity by CN is disturbance of central regulating mechanisms for breathing, but experimental evidence indicates that direct myocardial toxicity may also be a significant factor in the lethal toxicity of CN (Suzuki, 1968; Ballantyne and Bright, 1979a). The sensitivity of myocardial cytochrome *c* oxidase to CN is known from direct inhibitor studies (Camerino and King, 1966) and the low I_{50} of 2.74 μM (Ballantyne, 1977a).

30.6 ACUTE TOXICITY

30.6.1 Determinants of Acute Toxicity

As noted above the development of toxic effects from CN depends on an imbalance between the rate of absorption and its detoxification resulting in a progressive accumulation of CN in body tissues and fluids until this is quantitatively sufficient to adversely affect the vital biochemical pathways through which CN produces its toxic effects; i.e., the major determinants of the exhibition of CN toxicity are the rate of accumulation and the absolute magnitude of free (toxicologically active) CN at cellular target sites. To this end, many factors influence the bioavailability, biodistribution, detoxification, and bioelimination of CN, and their dynamic balance at any given time will govern the availability of free CN and hence the potential for toxicity to occur. Major determinants are as follows.

30.6.1.1 Rate of Absorption

The amount and rate of absorption of CN through a primary exposure route (i.e., the absorbed dose) depends on various factors, which include:

(a) The physicochemical properties of the molecule. Thus, HCN has a low molecular weight (MW), is nonionized, and readily diffuses; in contrast, KCN has a higher MW and, because it is ionized, may be absorbed at a significantly lesser rate. This is reflected simply in the difference in LD_{50} values; e.g., the acute i.m. LD_{50} values (with 95% confidence limits)

are 0.018 (0.017–0.020) mmol kg^{-1} for HCN and 0.050 (0.042–0.063) mmol kg^{-1} for KCN (Ballantyne et al., 1972).

(b) The exposure dose; i.e., the amount of CN available for absorption, which is a function of the amount of CN to which the organism is exposed, the exposure concentration and the exposure time, and the number and frequency of exposures.
(c) The route of exposure, because different routes may have different permeabilities. Thus, HCN vapor is readily absorbed through the pulmonary alveolar membrane, but skin will present a greater barrier to absorption. Additionally, the integrity of the absorbing membrane may be an important determinant; for example, cyanides are absorbed more readily through abraded than through intact skin (Ballantyne, 1984a, 1994a).

The absorbed dose is a prime determinant of the amount of CN available for distribution to the tissues by the blood stream. However, because there is a sequestration and binding of CN to the erythrocyte, and since CN in plasma is readily available for diffusion into the intercellular and intracellular fluids, the plasma concentration is the major quantitative determinant of the onset and severity of toxicity.

30.6.1.2 Differential Distribution of Cyanide

The differential distribution of CN to various tissues is clearly an important factor in toxicity because it determines the relative proportions of CN present at the detoxification sites and target tissues. Important in this respect is the route of exposure. Thus, inhaled or percutaneously absorbed CN will pass initially to the systemic circulation and target organs, with only a small proportion passing through the organs of detoxification, notably the liver. In contrast, a high proportion of CN dosed orally will pass through the liver and undergo first-pass detoxification. However, the factors involved are more complex than solely a consideration of hepatic transulfuration biodetoxification. For example, dietary variations that result in significant changes in hepatic rhodanese do not correlate with CN toxicity (Rutkowski et al., 1985), and extensive surgical or chemical injury to the liver does not increase the susceptibility of the mouse to the lethal toxicity of CN (Rutkowski et al., 1986). The influence of route is probably determined more by the relative influence of plasma transulfuration, erythrocyte sequestration, and the differential distribution to all tissues with a detoxifying biotransformation capacity. Differences in regional blood flow between tissues will clearly influence the rate of delivery of CN to specific tissues and thus the total organ or tissue dose. Also, the binding affinity of CN for cellular structures or macromolecules may influence the relative tissue and cellular distribution of CN. In general, CN is freely diffusible between blood plasma, interstitial fluid, and intracellular fluid compartments. However, as discussed above erythrocytes and certain intracellular macromolecules may be sites of sequestration.

30.6.1.3 Endogenous Detoxification

The rate of detoxification, in particular, enzymatic transulfuration, will clearly influence the rate of accumulation of body CN. As noted previously, with slow rates of absorption, the detoxification rate may equate with that of the absorption rate and signs of toxicity will not develop. As the rate of absorption increases, the rate of detoxification will not increase proportionately and there may an accumulation of CN; the rate of accumulation will obviously relate to the rate characteristics for the absorptive process. When there is a slow but continual increase in body CN, then a characteristic series of signs and symptoms will develop sequentially. However, an abrupt massive overdose may swamp the endogenous detoxification mechanisms and there will be prompt onset of severe toxicity with rapid death. Important determinants for endogenous detoxification potential are the rate of supply of CN to tissues having detoxification potential and the availability of substrate, in particular, sulfur donors for the transferase processes.

30.6.1.4 Miscellaneous Factors

Other important determinants for the development of toxicity are:

1. *Diurnal variation.* Baftis et al. (1981) investigated the variability in the lethal toxicity of i.p. KCN (72.5 mg kg^{-1}) to mice as a function of time, using a 12-h light-dark cycle (06.00–18.00 h/18.00–06.00 h). Mortality rate varied during the 24-h period, with peak activity at 16.00 h (83%) and a minimum at 06.00 h (43% mortality). There was also a circadian pattern in time to death; times were sooner at 2.00 h dosing and longest at 08.00 h dosing.
2. *Age.* Fitzgerald (1954) found that for adult male mice the s.c. LD_{50} of NaCN was about 5 mg kg^{-1}; in contrast, with neonatal mice the LDs were between 2.0 and 2.5 mg kg^{-1}.
3. The presence of *antidotal substances* will clearly influence the development of toxicity. For example, binding to methemoglobin (metHb), chelation with cobalt salts, or supplying sulfur donors will result in a shift in the equilibrium of CN from the intracellular to the extracellular compartment, and hence reactivation of inhibited cytochrome *c* oxidase.

The next section presents the quantitative acute toxicity of HCN vapor to laboratory animals and to humans by inhalation and by absorption across the skin. Details of the quantitative acute toxicity of HCN and other cyanides by other routes of exposure and absorption (p.o., i.p., s.c., i.v., p.c., and transocular) have been presented elsewhere (Ballantyne, 1983a, 1983b, 1984a, 1986, 1994a; Ballantyne et al., 1972).

30.6.2 Experimental Acute Inhalation Toxicity of HCN Vapor

Because of its low MW, poor ionization, and ready diffusion, HCN is readily absorbed across the lung. Published acute lethality data for various species and exposure times are given in Table 30.2. Examination of these data indicates that over an exposure period ranging from a few minutes up to an hour, there is a disproportionate relationship between the exposure time required to kill a given proportion of an exposed population and the exposure concentration needed to kill the same proportion. Thus, over the period of about an hour, as the lethal concentration is decreased the exposure time required to cause death increases, but not in proportion. The relationship between exposure time (*t*), exposure concentration (*C*), and the inhalation exposure dosage (*Ct*) required to cause death was studied in detail by Ballantyne (1984a, 1987a, 1994b). The results from some of these studies are illustrated in Figure 30.1. It can be seen that for the shorter exposure periods (a few seconds to a few minutes), as the exposure concentration required to produce a 50% mortality (LC_{50}) decreases, only relatively small increases in exposure time are required to attain 50% mortality; e.g., the exposure time for a 1229 mg m^{-3} LC_{50} is 1 min, and for a 493 mg m^{-3} LC_{50} is 5 min. This accords with the fact that under both of these exposure conditions it is likely that there is near saturation of the endogenous detoxification mechanisms. However, beyond about 5 min, proportionately longer exposure times are required to produce LC_{50} decreases; e.g., a 173 mg m^{-3} LC_{50} requires that there be an exposure time of 30 min. The relationship between LC_{50} and exposure time can be more clearly defined by examining the corresponding acute lethal inhalation dosages (LCt_{50}), as shown in Figure 30.2. This demonstrates that as the exposure time required to produce a lower LC_{50} increases, the LCt_{50} increases, almost in a linear manner. Thus, much higher LCt_{50} values are required to produce a given LC_{50} at the lower-concentration–longer-exposure conditions, than is the case for the higher concentration–shorter duration conditions. With the higher HCN airborne concentrations there is a marked concentration gradient across the alveolar membrane, facilitating the transfer and absorption of HCN that then enters the systemic circulation without a significant first-pass hepatic detoxification effect, and there is thus a rapid attainment of toxic tissue concentrations of CN. With lower HCN vapor concentrations there is a slower rate of titration of CN into the pulmonary circulation and a higher proportion of the dose is detoxified, resulting in a slower rise in the body burden of CN, and thus a relative delay to the onset of signs and a longer time to death. The LC_{50} versus exposure time curve (Figure 30.1) shows

an abrupt inflexion, which represents an important point in the absorption-detoxification scheme. At concentrations above this inflection there is a steep slope in the response line, indicating a significant reduction in the endogenous detoxification reserve. When deaths are examined over a long postexposure period (within 24 h after the inflexion point) the LC_{50} may be fairly constant (Levin et al., 1987). This is likely the result of an intermixing of mortalities due to cytotoxic hypoxia and to complications of this effect. Matijak-Schaper and Alarie (1982) found that the 30-min LC_{50} for Swiss–Webster mice was similar at 166 ppm for both intact mice and those dosed with HCN through a tracheal cannula, suggesting that in this species the absorption of HCN through the upper respiratory tract was not a significant factor in lethal toxicity.

Signs of HCN intoxication vary in both latency to onset and severity, depending mainly on the concentration of HCN vapor and exposure time. Typical signs resulting from experimental intoxication are rapid breathing, weak and ataxic movements, convulsions, loss of voluntary movement, loss of consciousness, decrease in breathing rate and depth, and breathing irregularities. In general, mortalities from HCN vapor occur either during exposure or within a short time of the end of exposure. Necropsy findings are few and consist mainly of congestion of various intra-abdominal viscera and scattered pleural and alveolar hemorrhages (Ballantyne, 1994b).

Using plethysmography, Matijak-Schafer and Alarie (1982) found that for the intact mouse the HCN vapor concentration needed to cause a 50% reduction in breathing rate (DC_{50}) by a 30-min exposure was 63 ppm. Below the DC_{50} asphyxiation was seen intermittently between short periods of normal breathing, but as lethal concentrations were approached, asphyxiation became continuous. With tracheal cannulated mice the DC_{50} was reduced to 34 ppm. In primates, Purser et al. (1984) noted that early during exposure to HCN in the range 102–156 ppm there was a marked episode of hyperventilation with significant increase in respiratory minute volume. This was followed by a slowing in the breathing rate, disturbance of consciousness, loss of muscle tone and reflexes, and occasional convulsions. At the end of the exposure period there was a rapid recovery.

An important clinical feature of the inhalation toxicology of HCN is the development of incapacitation; it is one of several factors that may impede escape from an atmosphere containing HCN. This may appear as muscle weakness, ataxia, and a semiconscious state that progresses into coma.

TABLE 30.2 Acute Lerthal Inhalation Toxicity of HCN Vapor to Various Species

Species	Sex	Exposure Time	Median Lethal Toxicity (95% confidence limits)	
			as LC_{50} (mg m^{-3})	as LCt_{50} (mg min m^{-3})
Mouse[a]	M	30 min	176 (129–260)	5280 (3870–7880)
Rabbit[b]	F	45 sec	2432 (2304–2532)	1824 (1728–1899)
	F	5 min	409 (321–458)	2044 (1603–2288)
	F	35 min	208 (154–276)	7283 (5408–9650)
Rat[b]	F	10 sec	3778 (371–4313)	631 (562–719)
	F	1 min	1129 (664–1471)	1129 (664–1471)
	F	5 min	493 (372–661)	2463 (1861–3301)
	F	30 min	173 (159–193)	5070 (4690–5497)
	F	60 min	158 (144–174)	9441 (8609–10399)
Rat[c]	NS	5 min	553 (443–689)	2765 (2215–3445)

[a] Matijak-Schaper and Alarie (1982).

[b] Ballantyne (1994b).

[c] Higgins et al. (1972); NS, not specified.

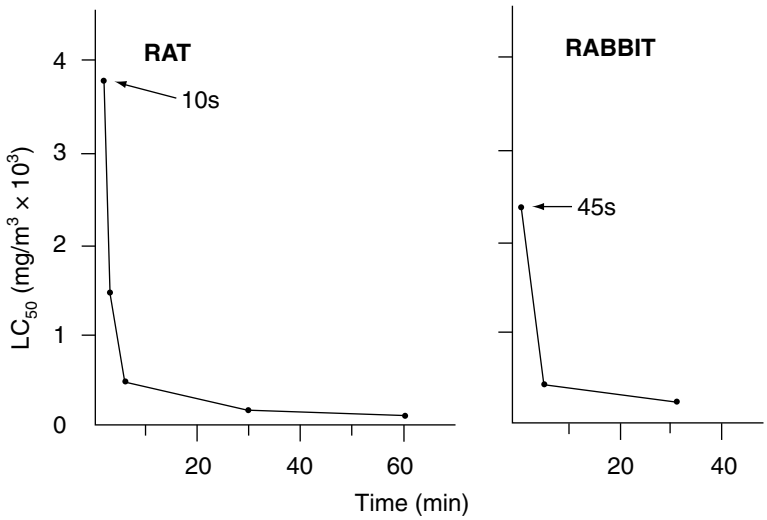


FIGURE 30.1 Relationship between exposure concentration (mg m^{-3}) and exposure time to causing a 50% mortality at a specified concentration (LC_{50}) in rats and rabbits exposed to HCN vapor. The slopes on the time–LC_{50} curves indicate a disproportionate relationship between time and concentration.

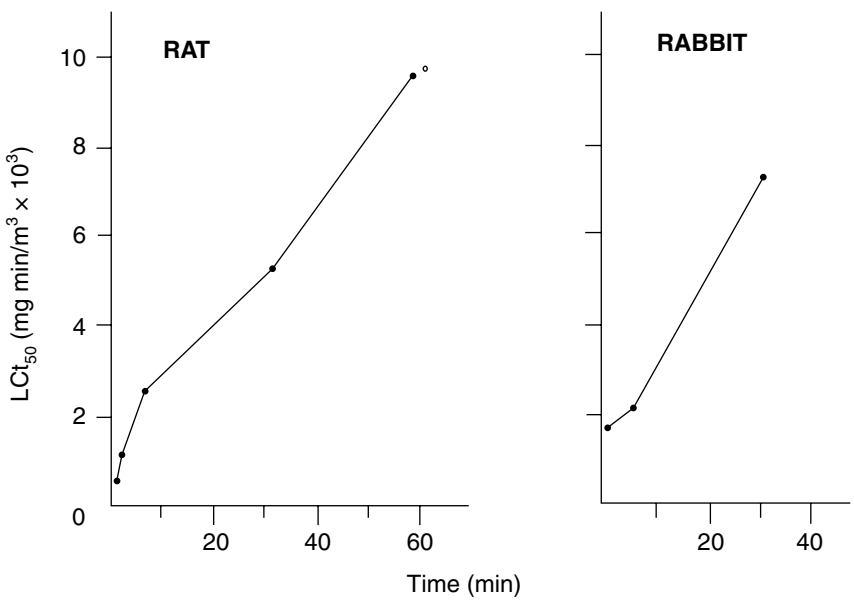


FIGURE 30.2 Relationship between lethal inhalation exposure dosage and time to cause a 50% mortality (LCt_{50}) in rats and rabbits. The individual points correspond to the exposure times and concentrations shown in Figure 30.1. The LCt_{50} increases progressively with time.

Over the range of HCN vapor concentrations, 102–156 ppm, used by Purser et al. (1984) there was a linear relationship between exposure concentration and the time to occurrence of hyperventilation and subsequent incapacitation ($r = 0.96$, $p < 0.02$). The slope on the relationship between exposure concentration and time to incapacitation was such that doubling the concentration from 100 to 200 ppm reduced the time to incapacitation from 25 min to 2 min. Thus a short period of exposure to concentrations of the order of 200 ppm (220 mg m^{-3}) HCN can cause the rapid onset of incapacitating effects. Rat studies have estimated the HCN vapor incapacitating concentrations are about 65% of the lethal concentration (Levin et al., 1987). In their studies Purser et al. (1984) found that for the first 10 min of the 30-min exposure period there was a linear correlation between HCN vapor concentration and blood CN ($r = 0.94$) if allowances were made for minute volume between different animals. After 10–15 min a plateau developed for blood CN. At the end of the 30-min exposure period there was no clear relationship between atmospheric HCN concentration and whole-blood CN concentration. Postexposure, blood CN remained unchanged for a 1-h recovery period. The average concentrations of blood CN for four monkeys were the following: during exposure, 10 min = 77 μmol l^{-1}, 20 min = 86 μmol l^{-1}, 30 min = 86 μmol l^{-1}; postexposure, 60 min = 85 μmol l^{-1}. Using HCN exposure concentrations of 71 and 183 ppm in rats, Chaturvedi et al. (1993) found the blood CN was proportional to both HCN exposure concentration and exposure time up to 35 min. They did not find a correlation between blood CN and the onset of incapacitation. In rats dying from acute inhalation of HCN vapor, the highest CN concentrations were found in blood, brain, heart, and lung, but liver CN was either low or absent (Ballantyne, 1994b). Details of blood and tissue CN concentrations following mortality from CN given by different routes of exposure have been presented by Ballantyne (1983a, 1983c, 1984a).

30.6.3 Human Acute Inhalation Lethal Toxicity of HCN Vapor

In 1931, Barcroft described a situation in which a man apparently survived a 15-min exposure to about 50–677 mg m^{-3} HCN vapor, but an accompanying dog became apneic. Bonsall (1984) described a case of industrial exposure of a man to HCN liberated from hydrazodi*iso*butyronitrile. After a 3-min exposure to (estimated) 500 ppm he collapsed and was fitted with breathing apparatus. He was subsequently treated with i.v. sodium thiosulfate, became paralyzed with convulsions, and was intubated, artificially ventilated, and given i.v. phenytoin. Weaned off the respirator at 48 h he was fully conscious at 36 h. Thus, through intensive medical management this individual survived a 3-min exposure to about 500 ppm HCN vapor. In other cases, exposure to 270 ppm HCN caused immediate death, 181 ppm HCN was lethal within 1 min, and 135 ppm was lethal after 30 min (Dudley et al., 1942). Moore and Gates (1946) estimated the absorbed lethal inhalation dosage for humans as 1.1 mg kg^{-1} based on the assumption that humans would be similar to several animals. Data available for i.v. LD_{50} values (in mg kg^{-1}) were 1.34 (dog), 0.81 (cat), 1.3 (monkey), 0.66 (rabbit), 1.43 (guinea pig), 0.81 (rat), and 0.99 (mouse). The detoxification rate of CN given i.v. to man was taken as 0.017 mg kg^{-1} min^{-1}. The LCt_{50} was calculated according to the formula:

$$K = VaC—Dt$$

Where, K = i.v. lethal dose (in mg kg^{-1}); C = total volume of breathed air (l kg^{-1}); a = fraction of inhaled vapor absorbed (70% in the dog); D = detoxification rate (mg kg^{-1} min^{-1}). Based on this approach, the timed LC_{50} values for a 70-kg man with a breathing rate of 25 l min^{-1} were calculated as shown in Table 30.3. These findings suggest, on the basis comparative data from other primates, that man is more resistant than monkeys. However, McNamara (1976) commented that it is doubtful if man is so much more resistant because the values for the monkey could also be assumed from the LCt_{50} values obtained from the Moore and Gates formula. Also, the experimental LCt_{50} values for HCN in animals increase with exposure time at a rate greater than calculated from the formula. This is not surprising since the general format of the Moore and Gates formula is similar to the Haber

equation (Haber, 1924), which assumes constancy of a fixed response for inhalation dosages over a certain range. Based on the following assumptions, McNamara (1976) re-evaluated the lethal inhalation toxicity of HCN vapor as shown in Table 30.4.

He assumed (1) that man has a susceptibility similar to that of the resistant goat or monkey, and (2) since (at that time) only mouse LCt_{50} data were available for 0.5 to 30 min, these values were used to estimate the relationship between LCt_{50} and time. The values were multiplied by four to obtain estimates of LCt_{50} for man at each interval, since the ratio of mouse:primate lethal inhalation toxicity is of the order of 1:4.

Based on available metabolic rate data, the following LC_{50} values were predicted by Hilado and Cummings (1977) for man, which are somewhat lower than the values calculated by McNamara (1976):

$$5\text{-min } LC_{50} = 680 \text{ ppm } (748 \text{ mg m}^{-3})$$

$$30\text{-min } LC_{50} = 200 \text{ ppm } (220 \text{ mg m}^{-3})$$

Landhahl and Herrmann (1950) estimated that following the inhalation of HCN vapor in the concentration range 0.5–20 mg m^{-3}, approximately 60% is retained through the lungs.

TABLE 30.3 Inhalation Toxicity of HCN Vapor to Humans Based on the Moore and Gates (1946) Formula and Calculated for a 70-kg Man with a Breathing Rate of 25 l min^{-1}. The Detoxification Rate for CN Was Assumed to Be 0.017 mg kg^{-1} min^{-1}

Time (min)	Calculated Lethal Inhalation Toxicity	
	as LC_{50} (mg m^{-3})	as LCt_{50} (mg min m^{-3})
1	4400	4400
3	1500	4500
10	504	5040
30	210	6300
60	140	8400

Table 30.4 Estimates of the Acute Lethal Inhalation Toxicity of HCN Vapor to Man by McNamara (1976)

Exposure	LCt (mg min m^{-3})			Approximate
Time (min)	1	50	90	LC_{50} (mg m^{-3})
0.5	1,117	2,032	3,480	4,064
1	1,930	3,404	5,705	3,404
3	2,546	4,440	7,526	1,466
10	3,888	6,072	11,491	607
30	11,992	20,632	35,443	688

30.6.4 Experimental Percutaneous Absorption of HCN Vapor

It is well documented that solutions of CN and solid cyanides applied to moist skin can readily penetrate the skin and produce intoxication (Ballantyne, 1994a). Also, HCN vapor can be absorbed across skin and can additively contribute to toxicity due to inhaled HCN, notably in the workplace. This stresses the need for skin as well as respiratory protection when overexposure to HCN is anticipated. Experimentally, Fairley et al. (1934) demonstrated that exposure of guinea pigs and rabbits to HCN in the atmosphere resulted in the p.c. absorption of sufficient amounts of CN to cause signs of toxicity and, if exposure were sufficiently prolonged, to cause mortality.

30.6.5 Percutaneous Absorption of HCN by Humans

When working for 8–10 min in an atmosphere containing 20,000 ppm HCN vapor but wearing respiratory protective equipment workers developed dizziness, weakness, and headache, probably due to the p.c. absorption of vapor (Drinker, 1932). Two cases of percutaneous HCN vapor poisoning, one in a firefighter wearing self-contained breathing equipment, were described by Steffens (2003). According to Dugard (1987) the rate of absorption of HCN vapor across the skin may be assumed to be proportional to the concentration in the atmosphere contacting the skin. Studies lead to a conclusion that total body surface contact (18,500 cm^2 for a 70-kg individual) with 1 ppm HCN by volume leads to the absorption of 32 μg CN h^{-1}. It has been estimated that the detoxification rate for a 70-kg individual is around 30 mg CN^{-1}. If the rate of absorption of CN exceeds this value then CN accumulates.

30.7 REPEATED EXPOSURE GENERAL TOXICITY OF CYANIDE

It is clear from the preceding discussions that animals and humans may tolerate potentially lethal (bolus) doses of CN if these are given over longer periods or in divided doses. Thus, Hayes (1967) found that rats tolerated 25 daily doses of KCN, each of which was equivalent to the acute p.o. LD_{50} (approximately 10 mg kg^{-1}) when these were given in the diet. Also, 90 daily doses, each of which was equivalent to 25 $\times$ LD_{50} (250 mg kg^{-1} day^{-1}), did not cause mortalities in rats when administered in the diet. Palmer and Olsen (1979) recorded increased liver weights, but no effects on body weight gain, in rats dosed with 200 mg l^{-1} KCN in drinking water for 21 days, which resulted in a daily intake of 30 mg KCN kg^{-1}. No such effects were seen with KCN in the diet (0.2 mg kg^{-1}; 20 mg kg^{-1} day^{-1}).

Rats receiving 1.075 mg KCN g^{-1} in the diet over 56 days showed no effects on body weight, food consumption, protein efficiency ratio (body weight gain/protein intake), or kidney and liver weights. Pigs receiving 1.25 mg KCN g^{-1} diet had a slight decrease in food consumption, but body weight was unaffected (Tewe and Maner, 1980, 1981a, 1981b). Rats having a diet containing 2.5 mg KCN g^{-1} (90 mg KCN kg^{-1} day^{-1}) had a slight reduction in food consumption and body weight gain (Tewe, 1982). Beagle dogs had NaCN incorporated in the diet (2.5 mg kg^{-1}) for 30–32 days, resulting in an oral intake of 6 mg NaCN kg^{-1} day^{-1}. There were no clinical signs, effects on food consumption, body weight or hematology, and no histopathology (American Cyanamid Company, 1959). In another study, dogs received NaCN by capsule up to 6 mg kg^{-1} day^{-1} for 15 months (Herrting et al., 1960). This resulted in immediate signs of toxicity postdosing and recovery within 30 min. The erythrocyte count was increased and serum albumin decreased. Degenerative changes were seen in cerebrocortical and cerebellar Purkinje cells. Baboons were given s.c. injections of KCN (1 mg kg^{-1}) for 9 months. The Hb concentration was increased, mean corpuscular Hb concentration decreased, and there was a low mean corpuscular volume (Crampton et al., 1979).

The potential for hepatonephrotoxicity was investigated by Sousa et al. (2002) who dosed adult male rats with KCN dissolved in drinking water at 0 (controls), 0.3, 0.9, 3.0, or 9.0 mg kg^{-1} day^{-1} for 15 days. Those given 9 mg kg^{-1} day^{-1} had a significant reduction in body weight gain over the study period, but there was no effect on water consumption. Plasma SCN concentrations were significantly ($p < 0.05$) increased in the KCN groups, but serum T_3 and T_4

concentrations were unaffected. However, histology of the thyroid glands revealed a dose-related increase in the number of cytoplasmic resorption vacuoles in the follicular colloid at all doses. Histology of the liver revealed cytoplasmic vacuolation of hepatocytes at 0.9 mg kg^{-1} day^{-1} and above, with some hepatocyte vacuolar degeneration at 9.0 mg kg^{-1} day^{-1}. In keeping with these findings, the serum aspartate aminotransferase activities (as mean ± SD; U l^{-1}) were significantly increased at 0.3 (80.1 ± 7.7), 0.9 (79.9 ± 6.2), and 3.0 (73.0 ± 11.1) mg kg^{-1} day^{-1}, but decreased at 9.0 mg kg^{-1} day^{-1} (43.2 ± 7.3) compared to the controls (60.3 ± 10.5). Kidneys from the 3.0 and 9.0 mg kg^{-1} day^{-1} groups showed congestion and cytoplasmic vacuolation of the proximal tubular epithelial cells. However, serum creatinine and urea concentrations were not significantly different from the controls, suggesting no alteration in renal function. Okolie and Osagie (1999) also experimentally investigated the potential for chronic hepatorenal lesions from 40 weeks of feeding a diet containing 702 ppm KCN; controls received a diet without added KCN. The results (summarized in Table 30.5) show that although food consumption and energy intake were increased in the KCN group, weight gain was reduced. This may be due to retarded muscle development as shown by Ibebunjo et al. (1992), who suggested that depressed growth rate associated with CN may be a consequence of methionine depletion leading to decreased protein turnover. Also, CN exposure and SCN production interferes with thyroid function and decreases tissue protein turnover (Hayase et al., 1987). CN consumption in the control diet was 9.0 ppm, resulting in a total CN intake of 0.39 mg day^{-1} $rabbit^{-1}$ compared with 36.5 mg day^{-1} $rabbit^{-1}$ for the 702 ppm KCN diet. The corresponding urine SCN concentrations were 10.8 ± 0.90 μmol dl^{-1} and 51.8 ± 2.4 μmol dl^{-1} ($p < 0.05$). These are consistent with the increased liver and kidney rhodanese activities of the KCN-fed rabbits (Table 30.6). Also the increased activities of lactate dehydrogenase in serum, liver, and kidney from KCN animals compared with the controls are consistent with a shift from aerobic to anaerobic metabolism in the KCN animals because of enhanced conversion of pyruvate to lactate (Padmaja and Pannikar, 1989a, 1989b; Singh et al., 1989). Biochemical evidence for hepatotoxicity was seen by significant increases in serum and decreases in liver sorbitol dehydrogenase, alkaline phosphatase, and glutamate-pyruvate transaminase, and for nephrotoxicity by the significant decrease in kidney alkaline phosphatase associated with a serum increase. The biochemical evidence for renal injury was supported by increases in serum urea and creatinine. Histologically there were focal areas of congestion and necrosis in the liver and foci of tubular and glomerular necrosis in the kidney. The findings suggest that chronic cyanide exposure, at least by the p.o. route, may result in hepatorenal injury. In a subsequent extension of the publication of the results, Okolie and Osagie (2000) reported the effects on heart, lung, and pancreas. Aspartate transaminase activities in heart and serum were unaltered, but alkaline phosphatase activity in heart and lung was significantly decreased (Table 30.6). Weekly measurements of serum amylase activity and blood glucose concentrations showed no significant differences between the KCN and control groups over the 40-week period. Histology of the pancreas and myocardium was normal, but lungs showed foci of pulmonary edema and necrosis. Thus, under the conditions of this study prolonged dietary consumption of sublethal doses of cyanide did not result in cardiac injury or any toxicity to the pancreas, but did cause systemic injury to the lungs. In a multispecies study (Soto-Blanco et al., 2001), rats received 0, 9, or 12 mg KCN kg^{-1} day^{-1} for 15 days; pigs were dosed 0, 2, 4, or 6 mg KCN kg^{-1} day^{-1} for 74 days; and goats received 0, 0.3, 0.6, 1.2 or 3.0 mg KCN kg^{-1} for 5 months. At the end of each dosing period, plasma samples were taken to measure SCN and glucose, and the pancreas was removed for histological examination. Lower body weight gains ($p < 0.05$) were noted in rats that received 9 and 12 mg kg^{-1}, and in goats from the 3 mg kg^{-1} group. Pigs had no differences in body weight gains. Plasma SCN concentrations were significantly higher ($p < 0.05$) in all experimental groups of all species compared to the controls. However, plasma glucose concentrations of all species were not significantly different from controls. Histological examination of the pancreas did not reveal any exocrine or endocrine abnormalities.

A 13-week study was conducted in which NaCN was administered in the drinking water to male and female F344/N rats and B6C3F_1 mice (Hébert, 1993). The drinking water NaCN concentrations were 0, 3, 10, 30, 100, and 300 ppm. Absorption of CN was confirmed by measured increases in urinary SCN. There were no adverse effects with respect to body weight, organ weights, hematology, clinical

TABLE 30.5 Food Consumption, Weight Gains, Feed Efficiency, and Thiocyanate Excretion in Control Rabbits and Rabbits Dosed with Cyanide in the Diet (702 ppm) for 40 Weeks

Measurement	Controls[a]	Cyanide[a]	p[b]
Food consumption (g rabbit-1 day^{-1})	44.1 ± 2.1	52.0 ± 1.7	<0.05
Energy intake (kcal day^{-1} rabbit^{-1})	101.4 ± 6.7	119.6 ± 3.9	<0.05
Weight gain (g rabbit^{-1})	919.0 ± 2.1	612.0 ± 1.8	<0.05
Feed efficiency (g body weight/g feed)	0.07	0.04	
Total cyanide intake (mg kg^{-1} rabbit^{-1})	0.39 ± 0.02	36.5 ± 1.2	<0.05
Urine thiocyanate (μmol dl^{-1})	10.8 ± 0.9	51.8 ± 2.4	<0.05

[a] Results as mean ± SE.

[b] Significance of difference between control and cyanide group.

Source: From Okolie and Osagie (1999, 2000).

chemistry, or histopathology. There was no evidence for thyroid or neurological damage. Concentrations of 100 ppm and above produced decreased water consumption, suggesting poor palatability. There were no changes in urinary pH, sorbitol dehydrogenase, or *N*-acetyl-β-D-glucosaminidase activity. With male rats there was a slight reduction in cauda epididymal weights in 30 ppm dosed male rats and mice, and in 300 ppm dosed rats the number of spermatid heads per testis was less than that of controls; sperm motility in all groups was marginally lower than in controls. NaCN at 100 and 300 ppm caused a significant increase, in female rats, of time spent in proestrus and diestrus relative to estrus and metestrus.

The effect of subchronic (30 days) low-dose exposure to KCN in drinking water was studied in Wistar rats (Pritsos, 1996), with particular reference to growth, mitochondrial damage, and ATP levels. The rats received 0, 50, 100, or 500 ppm KCN in drinking water. Body weight and food and water consumption were monitored over the study. At the end of the dosing period hepatic and cardiac mitochondrial functions were tested as mitochondrial respiratory control ratios, which are a measure of mitochondrial integrity and the ability of mitochondria to synthesize ATP (Rickwood et al., 1987). The measurement was made by monitoring mitochondrial O_2 consumption in the presence of substrate, state 4, and comparing the rate with respiratory rate in the presence of substrate and ADP, state 3. The ratio is obtained by dividing state 3 rate by state 4 rate. Hepatic and cardiac mitochondrial respiratory control ratios were decreased in a dose-related manner, being statistically significant at 100 and 500 ppm. There were no statistically significant differences in food consumption between the various groups. A dose-related decrease in body weight gain was measured, which was statistically significant for the 100- and 500-ppm groups. Cardiac, hepatic, and cerebral ATP levels were decreased in a dose-related manner, but only with statistical significance in the liver at 100 and 500 ppm and in the heart at 50 ppm. These findings indicate that subchronic dosing with CN causes mitochondrial dysfunction.

Studies on long-term repeated intake of cyanide through exposure to more complex cyanogenic materials (notably cassava) have indicated that chronic exposure effects of cyanide include goiter (Cliff et al., 1986), tropical ataxic neuropathy (Onsuntokun, 1981; Wilson, 1987), and epidemic spastic paresis (Howlett et al., 1990). Additionally, chronic occupational exposure to cyanide has also linked it to thyroid dysfunction (see section 30.14).

The toxicokinetics of CN by repeated dosing has been investigated by several workers. Okoh (1983) investigated the excretion of an acute dose of ^{14}C-CN in urine, feces, and expired air in rats

TABLE 30.6 Biochemical Results for Serum and Tissues from Control Rabbits and Rabbits Dosed with Cyanide in the Diet (702) ppm for 40 Weeks

Measurement[a]	Controls[b]	Cyanide[b]	p[c]
Serum urea (mmol l^{-1})	6.3 ± 0.5	9.3 ± 0.8	<0.05
Serum creatinine (mg $d1^{-1}$)	0.7 ± 0.1	1.2 ± 0.1	<0.05
LD	91 ± 3	143 ± 2	<0.01
Serum (U l^{-1})			
Liver (U g^{-1})	417 ± 7	785 ± 8	<0.01
Kidney (U g^{-1})	302 ± 9	405 ± 7	<0.05
Rhodanese (μg min^{-1} mg $protein^{-1}$)			
Liver	18.2 ± 1.6	38.4 ± 1.4	<0.01
Kidney	10.7 ± 1.3	28.6 ± 1.9	<0.01
SD	0.09 ± 0.02	3.02 ± 0.04	<0.01
Serum (U l^{-1})			
Liver (U g^{-1})	3.57 ± 0.15	1.83 ± 0.08	<0.01
GPT	10 ± 1	17 ± 2	<0.05
Serum (U l^{-1})			
Liver (U g−1)	3.1 ± 0.2	2.0 ± 0.3	<0.05
Alk Pase	10.0 ± 1.0	27.6 ± 2.3	<0.01
Serum (U l^{-1})			
Liver (U g^{-1})	98 ± 6	57 ± 5	<0.05
Kidney (U g^{-1})	143 ± 7	89 ± 6	<0.05
Heart (U g^{-1})	24.0 ± 3.1	19.0 ± 2.0	NS
Lung (U g^{-1})	68.0 ± 5.0	39.0 ± 3.5	<0.01
AT	14.0 ± 0.9	15.0 ± 1.2	NS
Serum (U l^{-1})			
Heart (U g^{-1})	4.3 ± 0.2	3.9 ± 0.3	NS

[a] LD, lactate dehydrogenase; SD, sorbitol dehydrogenase; GPT, glutamate pyruvate dehydrogenase; Alk Pase, alkaline phosphatase; AT, aspartate transaminase.

[b] Results as mean ± SE.

[c] Significance of difference between control and cyanide groups.

Source: After Okalie and Osagie (1999, 2000).

dosed daily with unlabeled KCN for 6 weeks (77 μmol rat^{-1} day^{-1}) and in control rats not receiving KCN in the diet. In rats receiving KCN the main route of elimination of ^{14}C was in urine, which counted for 83% of the total excreted radioactivity in 12 h and 89% of total radioactivity in 24 h. The major excretion metabolite was SCN and accounted for 71 and 79% of the total urinary activity in 12 and 24 h, respectively. The mean total activity excreted in expired air after 24 h was only 4%, and this did not change after 24 h. Of the total ^{14}C in expired air in 24 h, 90% was present as CO_2 and 10% as HCN. When these results were compared with those for ^{14}C given to control rats not fed KCN, it was demonstrated that the pattern of elimination of ^{14}C in both urine and expired air was not altered by subchronic dosing with unlabeled KCN. The toxicokinetics of subchronic p.o.

exposure to CN has also been studied in the rat by incorporating KCN into drinking water to give daily (24-h) doses of 40, 80, and 160 mg kg^{-1} for 13 weeks (Leuschner et al., 1991). Blood and plasma CN and SCN were measured at weeks 1, 3, 5, 7, 9, 11, and 13, and urine CN and SCN were measured at weeks 6 and 13. At 160 mg kg^{-1} 24 h^{-1} body weight gain and drinking water consumption were significantly reduced over most of the 13-week dosing period, some mortalities occurred; with 80 mg kg^{-1} 24^{-1} body weight and water consumption were decreased but food consumption was unaffected; 40 mg kg^{-1} 24 h^{-1} did not influence body weight gain or food consumption but drinking water consumption was reduced. The calculated daily-ingested doses of CN (with range) were 38.4 (31.8–46.6), 78.3 (72.9–1.3), and 143.9 (121.4–169.8) mg kg^{-1} 24 h^{-1}. A clear dose–response relationship was obtained for blood CN concentrations, and for each exposure dose CN concentrations remained relatively constant during the test weeks. Thus, for the high-dose KCN diets blood CN concentrations ranged from 16.0 to 25.5 nmol ml^{-1}, which are below lethal concentrations. Although CN could not be detected in the blood of control animals, low SCN concentrations were present in serum of controls in the range of 11.4–53.3 nmol ml^{-1}. In the KCN animals, the plasma SCN concentrations were more variable than the CN concentrations, but a dose–response relationship was still present. The plasma SCN range for the high dietary concentration dose was 341–877 nmol ml^{-1}. In the urine of controls CN could not be detected, but for the KCN-dosed animals there was a clear dose–response relationship for dietary SCN. The CN/SCN ratio in urine was 1/1000, and there was also a dose–response relationship for urinary SCN. At 6 and 13 weeks of dosing, approximately 11% of the dosed CN was excreted as urinary SCN, and only 0.003% as unchanged CN. These findings indicate that chronic cyanide dosing by the peroral route does not lead to substrate saturation of the CN detoxification pathway and does not alter the mode of CN excretion.

30.8 NEUROTOXICITY OF CYANIDE

30.8.1 Animal Studies

In experimental acute CN poisoning, CN concentrations in brain are consistently high and close to serum values, irrespective of the species (Figure 30.3) or route of exposure (Figure 30.4). The high CN concentration is in the brain parenchyma as well as the intracerebral vessels (Ballantyne et al., 1972). In general, there are no differences between the CN concentrations in white and in gray matter of the brain. Also, although still moderately high, the concentrations are lower than in cerebrospinal fluid (CSF) and blood plasma (Table 30.7; Ballantyne, 1975, 1983a, 1987a). CN appears to rapidly equilibrate across the neuronal plasma membrane and then slowly accumulates in mitochondria and membrane elements of the neuron (Borowitz et al., 1994).

Some *in vitro* studies have suggested that CN may produce certain effects by a direct effect on neurons rather than by an inhibition of metabolism. For example, Aitken and Braitman (1989) studied the effect of CN on synaptic and neural function in slices of guinea pig hippocampus. They found that CN (10–200 μM) rapidly depressed synaptic transmission between Schafer collateral-commissural fibers and pyramidal cells. Analysis of input/output curves revealed two components to the suppression: a decrease in excitatory postsynaptic potential generation and an increase in action potential threshold. One interpretation of these findings is that the rapidity of the effects is consistent with a direct, nonmetabolic effect on the CNS. However, most studies indicate that one or several biochemical effects are responsible for the neurotoxicity of CN. Major emphasis has been placed on the role of CN in producing an inhibition of cytochrome *c* oxidase activity. Thus, CN-killed animals have a highly significant decrease in cytochrome oxidase activity, shown both by biochemical measurements (Table 30.8) and histochemically by quantitative kinetic microdensitometry (Table 30.9). *In vivo* determinations of cytochrome *c* oxidase activity, undertaken by measurement of the levels of reduction of the terminal oxidase by reflectance spectrometry, showed in the cerebral cortex of rats receiving a sublethal dose of CN that a noncumulative, transient, and dose-dependent inhibition of the respiratory mitochondrial respiratory chain occurred (Piantadosi et al., 1983). The decreases

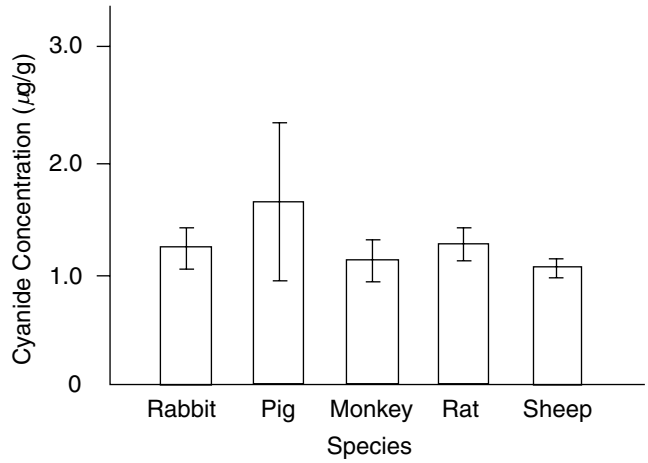


FIGURE 30.3 Concentrations of CN in brains from various species sacrificed by means of an intraperitoneal injection of KCN (8 mg kg^{-1}). Results presented as mean ± SD for $N = 6$ per species.

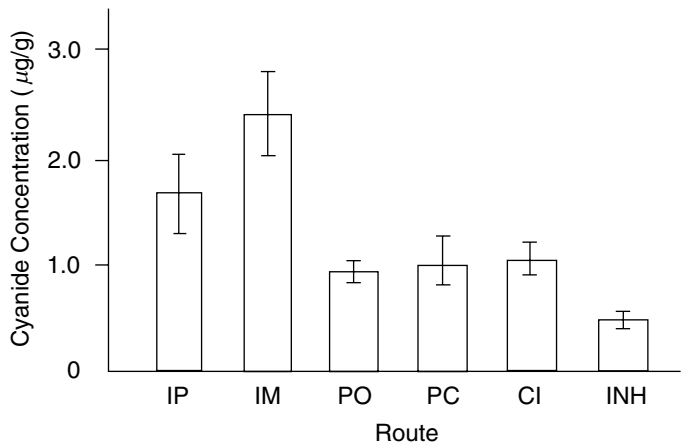


FIGURE 30.4 Concentration of CN in brains from rabbits sacrificed with HCN given by different routes of dosing. Inhalation exposure was 3 g HCN m^{-3} for 5 min; noninhalation doses were 5 × route LD_{50}. Results presented as mean ± SE for $N = 6$ animals per group. IP, intraperitoneal; IM, intramuscular; PO, peroral; PC, occluded cutaneous contact; CI, instillation into conjunctival sac; INH, inhalation.

in mitochondrial activity were accompanied by increases in regional cerebral HbO_2 saturation and blood volume. It was also demonstrated that CN inhibition of cytochrome *c* oxidase activity produced secondary Hb spectrum effects by preventing the unloading of O_2 in capillaries and that this interfered with the measurement of Hb absorbance changes. To overcome this, further studies were conducted using animals having an exchange transfusion with an oxygen-carrying isotonic, iso-oncotic perfluorochemical emulsion (Piantadosi and Silvia, 1986). It was found that the

TABLE 30.7 Concentrations of Cyanide in Blood Plasma, Cerebrospinal Fluid (CSF), and Brain White and Gray Matter of Sheep Killed with an Intramuscular Injection of KCN (10 mg kg^{-1})[a]

	Cyanide Concentration (μg g^{-1} or μg ml^{-1})			
	Plasma	CSF	Gray Matter[b]	White Matter[c]
Mean	1.46	1.57	0.89	0.99
SD[d]	0.38	0.43	0.29	0.22
Range	0.9–2.2	0.9–2.4	0.42–1.42	0.6–1.43

[a] After Ballantyne (1975). Statistical tests indicated the following significance of differences: Plasma versus CSF, $p < 0.1$; gray matter versus white matter, $p < 0.15$; gray matter versus CSF, $p < 0.0025$; white matter versus CSF, $p < 0.01$.

[b] Caudate nueleus.

[c] Subcortical white matter.

[d] Standard deviation.

TABLE 30.8 Result of Biochemical Measurements for Cytochrome *c* Oxidase (8 mg CN kg^{-1}; intramuscular) Rabbits from Two Separate Experiments

	Enzyme Activity (ΔE_{510} min^{-1} g^{-1}) as Mean ± SD[b]	
Experiment and Group[a]	Cerebral Cortex	Myocardium
Experiment 1		
Controls	1.55 ± 0.24	4.76 ± 0.28
Cyanide	0.80 ± 0.10	0.62 ± 0.13
p[c]-value	<0.01	<0.05
Mean decrease (%)	48	87
Experiment 2		
Controls	1.31 ± 0.27	4.95 ± 0.08
Cyanide	0.60 ± 0.24	1.19 ± 0.18
p-value[c]	<0.001	<0.001
Mean decrease (%)	54	76

[a] Data from: Experiment 1, Ballantyne (1977a); Experiment 2, Ballantyne and Bright (1979a). $N = 6$ animals per group.

[b] SD, standard deviation.

[c] Significance of difference between control group and cyanide group.

CN-induced transient increases in cytochrome *c* oxidase a_3 reduction level and subsequent redox recovery kinetics were similar in perfused and normal blood circulated rats. A dose-related suppression of electroencephalographic (EEG) activity was observed, with isoelectric conditions usually occurring after a 50% reduction in cytochrome *c* oxidase activity. Large doses of CN produced permanent EEG silence, but reversible effects could be achieved by careful titration with lower doses of CN. Also, pretreatment with sodium thiosulfate resulted in about a 4-fold protection of

TABLE 30.9 Cytochrome c Oxidase Activity Measured by Kinetic Microdensitometry in Cryostat Sections of Brain and Myocardium Taken from Control and Cyanide-Killed (8 mg CN kg^{-1}) Rabbits[ab]

	Enzyme Activity (ΔE_{550} min^{-1}) as Mean ± SE[c]	
Group[d]	Cerebral cortex	Myocardium
Control	0.016 ± 0.001	0.062 ± 0.001
Cyanide	0.008 ± 0.001	0.026 ± 0.001
p[e]-value	<0.001	<0.001
Mean decrease (%)	50	58

[a] Data from Ballantyne (1977a).
[b] Technique for kinetic microdensitometry by Ballantyne and Bright (1979a, 1979b).
[c] SE, standard error.
[d] $N = 6$ animals per group.
[e] Significance of difference between control and cyanide groups.

brain cytochrome *c* oxidase from CN-mediated redox changes. Thus, although the brain has a low thiosulfate-cyanide transulfurase activity (about 10% of liver [Borowitz et al., 1994]), sodium thiosulfate can prevent cerebral cytochrome *c* oxidase inhibition. This may be due to an extraneuronal action since Borowitz et al. (1994) noted that mice brain slices incubated with CN did not show an increase in SCN.

In addition to inhibition of cytochrome *c* oxidase, effects on other cerebral enzyme systems may contribute to the development of neurotoxic effects (Pettersen and Cohen, 1995). For example, inhibition of glutamate decarboxylase can result in a depletion of the inhibitory neurotransmitter γ-aminobutyric acid (GABA), possibly predisposing to the development of convulsions. Thus rats, guinea pigs, and mice given CN (40 mg KCN kg^{-1}, i.p.) had a 65–75% reduction in GABA concentration with a 106–110% increase in glutamic acid (Tursky and Sajter, 1962). Persson et al., (1985) also found that cyanide (5–20 mg NaCN kg^{-1}, i.p.) increased glutamic acid concentrations in cerebellum, striatum, and hippocampus (5–10 mg kg^{-1}), but higher doses decreased both glutamic acid and GABA. Cassel et al. (1991) demonstrated that decreased brain GABA levels were associated with increased susceptibility to convulsions in CN poisoning. Yamamoto (1990) found that GABA concentrations were decreased by 31% in KCN-treated mice exhibiting convulsions. The administration of α-ketoglutarate, with or without sodium thiosulfate, completely abolished the decrease in GABA and cyanide-induced convulsions. Although sodium thiosulfate alone also abolished the decrease in brain GABA, it did not protect against the CN-induced convulsions. These findings suggest that the depletion of GABA alone may not contribute to the development of convulsions induced by CN. Patel et al. (1992) demonstrated that in hippocampal cultures, NaCN-induced cytotoxicity is mediated primarily by activation of *N*-methyl-D-aspartate receptors. Also, Yamamoto and Tang (1998) found that when cerebrocortical neurons were exposed to KCN (0.01–1.0 m*M*) or *N*-methyl-D-aspartate (NMDA; 0.005–0.2 m*M*) for 24 h at 37ºC in 95% air/5% CO_2 environment, lactate dehydrogenase (LDH) efflux into the extracellular fluid was significantly increased in a dose-related manner and morphological changes were seen. The LDH efflux and morphological changes induced in cortical neurons by KCN were blocked by coexposure to 1.0 m*M* 2-amino-7-phosphonoheptanoic acid (a selective antagonist of NMDA), 1.0 m*M* melatonin (a potent hydroxyl and peroxyl radical scavenger), or 1.0 m*M* N^G-nitro-L-arginine (an inhibitor of nitric oxide [NO] synthase). These results suggest that activation of NMDA receptors and NO synthase and/or free radical formation may contribute to the neurotoxicity

induced by CN or NMDA. Yamamoto and Tang (1996a) found that the incidence of seizures and mortality produced by CN was reduced by intracerebroventricular preinjection of carbetapentane (a glutamate release inhibitor) or subcutaneous melatonin (a free radical scavenger). Cerebral lipid peroxidation produced by CN was also abolished by predosing with melatonin (Yamamoto and Tang, 1996b). In mice treated with KCN (7 mg kg^{-1}, s.c.) the brain showed elevated conjugated diene levels. Subcellular fraction studies showed that lipid peroxidation increased in the microsomal but not mitochondrial fraction (Ardelt et al., 1994). *In vitro* studies showed that the elevation of peroxidized lipids in rat brain cortical slices following incubation with 0.1 m*M* KCN was prevented by the omission of Ca^{2+} from the medium or pretreatment with diltiazem (a Ca^{2+} channel blocker). These findings suggest that both free radical formation and increased glutamate release may contribute to CN-induced neurotoxicity.

The functional interrelationship between CN and CNS dopamine may be complex. Cassel and Personn (1985) found that CN (5–20 mg NaCN kg^{-1}, i.p.) produced dose-related decreases in rat striatum dopamine (DA) concentrations, but noradrenaline concentrations were unaffected. In the olfactory tubercle the concentrations of both DA and noradrenaline increased postdosing. Thus, CN produced rapid regional changes in central catecholaminergic pathways. In mice given CN (6 mg KCN kg^{-1} twice daily for 7 days) there was central dopaminergic toxicity as evidenced by decreased numbers of dopaminergic neurons in the basal ganglia and decreased DA levels in the striatum (Kanthasamy et al., 1994a). Kiuchi et al. (1992) showed that perfusion of NaCN into the striatal region produced a transient and large increase in DA release associated with depletion of ATP. In severe acute CN intoxication there is decreased dopaminergic activity in the nigrostriatal area (Rosenberg et al., 1989) and lethal doses of CN decrease striatal DA and increase L-dihydroxyphenylalanine (DOPA) within 60 sec (Cassel and Persson, 1992). Subsequently, it was determined that whereas high doses of CN decrease striatal DA levels as noted above, low doses of CN increase rat striatum dopamine concentrations (Cassel, 1995). Low doses of CN probably affect the CNS without producing dramatic injury to neurons, while decreased levels of DA seen after high CN doses may reflect CN-induced effects in more seriously damaged neurons and of pathophysiological significance in intoxication (Cassel, 1995). Kanthasamay et al. (1994b) have shown that CN reacts nonenzymatically with the deaminated DA metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL) to form the cyanohydrin adduct 2-hydroxy-3-(3,4-dihydroxyphenyl) propionitrile (HPN). It was shown that incubation of mouse brain slices with CN (1 m*M*) generated 0.98 ng HPN 100 g^{-1} over 10 min, and also that HPN could be detected in brains of mice having injections of CN (15.6 μg) into the lateral cerebral ventricle. Additionally, after repeated doses of KCN [6 mg kg^{-1}, s.c., ×5], HPN was produced in the striatum (0.14 ng 100 mg^{-1}). Incubation of pheochromocytoma PC12 cells with HPN (500 μ*M* for 60 min) resulted in 23% of the cells being killed, and DA release was increased by 39.8%; uptake was partially blocked by the catecholamine uptake inhibitor imipramine. These findings suggest that CN reacts with the DA metabolite DOPAL to generate the biologically active cyanohydrin HPN that may be a factor in the neurotoxicity of CN, either by a direct neurocytotoxic effect or through the slow continual release of CN from the cyanogenic HPN. Also, CN produced increases acetylcholinesterase activity in cerebral cortex (57%), hippocampus (28%), and mesencephalon (18%) (Owasoya and Iramain, 1980).

The cytotoxic hypoxia of acute CN poisoning will affect the energy-dependent processes controlling cellular ionic homeostasis, and the ionic disequilibrium normally maintained between the intracellular and extracellular compartments may become disrupted (Maduh et al., 1993). In isolated cell preparations this cellular ionic disruption results in marked cellular acidosis and accumulation of cytosolic Ca^{2+} (Bondy and Komulainen, 1988; Li and White, 1977; Maduh et al., 1993; Nieminen et al., 1988). This may result in disturbances of Ca^{2+}-activated lipolytic enzyme activity, peroxidation of membrane phospholipids, changes in transmitter release and metabolism, and effects on other Ca^{2+}-modulating cell-signaling systems. Johnson et al. (1986) suggested that intraneuronal calcium may have an important role in mediating neurotoxicity They demonstrated that CN significantly increased whole-brain total calcium levels from 48.1 ± 1.8 (SE) to 66.5 ± 3.9 μg g^{-1} (dry weight)

within 15 min of dosing. CN-induced, centrally mediated tremors were correlated with the changes in measured whole-brain calcium. Pretreatment, 15 min before CN, with the calcium channel blocker diltiazem, prevented the rise in brain calcium and attenuated the cyanide-induced tremors. Yamamoto (1990) found that CN increased calcium concentrations by 32% in brain crude mitochondrial fractions from mice with convulsions. The increase in calcium was abolished by the combined administration of α-ketoglutarate and sodium thiosulfate, but it was not affected by sodium thiosulfate alone. These findings support the suggestion that calcium may play a significant role in mediating CN neurotoxicity. In other experiments by Johnson et al. (1986) CN caused a Ca^{2+}-dependent increase in conjugated diene production in mice brain lipids, indicating that peroxidation of the membranes had occurred. The effects noted depend on the dose of CN administered, because nontremorigenic doses did not affect total brain calcium or induce membrane lipid peroxidation. Pretreatment of KCN-dosed mice with either diltiazem (600 μg kg^{-1}, i.v.) or allopurinol (25 mg kg^{-1}, i.v.) blocked the generation of conjugated dienes. The results were interpreted as indicating that lipid peroxidation of lipid membranes plays a role in CN intoxication, and this action is related to altered regulation of neuronal Ca^{2+} homeostasis and activation of xanthine oxidase (Johnson et al., 1987). Procaine HCl provided significant protection against the lethal effects of KCN, which was further enhanced when given in combination with sodium thiosulfate and sodium nitrite. Procaine also antagonized the CN-induced whole-brain Ca^{2+} increase; KCN (7 mg kg^{-1}, i.p.) significantly increased whole-brain Ca^{2+} from 28.37 to 48.05 mg kg^{-1} dry weight within 30 min; this was prevented by pretreatment with procaine and maintenance of neuronal Ca^{2+} homeostasis (Jiang et al., 1998). The findings above suggest not only that calcium may be important in the pathogenesis of CN-induced neurotoxicity but also that Ca^{2+} antagonistic drugs may be useful adjunct drugs for the management of acute CN poisoning. Other comparative studies with calmodulin inhibitors (trifloperazine, chlorpromazine, and promethazine) given into the cerebral ventricles of mice before i.m. CN injections showed they were inhibitory to CN-induced convulsions and increased the CN LD_{50} value, but there was no positive evidence of a correlation between convulsions and mortality (Yamamoto, 1993a). Other different biochemical effects have been implicated as playing a role in the development of CN-induced encephalopathy. For example, Yamamoto (1989, 1993b) found that CN caused an increase in blood ammonia and brain neutral and aromatic amino acids, such as leucine, *iso*leucine, tyrosine, and phenylalanine. The loss of consciousness, hyperammonemia, and increased brain amino acid levels caused by CN were significantly inhibited by α-ketoglutarate. The findings suggest that the hyperammonemia and increase of brain neutral and aromatic amino acids may play a role in the loss of consciousness caused by CN. The possible involvement of caspase-3-like activity in CN-induced neuronal aptosis has recently been investigated (Gunaseker et al*.,* 1999). Exposure of cerebellar granule cells to KCN produced aptosis and an increase in caspase-3-like activity. The level of caspase-3-like activity with various concentrations of KCN reciprocally paralleled neuronal aptosis. Involvement of caspase-3-like activity was confirmed by the abolition of KCN-induced caspase-3 activity by the inhibitor z-VAD. Also, treatment of neurons with MK-801 (an NMDA antagonist) and an inhibitor of redox transcription factor NF-$_k$B (SN50 NF$_k$B) also blocked CN-induced caspase activity. To determine the link between caspase-3-activity and aptosis, cells were pretreated with agents that block the caspaselike proteases and MK-801 and SN50 NF$_k$B. Inhibition of caspase-3- like activity (z-VAD) and blockade of NMDA receptor activation and NF-$_k$B translocation all reduced aptosis, showing that caspase-3-like activity is increased in CN-induced aptosis. By using gel electrophoresis it was demonstrated that DNA fragmentation induced by KCN was blocked by the caspase-3-like protease inhibitor MK-801 and SN50 NF-$_k$B. These data suggest that CN induces aptosis by a NMDA receptor-mediated process leading to activation of caspase cascades. Gunesaker et al. (1996) concluded that CN initiates an excitotoxic-like reaction in cerebellar granule cells, which is mediated primarily by activation of the NMDA receptor; this led to Ca^{2+} influx, which stimulates concurrent generation of nitric oxide and reactive oxygen species resulting in lipid peroxidation and cell injury. In further studies cerebellar granule cells were used to investigate the early signaling events responsible for the induction of aptosis in CN-induced cell death (Gunaseker et al., 2000). Exposure to CN (100–500 μ*M*) for 24 h

induced maximum release of cytochrome *c* into the cytosolic fraction as assessed by Western blot. This was associated with enhanced caspase-3-like proteinase followed by aptosis. It was found that the induction of cytochrome *c* release into the cytosol occurred as early as 3 h of CN exposure, which preceded the initiation of caspase activity. Increase in cytosolic cytochrome *c* was blocked by pretreatment with uric acid, a scavenger of peroxinitrite, indicating that oxidative stress plays a role in the release of cytochrome *c* from the mitochondria. This process was initiated by NMDA receptor activation, since MK-801 blocked the elevation of cytosolic cytochrome *c*. However, the caspase-3 inhibitor Ac-DEVD did not prevent cytochrome *c* release, indicating that the induction of cytochrome *c* release is upstream to caspase activation in CN-induced cell death. Yamamoto (1995) proposed a hypothesis by which CN-induced convulsions depend on Ca^{2+}-calmodulin, nitric oxide, cyclic GMP, and protein kinase C. It was proposed that CN inhibition of mitochondrial cytochrome *c* oxidase leads to the depletion of ATP, which activates NMDA-sensitive glutamate receptors and causes increased synaptosomal Ca^{2+} levels. The ATP depletion also inhibits ATP-dependent Ca^{2+} efflux from presynaptosomes and leads to increased Ca^{2+} levels. The increase synaptosomal Ca^{2+} levels, working in conjunction with the regulatory protein calmodulin, turn on the synthesis of nitric oxide. The nitric oxide diffuses to adjacent cells where it activates guanylate cyclase, which synthesizes cyclic GMP. The latter activates protein kinase C. It was suggested that the activation of Ca^{2+}-dependent biological processes in the nerve endings may play an important role in the development of CN-induced convulsions, and that CN-induced neurotoxicity may be reduced by the inhibition of Ca^{2+}-dependent biological processes in the nerve terminal. Partly related to this suggestion Maduh et al. (1995) found that an inhibitor of protein kinase C [1-(5-*iso*quinoline-sulfonyl)-2-methylpiperazine (H-7)] partly protected against experimental NaCN lethal toxicity; they suggested that H-7 might be therapeutically effective in CN intoxication.

Physiological studies have indicated that marked cerebrovascular and blood perfusion changes may occur after cyanide dosing. Thus, slow i.v. infusion of KCN (0.05–0.1 mg kg^{-1} min^{-1}) increased cerebral blood flow in dogs by 130 and 200% with respective blood CN concentrations of 1.0 and 1.5 μg ml^{-1} (Pitt et al., 1979). Cerebral O_2 consumption was not affected at 1.0 μg ml^{-1}, but decreased to 75% of control values at 1.5 μg ml^{-1}. Increased cerebral blood flow caused by CN has also been demonstrated in various species by Russek et al. (1963). Funata et al. (1984), using a continuous infusion of NaCN, found an initial increase in local blood flow in white and gray matter after which acidosis developed and bradycardia occurred. This was followed by hypotension and decreased blood flow.

The studies above indicate that CN can access the CNS and produce biochemical and pathophysiological effects that may account for some of the toxic effects of CN. Various central neuropathological and neuropharmacological effects have indicated a central neurotoxic potential for CN. Several CNS degenerative changes have been seen histopathologically after acute and subchronic dosing with CN. Haymaker et al. (1952) showed almost exclusive gray matter necrosis in dogs if survival from acute CN intoxication were for >3 h; most susceptible regions were cerebral cortex, head of the caudate nucleus, putamen, globus pallidus, pulvinar of the thalamus, and cerebellar cortex. Lesions following repeated cyanide dosing have included degenerative changes in CNS ganglion cells and cerebellar Purkinje cells in dogs by 15 months peroral dosing (6 mg NaCN kg^{-1} day^{-1}; Hertting et al., 1960); degeneration of cerebrocortical neurons and cerebellar Purkinje cells, with myelin pallor in the corpus callosum of rats (22 weeks s.c. KCN, 1.43 mg kg^{-1} $week^{-1}$; Smith et al., 1963); necrotic lesions in the optic nerve and corpus callosum in rats (3 months s.c. NaCN, 1.16–3.24 mg kg^{-1}, 3 days $week^{-1}$; Lessell, 1971). Other studies have suggested a more selective involvement of white matter in CN encephalopathy (Brierley et al., 1976, 1977; Funata et al., 1984; Levine and Stypulkowski, 1959; Levine and Wenk, 1959). Hirano et al. (1967) ultrastructurally studied lesions of the corpus callosum produced by acute exposure to HCN vapor. They described fenestration of white matter as being caused by distension of cell processes, especially axons, with lytic changes in their cytoplasm. Earliest lesions were visible after 1 h and became established by 2 h. Lytic alterations were gradually succeeded by reactive changes at 24 h. Glial perikarya and myelin were well preserved in the early stages, but glial cells became more reactive and myelin was

destroyed later. Acute CN leukoencephalopathy has been histopathologically characterized as axonal swelling, separation, or destruction of myelin lamellae, intercellular edema, swelling of astrocytes, and glial necrosis (Funata et al., 1984). Microchemical analysis of myelin in rat experimental CN leukoencephalopathy supports a concept that myelin lesions are produced by a primary effect on glial cells followed by myelin degradation (Bass, 1968). Levine and Stypulkowski (1959) originally believed that leukoencephalopathy was the result of a direct effect of CN on oligodendroglial cells, but later Levine (1967) suggested that blood supply determined distribution of the lesions. In the cat, continuous infusion of NaCN produced severe damage in deep cerebral white matter, corpus callosum, pallidum, and substantia nigra (Funata et al., 1984). On the basis of morphological and physiological grounds they postulated that the circulatory depression that develops during extreme hypoxia decreases to a degree where metabolic demands cannot be met. The topographic selectivity of the leukoencephalopathy seems related to the characteristics of the cerebral vascular pattern, and the severity of the white mater lesions to the intensity of both the degree of hypoxia and hypotension during CN infusion, but not to the degree of acidosis, total dose of CN, or duration of infusion. The decreased venous pressure, combined with arterial hypotension, may be a factor in the pathogenesis of CN leukoencephalopathy because of the decreased perfusion pressure resulting in cerebral hypoperfusion (Ballantyne, 1987a). Neurobehavioral studies indicate that CN can alter behavior at concentrations that are not fatal. Mathangi and Namasivayam (2000) found that a one-month treatment of Wistar rats with KCN (2 mg kg^{-1}, i.p.) produced memory deficit in a T-maze test, with reduced concentrations of DA and 5-hydroxytryptamine in the hippocampus. Available data indicate that motor and cognitive functions may be affected, but the exact nature of such changes and the conditions under which they are manifest is currently uncertain (D'Mello, 1987).

30.8.2 Human Observations

In acute CN poisoning, the cerebral cytotoxic hypoxia, decreased brain ATP levels, increased brain ADP, increase in lactate and decrease in glycogen, are all factors resulting in some of the signs and symptoms characteristic of acute poisoning (MacMillan, 1989). These include disturbances of consciousness and perception and loss of central control functions, including those for the respiratory and cardiovascular systems.

Several cases, presented below, have been described with clinical neurological and neuropathological sequelae as a consequence of nonlethal acute CN poisoning. Lambert (1919) reported a patient who survived 16 days following accidental HCN vapor poisoning, and who had loss of cerebellar Purkinje cells and cerebrocortical gliosis. Finelli (1981) described a case of attempted suicide by a 30-year-old male who swallowed CN. Recovery was slow, and about 14 months after the poisoning episode he developed choreiform movements and dysdiadochokinesis of the left hand. However, one month later there was no objective evidence of brain damage or physical disability. Sixteen years after the original poisoning episode his mental status was normal, but he was mildly dysarthric, muscle tone was decreased in all limbs, mild athetoid movements were present in the upper extremities, and a left-hand dysdiadochokinesis was present. Computed tomography showed bilateral infarction in the globus pallidus and in the left cerebellar hemisphere. Utti et al. (1985) described an 18-year-old male who swallowed 975–1300 mg KCN in a suicidal attempt. He regained consciousness 7 h following O_2, sodium thiosulfate, and sodium nitrite, but recovered slowly with a personality change. Four months later, neurological examination revealed generalized rigidity and bradykinesia, with intermittent resting and postural tremor in the arms. There was unstable posture, with prominent antero- and retropulsion. A diagnosis of Parkinsonism was made, and anticholinergics were prescribed. Eighteen months after the original suicide attempt, he successfully took his own life with an overdose of imipramine and alcohol. At autopsy there were major destructive lesions in the globus pallidus and putmen, widespread lacunae were present in the striatum with a loose capillary network, glial fibers, and lipid-containing macrophages. The subthalamic nuclei were shrunken, showing some neurone loss with astrocyte proliferation. In the zona reticularis of the substantia nigra there was complete loss

of neurones with marked gliosis, but the area compacta was unaffected. Varnell et al. (1987) described two cases of death, confirmed by blood analyses, due to acute CN poisoning from the ingestion of cyanide-adulterated Excedrin capsules. Computerized axial tomography carried out within 3 h of collapse showed diffuse cerebral edema with diffuse loss of gray-white discrimination. Borgohain et al. (1955) described a 27-year-old female who developed persistent generalized dystonia following a suicidal attempt with KCN. Cranial computerized axial tomography showed bilateral putaminal lucencies. Magnetic resonance imaging (MRI) showed sharply delineated lesions corresponding to the two putamina. Visual evoked potentials, brain stem auditory evoked potentials, and evoked potentials on stimulation of the posterior tibial nerves were normal. Some improvement was noted on L-DOPA. Carella et al. (1988) described the case of a 46-year-old woman who ingested an unstated (unknown) amount of CN. She was hospitalized in light coma, reacted to nociceptive stimuli, and had a right Babinski reflex. At this time the CSF and a CT scan showed no abnormalities. She slowly partially recovered over the next two weeks, but still had some dysphonia, dysarthria, slight limb hypertonia, mild right hemiparesis, right Babinski, and diffuse hyperactivity of tendon reflexes. One year later she was hospitalized with speech and swallowing difficulties and unsteadiness of gait. CT scan at this time showed moderate cortical atrophy. Neuropsychological evaluation revealed impairment of spatial and visual memory, poor visuoperceptual performance and abstract reasoning. Five years after the poisoning incident she was readmitted with orolingual dystonia, having deviation to the right. There was marked anarthria and dysphonia. On voluntary eye closure she was frequently unable to open her eyes, and orbicularis oculi contraction was not evident. Spontaneous blinking was reduced. Moderate bradykinesia and slight spastic hypertonia were present, particularly on the right. A slight right hemiparesis and Babinski sign were present, and finger-to-nose testing was impaired on the left side. Standing and walking were wide based and unsteady. CSF examination was normal. An EEG showed diffuse irritative activity, most prominent in the left temporo-occipital leads. Visual evoked responses showed normal latency but decreased amplitude of P-100. Brain stem auditory evoked responses showed delay to the peak of VI wave. CT scan showed diffuse brain atrophy, most marked in the posterior cranial fossa, and all cerebral ventricles were dilated. Two hypodense areas in the basal ganglia were present. There were also low-density areas in the posterior portions of the cerebellar hemispheres and in the right parietal cortex. MRI confirmed atrophy of the cerebellum and cerebral hemispheres with marked ventricular enlargement. In another case, a man went into coma after ingesting NaCN (Grandas et al., 1989). He regained consciousness but was apathetic with reduced speech and loss of balance; dystonia and Parkinsonism developed during the following years. CAT revealed lucencies in the putamen and external globus pallidus. Feldman and Feldman (1990) described a case of a 28-year-old man who swallowed 800 mg of KCN in a suicide attempt and, after intensive antidotal and psychiatric treatment, developed severe Parkinsonian signs, including micrographia and hypersalivation. MRI revealed bilateral symmetrical basal ganglia abnormalities. Messing (1991) described the case of a 29-year-old who attempted suicide with 50 ml of 1% KCN (500 mg). When admitted to hospital he was apneic but recovered after 7 h. Parkinsonism developed in the following weeks, and regressed slowly in the second month after poisoning, with residual dysarthria, bradykinesia of the upper limbs, and brisk monosynaptic reflexes. Three weeks after intoxication a CAT scan was essentially normal, but there was hypodensity of the putamina after 5 months. Sharply delineated signal elevation in T2 corresponding to the two putamina was detected in MRI at 8 weeks and 5 months. Severe Parkinsonism developed in two men who ingested, respectively, 556 mg CN kg^{-1} (Utti et al., 1985) and 8.6 mg CN kg^{-1} (Rosenberg et al., 1989). Kales et al. (1997) reported a case of a 48-year-old male electroplater who developed neurobehavioral disturbances after a significant splash in the face with CN. When seen 6 months later, he had a paranoid psychosis with delusions of persecution and bilateral hand tremors. MRI demonstrated changes consistent with nigrostriatal degeneration.

Long-term and repeated exposures to CN have produced severe neurological effects including hemiparesis and hemianopia (ATSDR, 1997; Sandberg, 1967). During long-term occupational exposure to 15 ppm HCN there was fatigue, dizziness, headache, disturbed sleep patterns, parasthesiae, and

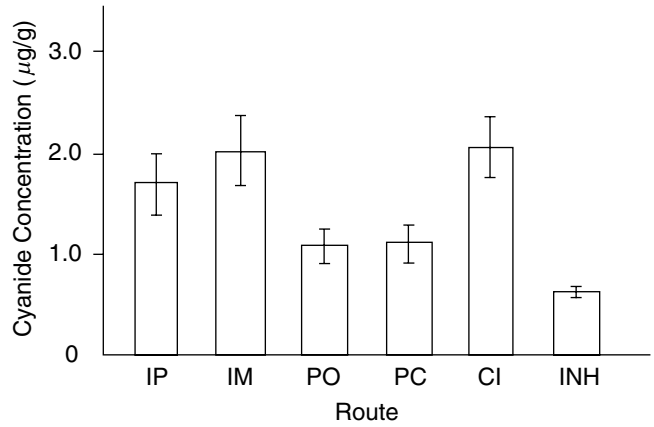


FIGURE 30.5 Concentrations of CN in myocardium of rabbits sacrificed with HCN dosed by different routes of exposure. Inhalation exposure was 3 g HCN m^{-3} for 5 min; noninhalation doses were given as 5 × route LD_{50}. Results presented as mean ± SE for $N = 6$ animals per group. IP, intraperitoneal; IM, intramuscular; PO, peroral; PC, occluded cutaneous contact; CI, instillation into conjunctival sac; INH, inhalation.

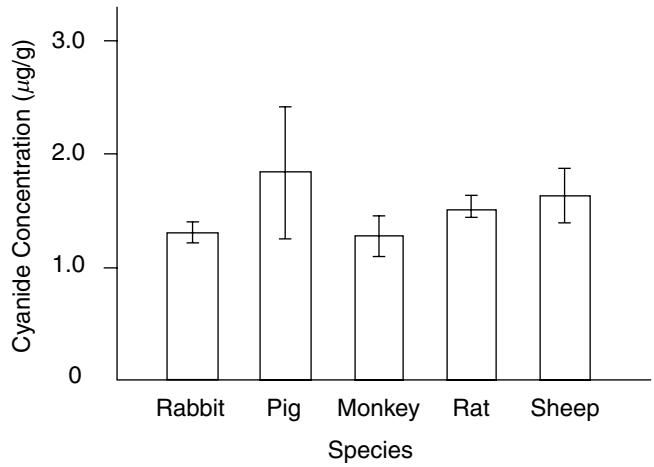


FIGURE 30.6 CN concentrations in myocardium from various species sacrificed by an intraperitoneal injection of KCN (8 mg kg^{-1}). Results presented as mean ± SE for $N = 6$ animals per group.

syncope (Blanc et al., 1985). Kumar et al. (1992) reported loss of immediate and delayed memory, decreased visual acuity, psychomotor ability, and visual learning in workers exposed to HCN.

Tobacco smoke contains significant amounts of CN and has been associated with tobacco amblyopia (Chisholm et al., 1967) in which there is increased plasma and urinary thiocyanate, and for which hydroxocobalamin and cyanocobalamin are therapeutic (Wilson, 1965, 1987). Smoking a pack of cigarettes may release 250–10,000 μg CN (Kulig and Ballantyne, 1993). Some investigators have also implicated CN as an etiological agent in other human neuropathies, including tropical ataxic neuropathy (Osuntokun et al., 1970) and Leber's optic atrophy (Towill et al., 1978). Tropical ataxic neuropathy is characterized by upper limb symmetrical hyporeflexia, lower limb symmetrical spastic paresis, spastic

dysarthria, diminished visual acuity, peripheral neuropathy, and cerebellar signs. Some epidemiological studies have associated ataxic polyneuropathy with higher intakes of cassava in endemic areas compared with nonendemic areas (Monekosso and Annan, 1964; Onsuntokun et al., 1979; Onobola et al., 1999, 2001). However, other studies have not associated ataxic polyneuropathy with high cassava intakes or exposure to CN (Oluwole et al., 2002; Onobola et al., 2001). Overall, the relationship between endemic ataxic polyneuropathy and exposure to CN from cassava foods remains unclear. The upper motoneuron disease Konzo, which occurs in rural African areas and is characterized by abrupt onset of variable degrees of symmetrical, isolated, nonprogressive, spastic paresis, has been associated with high dietary intake of CN from consumption of insufficiently processed cassava (Tylleskar et al., 1994).

30.9 CARDIOTOXICITY

That cardiac effects may be an important factor in the toxicity of cyanide is suggested by the high concentrations of cyanide to be found in the myocardium. Thus, consistently high concentrations are found by both different routes of dosing in the same species (Figure 30.5), and by the same route of dosing in different species (Figure 30.6). When cardiac mitochondria were exposed to ^{14}CN there was a time- and concentration-dependent uptake of CN (Wisler et al., 1991). Analysis of the findings indicated that there is an active or facilitated transport mechanism operating at lower CN concentrations, and passive diffusion of CN predominates at higher CN concentrations. Additionally, biochemical and enzyme cytochemical studies show a significant inhibition of myocardial cytochrome oxidase activity (Tables 30.8 and 30.9). In general, the degree of myocardial enzyme inhibition is greater than for cerebral cytochrome oxidase activity (Ballantyne, 1977a; Ballantyne and Bright, 1979a, 1979b). In keeping with this is the fact that, with rabbit tissue homogenates, the I_{50} for myocardial cytochrome oxidase is 2.74 μM and that for brain is 6.38 μM; the calculated pI_{50} values are 5.59 for myocardium and 5.20 for brain (Ballantyne, 1977a).

The direct evidence for cardiotoxicity and pathophysiological effect of CN on the myocardium comes from morphological, biochemical, and physiological studies in both experimental animal studies and human clinical observations. Electrocardiographic (ECG) changes have been noted by several observers. Leimdorfer (1950) carried out comparative ECG studies following i.v. NaCN in cats and monkeys and ethically questionable observations in humans. The latter were conducted on five schizophrenic patients (0.4–0.8 mg kg^{-1}), with the reasons for the studies not being stated. The ECG changes were similar in cat, monkey, and humans. After an initial brief (a few seconds) bradycardia there was tachycardia (a few minutes), followed by return of cardiac rate to preinjection values. At 20 sec postinjection there was increased T-wave amplitude and S-T segment elevation; the T-wave returned to normal within 15 min. Toxic doses in the cat (1–2 mg kg^{-1}) produced extreme and persistent bradycardia and arrhythmias; at 10 sec postinjection high biphasic T-waves appeared followed by negative T-waves, Wenckebach periods, incomplete heart block, and then complete heart block. Voltage became low, extreme bradycardia developed, and ventricular fibrillation occurred. Rats receiving i.p. KCN (10 mg kg^{-1}) demonstrated sinus arrhythmia, disappearance of T-waves, elevation of S-T segment, and terminal ventricular tachycardia and arrhythmia. With sublethal doses (2 mg kg^{-1}) there was slight bradycardia, T-wave suppression, and S-T segment elevation (Suzuki, 1968). In human cases of acute cyanide poisoning the following ECG changes have been noted: increased T-wave amplitude, progressive S-T segment shortening, and eventual origin of the T-wave high on the R-wave (DeBush and Seidel, 1969; Wexler et al., 1947).

Electrophysiological studies have shown that CN dosing causes a marked shortening of cardiac action potentials. Using isolated guinea pig ventricular myocytes and a microelectrode voltage clamp approach, it has been shown that shortening can be counteracted by glucose and is due to a marked increase in K^+ conductance (Van der Heyden et al., 1985). Given i.v. to dogs (2.5 mg kg^{-1}) CN produced an initial decrease in arterial blood pressure, hyperventilation, increased central venous

pressure, and bradycardia (Vich and Froehlich, 1985). This was followed by respiratory paralysis and increased blood pressure, and then by terminal apnea, progressive hypotension, profound bradycardia and hypoxic ECG changes.

For rats given acute i.p. KCN, ultrastructural changes were minimal in animals dying within 5 min, but with delayed (10–15 min) mortality there was sarcomere elongation, myofibril dissociation at the I-band, swelling and destruction of mitochondria, enlargement of the vesicles of the sarcoplasmic reticulum, and capillary endothelial cell swelling (Susuki, 1968). With sublethal injections (2 mg kg^{-1}), at 15 min there was slight swelling of the mitochondria and endoplasmic reticulum, and in those sacrificed at 1 h no mitochondrial ultrastructural pathology was seen.

O'Flaherty and Thomas (1982) found an increase in blood cardiospecific creatinine phosphokinase (CPK) activity 2 h after a 15-min exposure to 200 ppm HCN vapor. Ganote et al. (1976) found that CPK release from rat heart perfused with O_2-medium containing KCN occurred 30 min later than from heart perfused with N_2-medium with KCN. Baskin et al. (1987) studied the time course of events using a Landgendorff preparation with unpaced and paced (248–330 beats min^{-1}) guinea pig hearts. They were initially perfused with Krebs–Henseleit solution and subsequently with this solution containing NaCN (5×10^{-4} *M*). NaCN (5 min) reduced the contractile displacement 77.4 ± 5.7%, 87.4 ± 8.7%, and 56.3 ± 6.5% of the maximum postchallenge response at 0.5, 1.0, and 30 min, respectively, after CN dosing. Heart rate was 90.0 ± 3.4%, 89.5 ± 3.4%, and 78.7 ± 4.2% of the maximum response at 0.5, 1.0, and 30 min, respectively, after CN dosing. The onset of contractile displacement reduction by CN occurred more rapidly than electrical events (i.e., slowing of heart rates). In paced preparations a positive inotropic event that was independent of cardiac cycle length occurred within the first minutes. The CN-induced positive inotropy was inhibited by prior administration of DL-propanolol (1×10^{-5} *M*) a specific β-adrenergic antagonistic. The inhibitory effect of propranolol suggests that catecholamines may be responsible for CN-induced positive inotropy. Analysis revealed differential CN concentrations between five heart regions examined (right atrium, left atrium, left ventricle, right ventricle, and septum). The highest CN concentration was found in the left ventricle, which may be correlated with the regional cytochrome oxidase activity. Free CN accumulation in the heart appears to be frequency dependent, suggesting that at least a portion of the cardiac cycle uptake is mediated through an ion transport/permeability site. It was concluded that the time-dependent cardiac effects of CN appear to exhibit at least two components: (1) to exert an initial response on the β-adrenergic receptor, either directly or indirectly, and (2) to inhibit myocardial contractility through the inhibition of cytochrome oxidase. Kanthasamy et al. (1991) demonstrated that KCN (5 mg kg^{-1}, s.c.) produced a marked and sustained increase in catecholamines by sympathoadrenal stimulation. It was proposed that some of the cardiac and peripheral autonomic responses to CN are partially mediated by elevation of plasma catecholamines.

30.10 VASCULAR TOXICITY AND INFLUENCES ON VASCULAR REFLEXES

The ultimate effects of absorbed cyanide on the cardiovascular system is the result of complex interactions of various mechanisms which include, but not limited to, direct effects on the myocardium, direct effects on the vascular system, and adjustments in effector autonomic activity to the cardiovascular system. On the isolated aorta CN can cause either contraction or relaxation depending on the CN concentration and the species investigated (Robinson et al., 1984, 1985a, 1985b). With rabbit aorta, contractions start at 10^{-11} *M* and reach a maximum at 10^{-5} *M;* at concentrations between 10^{-5} and 10^{-3} *M* relaxation occurs (Robinson et al., 1985a). The mechanism of antagonism of noradrenaline-induced contractions of the rabbit aorta by CN was studied by Robinson et al. (1985b). Ouabain did not alter the relaxant action, suggesting that stimulation of the (Na^+-K^+) ATPase is not involved. Also, verapamil did not alter the CN antagonism of noradrenaline-induced aortic strip contraction and the CN antagonism of high K^+ concentrations and noradrenaline contractions to the same extent, suggesting

the effect was not related to involvement of either intracellular or extracellular Ca^{2+} stores. However, with lower concentrations of K^+ ($<4 \times 10^{-2}$ *M*) CN caused a concentration-dependent potentiation of K^+-induced contractions. They also studied the effects of various pharmacologically active agents on CN-induced contractions. Ouabain and verapamil enhanced contractions, but atropine, pyrilamine, 2-bromolysergic acid diethylamide, and phentolamine did not alter CN-induced contractions. In contrast, 4,4′-disothiocyano-2,2′-stilbene disulfonic acid (DIDS) or chlorpromazine partially reduced strip contraction. The findings may possibly have the following relevance.

1. CN-induced vascular contractions are probably not due to stimulation of muscarinic, serotoninergic, or α-adrenergic receptors.
2. If the effects on the coronary arteries are similar to those noted on the aorta, hypoxia-induced depolarization could enhance CN-induced coronary artery vasoconstriction and thus contribute to toxicity by facilitating myocardial ischemia.
3. Chlorpromazine or DIDS may be therapeutically effective in reducing lethality by inhibiting coronary vasoconstriction.

Paulet (1955) using normally innervated and bilaterally vagotomized animal preparations provided evidence for cardiac failure in acute cyanide poisoning and implicated involvement of the following major factors: direct myocardial toxicity, central vagal stimulation, and inhibition of central sympathetic activity. Studies on cats by Tanberger (1970) confirmed a dose-dependent stimulation of parasympathetic activity but also showed a stimulation of sympathetic activity. Since the changes in autonomic activity were observed after bilateral vagotomy and bilateral elimination of the carotid sinus, it was postulated that they represent effects primarily on the CNS. However, other workers described a reduction in vagal cardiovascular tone coupled with increased sympathoadrenal activity, resulting in increased cardiac output and arterial blood pressure (Krasney, 1970).

It is known that carotid and aortic chemoreceptors are important mechanisms for the reflex adjustment of the autonomic activity leading to regulation and homeostasis in the cardiovascular system (Korner, 1959; Kahler et al., 1962; Daly and Scott, 1964). Using a sublethal intra-aortic injection of NaCN in conscious dogs (0.3 mg kg^{-1}) Krasney (1971) found that CN caused abrupt increases in cardiac output, heart rate, and arterial blood pressure, but systemic vascular resistance was unchanged. A reflex hyperventilation accompanied these changes. After section of the carotid and aortic depressor nerves, there were also increases in cardiac output, heart rate, and arterial pressure; systemic vascular resistance initially decreased and then returned to control values. Ventilation was unchanged. These findings contrast with those in anesthetized, sinoaortic denervated dogs, where there is a decrease in blood pressure and vascular resistance. Thus, in the intact conscious dog, chemoreceptor reflexes are not essential for the increases in cardiac output and blood pressure that occur in response to the CN-induced cytotoxic hypoxia. Additionally, in the sinoaortic denervated animal the cardioaccelerator and vasoconstrictor responses to CN are abolished by surgical or pharmacological autonomic blockade, or by cervical cord transection (Krasney, 1967, 1970; Krasney et al., 1966). These results suggest that the major sites of the initiation of the circulatory responses to CN are outside the sinoaortic reflexogenic zone and probably within the CNS (Krasney, 1971). Studies on fetal lambs injected with NaCN *in utero* led to the conclusion that the aortic and carotid bodies are active and their stimulation leads to cardiorespiratory responses characterized by slowing of the fetal heart rate, respiratory effort, and no consistent change in arterial pressure (Itskovitz and Rudolph, 1987).

30.11 RESPIRATORY SYSTEM AND RESPIRATORY REFLEX RESPONSES TO CYANIDE

Bhattacharya et al. (1994) compared the effects of KCN dosed sc (0.5 and 1.0 LD_{50}) and inhalation of HCN vapor (55 ppm; 60.6 mg m^{-3}) for 30 min. Both routes of exposure resulted in increased airflow, transthoracic pressure and tidal volume accompanied by significant decrease in pulmonary

phospholipids. HCN inhalation also produced a direct effect on the pulmonary cells as evidenced by decreased compliance.

An early and characteristic feature of acute CN intoxication is the development of tachypnea and hyperpnea, resulting in an increased tidal volume. This may clearly enhance the inhalation dosage of HCN during the early stages of vapor exposure. The effect is generally believed to be due to the stimulation of aortic and carotid body chemoreceptors following accumulation of acid metabolites at these sites from inhibition of cytochrome *c* oxidase (Comroe, 1974). The mechanism by which hypoxia leads to increased afferent sinus nerve activity is not fully understood; however, the glomus cell, a secretory cell in apposition to the afferent nerve ending, appears to play an essential role in the transduction process. Thus, chemosensitivity is abolished following destruction of the cells (Verna et al., 1975), and chemosensitivity following sinus nerve section correlates with reinnervation of the glomus cells (Ponte and Sadler, 1989). Glomus cells of the rat carotid body are not homogeneous in their electrophysiological characteristics, and there are at least two populations (Donnelly, 1993). They differ in their voltage-dependent membrane currents as well as resistance and capacitance, but neither generates repetitive action potentials. The subtypes rapidly respond to CN-induced cytotoxic hypoxia and may mediate separate roles in the organ response to chemostimulation. Acker and Eyzaguirre (1987), by studying light absorbance changes in the mouse carotid body during CN intoxication, obtained evidence that cytochromes other than *c* and *aac* might have high sensitivity to P_{O_2} changes. Additionally, the general development of blood acid–base changes, particularly lactate acidosis, may also be a significant factor in the respiratory responses to CN intoxication. Levine (1975) infused NaCN solution distally into the upper abdominal aorta of anesthetized dogs and was thus able to induce cytotoxic hypoxia in distal tissues without stimulating the aortic or carotid chemoreceptors. Using a dose rate of 0.12 mg kg^{-1} min^{-1} given over 10 min resulted in decreases in O_2 consumption (46 $\pm$ 6 SE%), increased arterial blood lactate (5.25 $\pm$ 0.92 mmol l^{-1}), increased arterial lactate/pyruvate ratio (50.4 $\pm$ 14.3), increased minute volume (228 $\pm$ 36%), decreased arterial P_{CO_2} (21 $\pm$ 2 mmHg), and increased arterial blood pH (0.06 $\pm$ 0.01). In another series of experiments involving aorticocarotid denervation, metabolic and ventilatory changes also occurred after the intra-aortic infusion of NaCN solution, suggesting any recirculating CN capable of stimulating aorticocarotid chemoreceptors was limited. Also, in experiments involving perfusion of the heads of vagotomized dogs by other donor dogs, the intra-aortic infusion of NaCN still produced significant ventilatory changes. The studies above indicate that in addition to aorticocarotid chemoreceptor stimulation, intra-aortic CN may stimulate ventilation by other mechanisms.

It has also been suggested that the carotid body chemoreceptors may play a role in glucose homeostasis (Alvarez-Buylla and Alvarez-Buylla, 1988, 1994). Carotid chemoreceptor stimulation with cyanide results in a rapid hyperglycemic response. Measurement of rat hepatic venoarterial glucose difference indicated that carotid body chemoreceptor stimulation with a bolus of NaCN (5 μg 100 g^{-1}) produced an immediate increase in hepatic glucose output. The same dose was ineffective on glucose mobilization after bilateral adrenalectomy or after neurohypophysectomy. Reflex glucose mobilization was maintained after adenohypophysectomy or in adrenalectomized rats after adrenal autotransplantation (Alvarez-Buylla et al., 1997). These findings indicate that the neurohypophysis and adrenals are necessary for the hyperglycemic response to carotid body chemoreceptor stimulation by CN.

The depression of respiration caused by CN may be mediated through the brain stem, notably the ventral medulla. Mitra et al. (1993) found that microinjection of NaCN into the ventrolateral region of the cat medulla caused a depression of phrenic nerve amplitude and elevated cervical sympathetic tone and blood pressure. These findings indicate that the respiratory depression and vasomotor excitation produced by CN is limited to discrete regions in the intermediate area ventrolateral medulla, with dissociation of respiratory and vasomotor responses. Haxhiu et al. (1993) also found that topical application of NaCN to the intermediate area of the ventral surface of the medulla decreased activity in the phrenic nerve and respiratory muscles, and increased blood pressure.

In contrast, intrathecal (C_5-T_3) administration of NaCN increased electrical activity of the respiratory muscles but also caused increased arterial blood pressure. Thus, these data indicate that CN exerts site-specific qualitatively different responses along the neuraxis with respect to respiratory activity; at the ventral medullary surface it causes respiratory depression, but acting on spinal neurones it causes increased respiratory motor activity. However, at both levels (medulla oblongata and spinal cord) it causes increased sympathetic activity and increased blood pressure. The possible effect of peripheral chemoreceptor activity on the neural responses of the intermediate ventral medullary area was studied by Carroll et al. (1996). It was found that i.v. NaCN caused a dose-dependent rapid decline in intermediate ventral medullary surface neural activity, which was eliminated by bilateral carotid sinus denervation. This is consistent with the possibility that superficial neurone populations in the intermediate area receive inhibitory influences from carotid chemoreceptors.

30.12 DEVELOPMENTAL TOXICITY OF CYANIDE

There are relatively few studies on the developmental toxicity of simple cyanides in comparison with studies with more complex cyanogenic materials (Ballantyne, 1987a). In the female albino rat, i.p. KCN dosed at 3 mg kg^{-1} over gestation days (gd) 0–15 produced 5% lethality, growth retardation in 20%, and a 3% incidence of meningocoele (Singh, 1982). NaCN was given by slow infusion from subcutaneously implanted osmotic minipumps to pregnant in Golden Syrian hamsters (Doherty et al., 1982). Preliminary studies indicated that 0.0125 mmol kg^{-1} h^{-1} did not produce anomalies, but at a dose rate of 0.13 mmol kg^{-1} min^{-1} or greater 10% resorptions and some maternal mortality occurred. Based on these findings the doses employed in the definitive study were 0.126, 0.1275, and 0.1295 mmol kg^{-1} min^{-1} given over gd 6–9; controls received distilled water. The total CN doses given over the infusion period were 30–40 times the acute s.c. LD_{50} of NaCN to hamsters (7.4 mg kg^{-1}; 0.15 mmol kg^{-1}). Major findings were:

1. At 0.1295 mmol kg^{-1} min^{-1} maternal toxicity was apparent at 36–48 h and included excess salivation, breathing difficulty, incoordination, and hypothermia; most animals lost weight between gd 6 and 9. There was only mild maternal toxicity at the two lower dosages.
2. There were significant increases in resorptions. The incidence of total resorptions for total implantations was: controls, 10%; low dose, 62%; mid dose, 72%; high dose, 83%.
3. Crown-rump length was reduced in the NaCN groups.
4. The incidence of malformations was: controls, 4 of 75 (5%); low dose 16 of 26 (62%) $p < 0.05$; mid dose, 10 of 23 (43%) $p < 0.05$; high dose, 1 of 5 (7%). The most common anomalies were neural tube defects (nonclosure, encephalocele, and exencephaly).
5. Groups of antidotal controls were also used: the 0.126 and 0.127 mmol kg^{-1} min^{-1} groups were given, respectively, 0.03 and 0.04 mmol kg^{-1} h^{-1} sodium thiosulfate. This prevented the development of maternal toxicity. Resorption rates were significantly reduced compared with the NaCN-alone groups; 30% for the 0.126 mmol kg^{-1} min^{-1} + thiosulfate group and 8% for the 0.127 mmol kg^{-1} min^{-1} + thiosulfate group ($p < 0.05$ compared with the NaCN-alone groups). Crown-rump lengths were not different from the controls. Malformations were very significantly reduced at 0.126 mmol kg^{-1} min^{-1} + thiosulfate 1 of 49 (2%) and 0.127 mmol kg^{-1} min^{-1} + thiosulfate (1%). Both were neural tube defects. The decreased incidence of maternal toxicity and fetal malformations in the thiosulfate groups was associated with significantly lower whole blood CN concentrations, as shown for the 0.0126 mmol kg^{-1} min^{-1} groups; NaCN alone blood CN = 26.5 ± 18.5 SD μmol l^{-1}, and NaCN + thiosulfate blood CN = 1.92 ± 1.98 μmol l^{-1}. These findings indicate that cyanide given by slow s.c. infusion at the period of maximum organogenesis is embryofetotoxic and teratogenic.

30.13 REPRODUCTIVE TOXICITY OF CYANIDE

Reproductive performance was investigated in rats by Tewe and Maner (1981a) by incorporating KCN (0.5 or 1.25 mg CN g^{-1}) into a basal diet that contained low HCN cassava (0.021 mg HCN g^{-1}). Controls received basal diet (0.21 mg HCN g^{-1}). Diets were fed for 20 days before parturition, during pregnancy, through lactation, and in the postweaning period. No significant differences were noted between the control and treated groups with respect to gestational weight gain, litter size, pup birth weight, food consumption and body weight changes during lactation, maternal liver and kidney weight, weanling weights, or offspring mortality. In a study with pigs, involving feeding a diet containing 0.03, 0.28, or 0.52 mg CN g^{-1} throughout gestation and lactation, there were no differences with respect to litter size or birth weights. There were reduced organ/body weight ratios for the thyroid gland, spleen and heart (Tewe and Maner, 1981b). In a subchronic (13 weeks) drinking water study, male and female F344/N rats and $B6C3F_1$ mice were dosed with 0, 3, 10, 30, 100, and 300 ppm NaCN. Male rats and mice given 300 ppm NaCN had slight reduction in cauda epididymal weights. Male 300 ppm rats had lower numbers of spermatid heads per testis, and in all NaCN groups sperm motility was marginally reduced. NaCN did not produce effects estrus cyclicity in female mice, but 100 and 300 ppm female rats had a significantly longer time in proestrus and metestrus compared with estus and metestrus. It was considered that these effects were insufficient to decrease fertility (Hébert, 1993).

30.14 EFFECTS OF CYANIDE ON THYROID ACTIVITY

Some animal studies have suggested that CN may adversely affect thyroid gland function. For example, Sousa et al. (2002) male rats were dosed with KCN in the drinking water at 0, 0.3, 0.9, 3.0, and 9.0 mg kg^{-1} day^{-1} for 15 days. This resulted in an increase in the number of resorption vacuoles in the follicular colloid at all doses of KCN, but serum T_3 and T_4 concentrations were not significantly different from the controls. Philbrick et al. (1979) found decreases in plasma T_4 and T_4 secretion associated with increases in thyroid gland weights in weanling rats exposed to 1500 ppm CN or 2240 ppm SCN in the diet for 11 months. The possible effect on thyroid gland function of free CN and of the cyanogenic glycoside cassava added to the diet of dogs was investigated by Kamalu and Agharanya (1991). Three groups of dogs were fed nutritionally balanced diets of (a) rice, (b) cassava, and (c) rice plus HCN for 14 weeks during which time samples were taken for the sequential measurement of plasma SCN and serum T_3. At the end of the 14-week period plasma phenylalanine and tyrosine and thyroid gland weights were measured, and the thyroid glands were removed for histological examination. Plasma SCN was not detectable in control dogs, but those consuming cassava and CN generated significant amounts. A negative correlation was obtained between serum T_3 and plasma SCN, showing that T_3 decreases as SCN increases in the cassava and HCN groups. Mean thyroid gland weights (±SE) at 14 weeks for the rice controls, cassava, and rice + HCN groups were, respectively, 173 ± 13.3, 119 ± 13.0, and 212 ± 38.5 g kg^{-1}. Thus there were significant thyroid gland enlargements for the rice + CN group compared with the cassava group ($p < 0.005$) but neither was significantly different from the rice-alone group. Serum T_3 (± SE) levels at 0, 3, 5, and 14 weeks were as follows:

Rice controls	0.90 ± 0.09, 1.05 ± 0.09, 1.14 ± 0.08, 1.25± 0.05* nmol l^{-1}
Cassava	1.28 ± 0.16, 1.18 ± 0.02, 1.87 ± 0.06, 1.78 ± 0.04* nmol l^{-1}
Rice + HCN	0.87 ± 0.08, 0.90 ± 0.15, 0.90 ± 0.12, 0.56 ± 0.08 nmol[#] l^{-1}

*Significant difference from value at 0 week ($p < 0.02$)

[#] Significant difference from rice and cassava groups at 14 weeks ($p < 0.05$)

Rice controls thus showed a slowly progressive increase in serum T_3 and were 40% higher at 14 weeks than at 0 weeks. In the cassava group there was an initial decrease (at 3 weeks) in serum T_3,

and thereafter an increase to 38.8% at 14 weeks over the value at 0 weeks. In the rice + HCN group the serum T_3 level was unchanged for the first 5 weeks, but by 14 weeks the value had decreased 36% below the O-week value and was significantly lower than the values for control or cassava dogs at 14 weeks ($p < 0.05$). Histology of the thyroid glands showed them to be normal in the rice and cassava groups, but in the HCN group there was a large variation in the size of the lumina of follicles, and colloid was sparse and pale staining. The epithelium of the follicles was thickened, cuboidal, and tended to be multilayered. In the cassava group, the relatively low-mean thyroid gland weight, rise in serum T_3, and normal thyroid histology suggests the animals have essentially normal thyroid function despite high plasma SCN. This suggests that when cassava diet is taken with a nutritionally balanced diet, despite generating SCN, SCN has no deleterious effect on thyroid gland function. In contrast, dogs consuming free CN developed hypothyroidism and goiter.

Several occupational exposure studies have shown that repeated exposure to CN might have an adverse effect on thyroid gland function. Thus, mean TSH levels were significantly higher, than controls, in workers exposed to 15 ppm HCN in a silver-reclaiming facility. T_3 levels were also increased (Blanc et al., 1985). Banerjee et al. (1997) evaluated serum SCN concentrations of 35 nonsmoking workers in a cable industry who worked in an electroplating process. The mean serum SCN concentration (±SD) of these workers was 316 ± 15 μmol l^{-1} compared with 35 nonexposed controls of 90.8 ± 9.02 μmol l^{-1} ($p < 0.01$). Serum thyroid hormone levels were; T_4-controls 6.09 ± 0.601 μg dl^{-1}, exposed 3.81 ± 0.318 μg dl^{-1} ($p < 0.05$); T_3-controls 111.0 ± 9.3 ng dl^{-1}, exposed 57.2 ng dl^{-1} ($p < 0.05$); TSH-controls 1.20 ± 0.301 μU ml^{-1}, exposed 2.91 ± 0.29 μU ml^{-1} ($p < 0.05$). Serum T_4 was negatively correlated with serum SCN ($r = -0.363$, $p < 0.05$), and serum TSH positively correlated with serum SCN ($r = 0.354$, $p < 0.05$). The findings suggest that occupational CN exposure results in impaired thyroid function. Some authors believe that the thyroid effects are mediated by SCN, which inhibits both the uptake and utilization of iodine by the thyroid gland (Vander Laan and Bissell, 1946; Ermans et al., 1972; Solomonson, 1982; Fukayama et al., 1992).

30.15 GENOTOXIC AND ONCOGENIC POTENTIAL OF CYANIDE

KCN did not produce reverse mutations in *Salmonella typhimurim* strains TA98, TA100, TA1535, TA1537, or TA1538 up to 3 × 10 mmol $plate^{-1}$ (above which bacterial toxicity occurred) either in the presence or absence of metabolic activation (DeFlora, 1981). A *S. typhimurium* study (TA97, 98, 100, and 1535), with and without metabolic activation, did not increase the number of revertant colonies at five doses ranging from 1.0 to 333 μg $plate^{-1}$ (Hébert, 1993). Negative bacterial mutagenicity studies have also been reported for CN by Reitveld et al. (1983) and Owais et al. (1985). HCN was marginally mutagenic in *S. typhimurium* TA100, the effect being reduced 60% by S-9 liver homogenate metabolic activation (Kushi et al., 1983). CN did not induce DNA strand breaks in cultures of mouse lymphoma cells (Garberg et al., 1988).

In a chronic study groups of 15 male and 15 female rats were maintained for 104 weeks on diets containing 0.07 and 0.09 mg HCN kg^{-1}. These were equivalent respectively to 3.2 and 7.8 mg kg^{-1} day^{-1} (males) and 4.3 and 10.4 mg kg^{-1} day^{-1} (females) (Howard and Hanzal, 1955). There were no effects on growth rate, no signs of toxicity, and no histopathological findings. Philbrick et al. (1979) fed groups of 10 male weanling rats with diets containing 1.5 g KCN g^{-1} for 11.5 months; equivalent to 30 mg kg^{-1} day^{-1}. There were no mortalities of signs of toxicity. Positive findings were reduced body weight gain, decreased plasma T_4 levels, and decreased rate of T_4 secretion. There was no definitive peripheral or central neurohistopathology.

The currently available data suggest that cyanide does not have an oncogenic potential. Indeed, and conversely, Perry (1935) found that exposure of rats to HCN vapor at concentrations close to lethal retarded the growth of implanted Jensen rat sarcomas. Also, Brown et al. (1960) found that i.p. CN prolonged the lives of mice transplanted with Erlich ascites tumor and sarcoma 180. However, carefully controlled clinical trials with the cyanogenic amygdalin laetrile showed it to be ineffective as an anticancer treatment (Moertol et al., 1982).

30.16 ON THE ODOR OF HCN VAPOR

The odor of HCN vapor is usually likened to that of bitter almonds; the latter releases HCN from the cyanogenic glucoside amygdalin in the seeds (Poulton, 1988). On occasion it may be the initial clue as to the cause of poisoning in cases of intentional (homicidal or suicidal) poisoning (Chin and Calderon, 2000). Detection levels are often cited to be approximately 1 ppm (Guatelli, 1964), but in those who can detect the odor there is a wide distribution of detection levels by individual subjects (Brown and Robinette, 1967). A range of 0.5 to 5.0 ppm has been cited by Kulig and Ballantyne (1993). However, some individuals are not able to detect the odor of HCN. The ability to smell CN odor is a genetically determined trait that is absent in 2–45% of different ethnic populations (Ballantyne, 1987a). Kirk and Stenhouse (1953) proposed that CN anosmia is a sex-linked Mendelian recessive characteristic with males being more affected than females. A higher proportion of CN anosmic men was also found in the results of several studies (Kirk and Stenhouse, 1953; Fukumoto et al., 1957; Sayek, 1970). However, Huser et al. (1955) consider the trait to be neither recessive nor sex-linked, and Brown and Robinette (1967) do not consider CN anosmia to be a simple segregating genetically controlled trait.

The inability to smell the odor of CN may raise several practical problems, such as presenting obstacles in the early or confirmatory diagnosis of acute CN poisoning (section 30.18) and also leading to potentially hazardous working conditions where CN may be present. For example, Gonzalez (1982) described a case in which a chemist was poisoned with CN and in doing so spilled some material on the floor. The coroner's officer detected the odor at the scene, but by the time he arrived several police officers already present had become symptomatic. Curry (1963) described a case in which a felon entered a building and in doing so accidentally knocked the top from a container of CN fumigation powder. Pursuing police officers, who did not recognize the CN odor, became dizzy and required hospitalization. The potential biohazard to personnel involved in an autopsy of a case of poisoning by the suicidal ingestion of CN was shown by the demonstration that in samples of blood taken from staff within 10 min of the completion of the autopsy the blood CN concentrations were increased (Andrews et al., 1989). A recommendation that a respirator be worn during possible CN autopsies, or that the stomach should be opened in a fume cupboard, has been questioned on the basis that some cases of CN poisoning might be missed by those who cannot smell the CN odor (Fernando and Busuttil, 1991). As a deliberate precaution against absorbing CN from a victim, Nolte and Dasgupta (1996) reported the case of a 32-year-old male who committed suicide with KCN. The autopsy was conducted in a negatively pressurized isolation room, and the stomach was opened under a biosafety cabinet hood. CN measurements from the victim were: blood, 5.7 $\mu g\ ml^{-1}$; stomach contents, 655 $\mu g\ ml^{-1}$. No CN was detected in the blood of the three prosectors before or after the autopsy.

It is important that those who encounter, or may encounter, CN during their work should know if they can detect the odor of CN in the atmosphere. The inability to detect the CN odor may be a source of worry or concern to those who need this sense from an occupational perspective. Indeed Nicholson and Vincenti (1994) described a case of phobic anxiety in a 29-year-old industrial process worker who was unable to smell the odor of HCN.

30.17 CLINICAL TOXICOLOGY

30.17.1 Acute Exposure: Signs, Symptoms, Investigation, and Diagnosis

If large amounts of CN are absorbed quickly, and particularly by peroral and inhalation routes, collapse is rapid in onset or almost instantaneous, and often accompanied by severe convulsions with death following quickly. Less severe exposures produce the symptoms listed in Table 30.10; the number of these present, their order of appearance, and their intensity depend on various factors, including the route, exposure concentration (dosage), duration of exposure, rate of absorption of CN,

TABLE 30.10 Symptoms of Acute Cyanide Exposure (Poisoning) in Humans

Weakness
Fatigue
Headache
Anxiety
Restlessness
Palpitations
Confusion
Dizzyness
Vertigo
Dyspnea
Nausea
Nasal irritation (respiratory exposure)
Precordal pain

TABLE 30.11 Physical Signs of Acute Cyanide Poisoning in Human

Initial increase in breathing rate and depth, which later becomes slow and gasping
Vomiting
Diarrhea
Facial flushing
Transient increased blood pressure
Tachycardia followed by bradycardia
Cardiovascular collapse
Epistaxis
Convulsions
Loss of consciousness
Urinary and fecal incontinence
Cyanosis
Areflexia
Mydriasis and sluggish or unresponsive pupils
Decerebrate rigidity
Cardiac arrest

and physical mode of presentation. Physical signs of exposure are listed in Table 30.11. The presence cyanosis indicates that respiration has ceased or has been inadequate for several minutes. If there is exposure to low atmospheric concentrations of HCN all the signs and symptoms characteristic of CN intoxication may appear in progression. However, with massive doses absorbed over a short period, many of the typical signs and symptoms may not develop and there is a rapid onset of intoxication with convulsions, coma, collapse, and death. Thus, depending on the magnitude, degree,

duration, severity of exposure, and time elapsed, the presentation of cases of HCN vapor poisoning will be variable. Some patients, at one end of the clinical spectrum, may be conscious and complain of only a few symptoms with hyperpnea usually a presenting sign (Hall and Rumack, 1986), but at the severe end of the spectrum patients may present in coma with areflexia, mydriasis, and unresponsive pupils.

The early development of tachypnea usually accompanied by hyperpnea, which results in an increased tidal volume, is usually ascribed to the stimulation of carotid and aortic chemoreceptors resulting from the accumulation of acid metabolites following cytochrome *c* oxidase inhibition. However, the blood acid–base changes, particularly lactate acidosis, may also play an important role in breathing changes.

Tachycardia is often present initially followed by bradycardia. Third degree heart block may occur (Wexler et al., 1947; Lee-Jones et al., 1970). The ECG (EKG) often shows increased T-wave amplitude, progressive shortening of the S-T segment, and eventual origin of the T-wave high on the R-wave (Wexler et al., 1947; DeBush and Seidl, 1969). Deviation of the S-T segment has also been described following nonlethal injection of NaCN (Leimdorfer, 1950). The blood pressure may be raised initially, but hypotension develops with increasing intoxication.

An important clinical biochemical effect that needs therapy is the development of metabolic (lactate) acidosis, which when marked and sustained may account for several of the signs, symptoms, and complications of acute CN poisoning (Graham et al., 1977). It must be noted that the following also need to be considered in the differential diagnosis of anion gap metabolic acidosis: methanol, paraldehyde, phenformin, iron, isoniazid, lactate, ethylene glycol, salicyclate intoxications, uremia, and diabetic ketoacidosis (Chin and Calderon, 2000).

Venous blood removed from victims of acute cyanide poisoning may be bright red in appearance because of the cytotoxic hypoxia resulting in reduced O_2 extraction in tissues and a resultant decreased arteriovenous oxygen difference. For example, Johnson and Mellors (1988) recorded a case of a 30-year-old male who attempted suicide by swallowing about 3 g of NaCN. He was successfully treated with amyl nitrite, sodium nitrite, and sodium thiosulfate. Blood gas analysis values during the acute phase of poisoning were Pao_2 256 mmHg (99.7% saturation), Pvo_2 84 mmHg (95.4% saturation), with an A-V O_2 difference of 1.4 ml dl^{-1}; during recovery the values were Pao_2 81 mmHg (96.3% saturation), Pvo_2 30 mmHg (59.7% saturation) with an A-V O_2 difference of 8.1 ml dl^{-1}. Nakatani et al. (1993) also found a high Po_2 and Hb saturation in venous blood early in a case of cyanide poisoning (Pvo_2 = 98 torr, O_2 saturation 95.6%) and which progressively decreased with treatment. However, some regard venous O_2 saturation measurements as giving equivocal results or are not diagnostic of acute CN poisoning (Curry and Patrick, 1991; Yeh et al., 1992). Some authors consider this reduced A-V O_2 difference as an important clinical diagnostic feature, which may also be detected on ophthalmoscopy as a similar appearance to the coloration of blood in the retinal arteries and veins. Nakatani et al. (1992) found the arterial blood ketone body ratio (acetoacetate/β-hydroxybutyrate [AKBR]), which reflects the redox state of hepatic mitochondria, to be useful as a measure of the efficacy of treatment for CN poisoning.

Reported complications of acute CN poisoning have included pulmonary edema (Graham et al., 1977), acute real failure (Mégarbane and Baud, 2003), rhabdomyolysis (Brivet et al., 1983), central nervous system degenerative changes, and early diffuse cerebral edema (Fligner et al., 1987; Varnell et al., 1987). Possible mechanisms for the pathogenesis of pulmonary edema have included:

1. Local injury to pulmonary capillary endothelium and alveolar epithelium. However, the rate of resolution after diuresis suggests that this is not a significant factor.
2. Neurogenic effect following CNS injury.
3. Direct effect on the myocardium, leading to left ventricular failure and increased pulmonary capillary pressure. Graham et al. (1977) believe this to be the most likely mechanism.

30.17.2 Repeated Exposure Effects

Symptoms reported as a result of repeated exposure to CN are often similar to those noted with acute intoxication (Hathaway et al., 1996). These notably include weakness, fatigue, nausea, headache, confusion, dizziness, and vertigo. In a report of a study with 36 former workers in a silver-reclaiming facility who were chronically exposed to CN there were residual symptoms up to 7 months in some following cessation of exposure; frequent symptoms were headache, eye irritation, ready fatigue, loss of appetite, epistaxis (Blanc et al., 1985). Saia et al. (1970) compared 22 controls with 40 individuals who were employed near CN vats; the latter had twice the incidence of insomnia, tremors, dermatitis, epistaxis, and vertigo.

El Ghawabi et al. (1975) studied 36 male electroplaters, age range 30–50 years, from three factories in Europe. The respective mean (± SD) HCN concentrations in the workplace were 8.1 ± 8.3, 6.4 ± 6.9, and 10.4 ± 10.9 ppm. The following (with numbers of individuals affected) were recorded: headache (29), weakness (28), giddiness (28), throat irritation (16), vomiting (16), breathing difficulties (16), precordial pain (7), excess salivation (3), and visual problems (3). Of the 36 males, 20 had mild to moderate thyroid enlargement, and the exposed had a significantly higher thyroid uptake of ^{131}I at 4 and 24 h. Also, the exposed individuals had higher Hb concentration and lymphocyte counts. Urinary SCN increased about the middle of the working week and plateaued during the latter part of the week. Carmello (1955) studied 13 HCN fumigators who had episodes of acute CN poisoning with loss of consciousness. There was a high incidence of disturbances of equilibrium, vertigo, rapid eye movements, and precordial pain. Eleven had hypertrophic gastritis. Radojicic (1973) studied 43 CN-exposed electroplaters and annealers in Yugoslavia, most of who complained of fatigue, headache, hand and foot trauma, and nausea. Exposed workers had a higher urinary SCN excretion compared with a control group of 20 workers. Chandra et al. (1980) studied 23 male workers in an electroplating and casehardening factory in India. General workplace HCN concentrations ranged from 0.2 to 0.8 mg m^{-3} (average, 0.45 mg m^{-3}), with a breathing zone range of 0.1–0.2 mg m^{-3}. Blanc et al. (1985) studied 36 former workers in a silver-reclaiming factory, with a median time since last employment as a silver extractor of 10.5 months (range, 7–30 months). During employment they had a high incidence of headache, dizziness, nausea, eye irritation, anorexia, epistaxis, and ready fatigue (incidence, about 50% and up). There was a significant decrease in the incidence of these symptoms after the cessation of exposure. None had a palpable thyroid gland enlargement or focal neurological deficits. There was a tendency for slight decrease in serum vitamin B_{12} and folate concentrations and an increase in serum TSH. Neurological effects of repeated exposure to CN, including from cyanogens and from tobacco smoke, have been briefly discussed in section 30.8.2.

30.17.3 Management of Acute Cyanide Poisoning

Some authors have noted that patients can occasionally recover spontaneously and rapidly from acute cyanide poisoning, as judged by clinical findings and sometimes blood CN concentration (Graham et al., 1977; Edwards and Thomas, 1978). Based on such considerations, it has been suggested that the decision to give antidotes should be based on the changing clinical condition of the patient, particularly the level of consciousness (Peden et al., 1986; Bryson, 1987). However, this type of advice generally comes from experienced clinical or occupational health physicians. It is given because some antidotes may produce serious adverse health effects and in this respect is appropriate for those who have the necessary clinical experience. However, a decision not to give antidotes should only be taken by the general physician or receiving room (casualty department) officer after receiving verbal advice following immediate contact with a Poison Control Center. The decision to withhold antidotes should not influence the use of safer support measures. The management of acute cyanide poisoning may be considered in order as first-aid treatment, medical support management, and antidotal treatment.

30.17.3.1 First-Aid Treatment

An appropriately trained person who has prompt access to the CN workplace should carry out first-aid treatment. This person, who should be wearing the appropriate protective clothing and equipment, should ensure the following:

(a) The affected individual is promptly transferred to a clean environment, contaminated clothing removed, and any other aspects of decontamination carried out (e.g., flushing the skin). An emergency situation should be declared so that other emergency and support personnel can be called to the site.
(b) If breathing has ceased, or is labored, then artificial ventilation should be considered by the first-aider. This can be by the Holger–Nielsen method or, preferably, by use of a mask with a hand-squeezed ventilation bag (Ambu bag). The use of mouth-to-mouth ventilation is to be avoided because of the likelihood of secondary poisoning in the first-aider (Thompson and Bayer, 1983; Lafin et al., 1992; Sternbach, 1992).
(c) If ampoules of amyl nitrite are available, and the patient is breathing, one should be broken into a tissue and placed under the nose of the affected individual for about 15 sec. This can be repeated every 5 min if necessary. It should be ensured that these ampoules are in-date, or else they may explode on crushing with spreading of glass splinters due to internal pressure buildup, or there may have been loss of amyl nitrite due to decomposition. It has been recommended that ampoules are stored below 15ºC, and the shelf-life should be taken as 6 months from the day of receipt (Beasley et al., 1978). It should be remembered that if amyl nitrite is being used in the presence of 100% oxygen, this is an explosive mixture and appropriate must be care taken. Otherwise, antidotes should not be given until medically qualified physicians arrive (not paramedics) or the subject reaches a receiving room (casualty department).
(d) If breathing is difficult, 100% oxygen from a cylinder should be supplied by mask.
(e) If cardiac arrest occurs then external cardiac massage should be started, providing the first-aider is adequately qualified to do so.
(f) As early as is possible a sample of venous blood should be collected into an anticoagulated tube and tightly sealed for subsequent CN analysis.

30.17.3.2 Supportive Medical Management

A physician should supervise the following:

(a) Ensure that the airway is patent and aeration is adequate. This may require the use of endotracheal intubation. Also if ventilation is insufficient, or breathing has stopped, it may be necessary to use mechanically assisted ventilation.
(b) Although the basic mechanism of toxicity in acute CN poisoning is cytotoxic hypoxia, there is data to indicate that the use of oxygen is valuable as an adjunct to treatment. Thus, it is generally believed by many that although normobaric 100% O_2 has minimal effect on CN poisoning (Litovitz, 1987), it acts as an adjunct (synergistically) with other antidotes (Holland and Kozlowski, 1986; Beasley and Glass, 1998; Kulig and Ballantyne, 1993). It is said to be particularly effective as an adjunct to sodium nitrite-sodium thiosulfate and, to a lesser extent, with thiosulfate alone (Sheehy and Way, 1968; Burrows and Way, 1977; Litovitz, 1987). Although it might be anticipated that hyperbaric O_2 (HBO) would be more effective, it is uncertain if HBO offers any clinical advantage over normobaric O_2 either alone or in combination with other antidotes (Litovitz, 1987; Gorman, 1989; Kulig and Ballantyne, 1993; Salkowski and Penney, 1994; Tomaszewski and Thom, 1994). However, when antidotal treatment is refractory, and where available, HBO should

be considered as a treatment option (Goohhart, 1994). The arterial ketone body ratio (AKBR) has been shown to be useful as a measure of the efficacy of treatment for CN poisoning by Nakatani et al. (1992). The AKBR, which is the ratio of acetoacetate/β-hydroxybutyrate in arterial blood, reflects the redox state in hepatic mitochondria and is closely correlated with electron transport and O_2 utilization. In CN poisoning, and during recovery, as the Pvo_2 decreases, the AKBR increases.

(c) A plasma lactate concentration of 10 mmol l^{-1} or greater in fire victims without severe burns, and 8 mmol l^{-1} in pure CN poisoning, is a sensitive indicator of CN poisoning (Mégarbane and Baud, 2003). It is important to reverse the acid–base imbalance of lactate acidosis by the use of i.v. bicarbonate.

(d) With severe cases it may be necessary to use anticonvulsants.

(e) Cardiovascular complications may require the use of atropine, i.v. fluids, and vasopressors.

30.17.3.3 Antidotes

Acute cyanide poisoning is one of the relatively few toxic illnesses for which specific antidotes exist. Indeed a very large number of differing antidotes have been proposed, some of which are experimental only, but several others are used clinically and have appropriate governmental approval for use in the treatment of acute CN poisoning. Despite the fact that acute CN poisoning is relatively uncommon, and the majority of medical practitioners will not see a case during their professional life, the reason for the large number of antidotes available is partly because the mechanism of lethal toxicity of CN intoxication is well understood and this makes the approach to antidote development of considerable academic and applied interest, and partly because CN is often regarded as being a potentially rapidly acting chemical warfare agent for which stockpiles of antidote are required.

The major CN antidotes that have been investigated or developed experimentally, and also those that are currently accepted for use in the treatment of human acute CN poisoning, are listed in Table 30.12. The following account only briefly summarizes CN antidotes; more detailed reviews are to be found by Marrs, 1987, 1988; Meredith et al., 1993. The most convenient way in which to classify CN antidotes is into the following three main groupings, based mainly on mechanistic grounds:

(a) Those that enhance the biological biodetoxification mechanisms of converting CN to less toxic products; these are thus predominantly agents for facilitating SCN formation, either as sulfur donors or enzymes (mainly rhodanese).

(b) Materials that can either directly capture and complex CN or indirectly result in the formation of capture and complexing agents; both will result in the sequestration of CN and limit its availability to exert toxic effects. Principal among these are the direct agents cobalt salts, cyanohydrin formers and methemoglobin; and the indirect agents methemoglobin generators.

(c) Miscellaneous and adjunct antidotes that have been developed or suggested for counteracting certain specific toxic mechanisms or signs. These have included Ca^{2+}-antagonists and anticonvulsants, and thus some are more specifically to be regarded as supportive. The principal CN antidotes that have been clinically used and those developed experimentally are shown in Table 30.12, and considered briefly below.

The *sulfur donors,* of which sodium thiosulfate is the most frequently used, are believed to accelerate the endogenous sulfurtransferase mechanisms to form SCN from CN. Sodium thiosulfate is frequently used in combination with other antidotes having different modes of antidotal action; e.g., with sodium nitrite or 4-dimethylaminophenol (4-DMAP). In general, sodium thiosulfate is used as second-line treatment because it is believed by some that it is slow acting, due possibly to the slow penetration of mitochondria, although there are grounds to question this assumption (Marrs, 1987).

TABLE 30.12 Cyanide Antidotes Used Clinically and developed experimentally

Major Class	Subclass	Example
Biodetoxification enhancement	Sulfur donor	Sodium thiosulfate[a]
		Sodium ethanethiosulfonate
		Sodium propane thiosulfonate
		Sodium tetrathionate
		Cystine
		Thiocystine
		Mercaptopyruvates
	Enzymes	Rhodanese
Direct complex and capture agents	Cobalt compounds	Dicobalt edetate[a]
		Cobalt histidine
		Cobalt chloride
		Cobalt acetate
		Sodium cobaltinitrite
		Cobamide
		Hydroxocobalamin[a]
	Cyanohydrin formers	Pyruvates
		α-Ketoglutarates
		D,L-Glyceraldehyde
		Glucose
		Mercaptopyruvates
	Methemoglobin	Stroma-free methemoglobin
Indirect complex and capture agents	Methemoglobin formers	Sodium nitrite[a]
		Amyl nitrite[a]
		4-Dimethylaminophenol[a]
		4-Aminopropiophenone
Adjunts and miscellaneous	Ca^{2+} antagonists	Flunarizine
		Diltiazem
		Verapamil
	Others	Chlorpromazine
		Phenoxybenzamine
		Centrophenoxine
		Etomidate
		Naloxone

Alternative sulfur donors to thiosulfate have only marginal advantage, and less is known of their toxicity.

Rhodanese is unstable when derived from liver, but derived from cultures of *Thiobacillus denitrificans* it is more stable and has been investigated in experimental animals (Meredith et al., 1993). It has been successfully used antidotally in experimental CN poisoning (Pronczuk de Garbino and Bismuth, 1981) but not employed clinically. The possible use of rhodanese by encapsulation in carrier erythrocytes has been investigated (Leung et al., 1991).

On the basis of the evidence indicting that CN toxicity (notably neurotoxicity) may involve a loss of mitochondrial energy metabolism, and is associated with increase in cytosolic fee Ca^{2+}, the use of *Ca^{2+}-antagonists* in the management of CN poisoning has been proposed. Although there is experimental therapeutic and mechanistic evidence for this concept (Maduh et al., 1993), clinical studies have not been conducted.

Stroma-free methemoglobin is a direct-acting CN capture agent, binding it to form cyanmethemoglobin (CNmetHb) and thus sequestering the CN and reducing its availability for exerting toxicity, and avoids the problems with indirect metHb capture agents that reduce the blood O_2-carrying capacity. Although metHb is rapidly cleared from the vascular system, the overall experimental evidence is that it may be an effective and nontoxic antidote in acute CN poisoning (Boswell et al., 1988).

Cyanohydrin formers also represent direct-capture agents for CN. The CN ion reacts with carbonyl groups to form cyanohydrins, and results in a sequestering action. Among cyanohydrin formers, α-ketoglutararic acid has shown promise experimentally as a CN antidote (Moore et al., 1986; Hume et al., 1995), but there is a possibility for an additional antidotal activity by decrease in convulsions (Yamamoto, 1990).

Methemoglobin generators are indirectly acting CN antidotes that lead to the formation of metHb that binds with and sequesters CN as CNmetHb. MetHb does not have a higher affinity for CN than cytochrome oxidase, but there is a much larger potential source of metHb than there is of cytochrome oxidase, and the efficacy of metHb formation is thus primarily the result of mass action. A drawback of the metHb generation is that this results in impairment of O_2 transport to tissues. Amyl nitrite generates only small amounts of metHb by vapor inhalation (Bastian and Mercker, 1959), but is still recommended by many for use in the first-aid treatment of acute CN poisoning. Artificial respiration with amyl nitrite broken into an Ambu bag was reported as being lifesaving in dogs severely poisoned with CN and before any significant metHb formation occurred (Vick and Froelich, 1985). The vasogenic effects of amyl nitrite may play a role in its antidotal effect in CN poisoning. Sodium nitrite is given by i.v. injection to induce metHb, which competes with cytochrome oxidase for CN; CNmetHB is produced and cytochrome oxidase activity restored. Nitrites can cause adverse cardiovascular effects because of the vasodilation and hypotension they induce. It is generally recommended that the metHb levels be kept below 35–40% which is the range in which significant O_2-carrying can be reduced by methemoglobinemia alone (Hall and Rumack, 1986; Kirk et al., 1993; Kulig and Ballantyne, 1993). Excess metHb may be corrected with either methylene or toluidine blue, or exchange transfusion. Patients with glucose-6-phosphate dehydrogenase deficiency are at risk from nitrite therapy because of the likelihood of hemolysis. Another methemoglobin generator is 4-dimethylaminophenol (4-DMAP) which sets up a catalytic cycle in the erythrocyte in which oxygen oxidizes DMAP to *N,N*-dimethylquinoneimine which oxidizes Hb to metHb (Kiese, 1974). It is generally considered that 4-DMAP acts more rapidly in producing metHb than does sodium nitrite and generates metHb within a few minutes of injection (Weger, 1983). 4-DMAP treatment is also contraindicated in those with glucose-6-phosphate dehydrogenase deficiency. *p*-Aminopropiophenone is also a metHb generator but is likely to be inferior to that of 4-DMAP because (at least by gavage) the action of *p*-aminopropiophenone is slower in onset than that of the aminophenol. However, it has been shown experimentally to be a successful CN antidote (Bright and Marrs, 1987).

Cobalt compounds act as direct CN-binding agents and thus sequester CN and limit its access for exerting toxicity. The cobalt cyanide complexes are excreted in urine (Frankenberg and Sörbo,

1975), but inorganic cobalt compounds are somewhat toxic and possibly too much so for clinical use (Paulet, 1961); they cause circulatory disturbance and may produce myocardial injury (Marrs, 1987). Hydroxocobalamin binds strongly to CN to form cyanocobalamin and does not interfere with O_2 transportation. Several studies have confirmed the efficacy of hydroxocobalamin in experimental CN poisoning, and its use has been advocated on the basis of low toxicity (Pontal et al., 1982; Borron and Baud, 1996), although urticara and vascular collapse have been reported following its administration (Dally and Gaultier, 1976). Since 1 mole of hydroxocobalamin only binds 1 mole CN (Lovatt Evans, 1964) and its MW is high, large volumes of solution are needed to treat substantial poisoning. Detoxification of 1 mmol CN (equivalent to 65 mg KCN) requires 1406 mg hydroxocobalamin (Meredith et al., 1993). Therefore, standard ampoules of hydroxocobalamin (containing 1–2 mg) are ineffective. However, in some countries a formulation of 4 g hydroxocobalamin is available that can be reconstituted for i.v. use. It has found particular use in the treatment of cyanogen poisoning, notably sodium nitroprusside. Dicobalt edetate has found use in several countries, but there have been reports of adverse reactions to its use including vomiting, facial edema, urticaria, collapse, chest pains, anaphylactic shock, hypotension, ventricular arrythmias, and convulsions (Hilmann et al., 1974; Naughton, 1974; Tyrer, 1981). This has led to recommendations that it should be used only with clearly established cases of acute CN poisoning, and even then with caution (Meredith, 1993; Pontal et al., 1982).

Certain *vasogenic compounds,* by themselves not antidotal, may have a potentiating effect on mechanistically antidotal compounds. For example, chlorpromazine potentiated the antidotal effect of thiosulfate (Way and Burrows, 1976), and phenoxybenzamine, an α-adrenergic blocking agent, also potentiated thiosulfate.

Currently, of all the potentially available antidotes for acute CN poisoning discussed briefly above, the following are used clinically. It is particularly notable that there is a geographical variation in the specific antidotes used. This is partly explained by the country in which the antidote was developed, in part by the differing experience of clinicians in different countries, to some extent by the attitude and approach of the regional appropriate government department responsible for the control of medicines, and in some countries by a silly autocratic attitude by some clinical toxicologists. Dicobalt edetate (Kelocyanor) is used in the United Kingdom (Tyrer, 1981) and Australia (Worksafe Australia, 1989). In the USA, and partly because of the early development of the antidotal combination in that country, sodium nitrite and sodium thiosulfate are recommended. In Germany, DMAP was developed and is used there (Steffens, 2003). Hydroxocobalamin is used in France (because of low toxicity) for CN poisoning (Mégarbane and Baud, 2003), and in many countries for control of the slow cyanogenesis during sodium nitroprusside infusions and for treatment of smoke inhalation victims. The use patterns are generally as follows.

(a) Dicobalt edetate is used if the patent is unconscious or lapsing into unconsciousness, and the contents of one ampoule (300 mg in 20 ml glucose) are given by slow i.v. injection over about 3–4 min (Beasley et al., 1978; Tyrer, 1981). Given i.v. it may cause cardiac arrythmias.
(b) With sodium nitrite (often given with sodium thiosulfate) initially give 300 mg i.v., as 10 ml 3% solution at 2.5–5.0 ml min^{-1}. Pediatric dose is approximately 0.2 ml kg^{-1}.
(c) Sodium thiosulfate used as 12.5 g i.v., given as 50 ml of 25% solution. Pediatric dose is 1.65 ml kg^{-1}.
(d) 4-DMAP given as 5 ml of 5% solution (250 mg or 3–4 mg kg^{-1}).
(e) Hydroxocobalamin as 10 ml of 40% (4 g) given i.v.

30.18 OCCUPATIONAL TOXICOLOGY AND INDUSTRIAL HYGIENE

Exposure to HCN is principally by vapor inhalation, although some percutaneous absorption may occur from both liquid and vapor in contact with the skin. Such exposures may occur during the

use of HCN for industrial syntheses, in applications where HCN is used directly (e.g., fumigation), and in processes where HCN may be released in certain occupational settings (e.g., electroplating, mineral extraction). The possible exposure of firefighters to HCN from combustion processes is discussed below (section 30.19.4).

The current Threshold Limit Value (TLV) recommended by American Conference of Governmental Industrial Hygienists (ACGIH) (2003) for HCN is 4.7 ppm as a ceiling value with a skin absorption notation. The critical effects used to establish this value were noted to be CNS, anoxia, lung, and thyroid gland. A Biological Exposure Index (BEI), based on SCN formation, has not been recommended, in part because of the variability in serum and urine SCN concentrations according to personal and ethnic variations; e.g., the effect of CN in cigarette smoke and cyanogens in foods. The characteristic almond odor of HCN, which has a lower detection threshold around 0.5 ppm (Kulig and Ballantyne, 1993) is not a good warning of potential exposure below the TLV because of the wide distribution of the threshold values for individuals able to smell cyanide vapor (Brown and Robinette, 1968), and because up to 40% of individuals cannot detect this odor (see section 30.16). However, for detection of HCN in the atmosphere Draeger tubes are available. Also, and much more suitable, are intermittent of continuous air samplers for quantitative HCN detection that can be linked to alarm systems.

Although a BEI has not been recommended, methods are available to measure blood CN concentrations, and SCN in serum or urine, and can be used as biomarkers of exposure (ATSDR, 1997). Whatever methods are used they must be carefully conducted and the findings interpreted with caution. Thus, blood CN measurements should be conducted using whole blood and undertaken as soon as possible after collection of samples. Whole-blood thiocyanate measurements may not be a good measure of exposure to CN because of recovery problems (Ballantyne, 1977c). Plasma, serum, or timed urine SCN specimens give a more reliable index of CN exposure. However, as a biomarker of occupational exposure, the results of SCN measurements need to take into the possible contribution from other sources of CN exposure, including smoking and dietary cyanogens. There are no occupationally (industrial hygiene) useful biomarkers of effect of exposure to CN (ATSDR, 1997).

Due to the acute toxicity of HCN and its rapidity of action, in the workplace it is necessary to ensure that there very careful attention is paid to protective and precautionary measures, which should include the following:

(a) Those who work with CN, and their immediate management, should be thoroughly educated about the CN compounds used and handled; there should be familiarity with the chemistry (physicochemical properties), handling of materials, safety precautions, protective and precautionary measures (as outlined below), recognition of intoxication, emergency and first-aid procedures, escape routes from the CN work area, cleanup and disposal measures, and relevant literature (e.g., MSDSs). This familiarity should be maintained and kept current by periodic lectures and training sessions.

(b) In the laboratory, work with HCN should be carried out in a well-ventilated flow-through fume cupboard. In the workplace, the ventilation should be sufficient to maintain the atmospheric HCN concentration as far below the TLV as possible. The vapor concentration should be measured frequently or, preferably, continuously. Protective equipment and clothing for routine use should be readily available, including aprons and rubber or PVC gloves to protect skin, because of the known potential for percutaneously absorbed HCN to add to the body burden of CN from that due to inhalation exposure. Emergency protective equipment should be readily available and its integrity and function periodically confirmed; this includes full chemical suit, impermeable gloves, and respirator (which should be full-face and of the absorbent canister type or, preferably, air-supplied).

(c) All those working with, handling, or having managerial responsibility for cyanides, should be aware of whether they can detect the odor of HCN in the atmosphere.

(d) No person should work alone while handling CN compounds. There should always be an additional person nearby, designated a safety officer, who can view the operation at a distance

sufficient to raise an alarm, to allow protective clothing and equipment to be donned, and to undertake the initial rescue and possible resuscitation measures. Thus, ideally this person should be trained in the emergency measures for a CN-leak and, desirably, he should also be trained in first-aid measures to treat acute CN poisoning (Jenks, 1983a).

(e) One individual at or near the CN workplace area, ideally the safety officer, should be fully trained in general first-aid measures and specifically in the first-aid management of acute cyanide poising; this individual should undergo periodic retraining. The first-aid treatment of acute CN poisoning is discussed above (see section 30.17.3.1).

(f) First-aid equipment and necessary medicines for the local treatment of acute CN should be readily available close to the CN handling area. These should include mask with manual inflator for artificial ventilation, oropharyngeal airway, oxygen cylinder with mask, and in-date ampoules of amyl nitrite (kept at 15ºC or lower). A water spray should be nearby in case it is necessary to decontaminate the skin of the affected individual. Antidotes should be available in a sealed container that is marked "ANTIDOTES—FOR MEDICAL USE ONLY". This should be given to a suitably qualified individual only (not a paramedic) who may attend the site, or on arrival at the hospital.

There should be provision for random audits of the training sessions, work practices, and the supply and maintenance of protective and first-aid equipment,

30.19 HCN AS A PRODUCT OF COMBUSTION AND A SMOKE INHALATION HAZARD

30.19.1 General Considerations on Combustion

Mortality and morbidity as a result of being exposed to a fire may be due to one or more factors including direct physical trauma, averse physiological effects from heat stress, primary thermal burns and secondary complications, oxygen depletion, inhalation of toxic vapors, gases and particulates which may produce local respiratory tract injury or be absorbed and cause systemic toxicity (Silcock et al., 1978; Coleman, 1981; Peters, 1981; Norris and Ballantyne, 1999). A variety of factors may impede escape from a fire and thus enhance the likelihood of further exposure and thus increase the risk of death or injury. Major factors which hinder escape include physical barriers and physical injury, fear, panic, an obscuring smoke, prior use of alcohol or narcotic drugs, the presence of peripheral sensory irritants in the atmosphere, and the presence of airborne materials that become absorbed and result in CNS behavioral effects or disturbance of consciousness (Ifshin, 1977; Ballantyne, 1981, 1987b; Woolley, 1982; Kaplan et al., 1984; Purser and Grimshaw, 1984; Norris and Ballantyne, 1999;). Peripheral sensory irritant materials may be produced by combustion processes and cause ocular effects that can produce distracting and harassing effects (Ballantyne, 1999); typical examples of sensory irritants that may be in a fire atmosphere include acrolein, sulfur dioxide, hydrogen chloride, aliphatic aldehydes, and various inorganic and organic acids. Also present in the fire atmosphere may be substances that can be inhaled, absorbed, and exert adverse effects on the CNS, such as behavioral changes and disturbances of consciousness (Purser, 1966); these may include HCN and CO. Hypoxic hypoxia, due to depletion of atmospheric O_2 or secondary to pulmonary injury, may result in impaired judgment, impairment of coordination, and altered conscious level.

HCN is now well known to be, in certain situations, a causative and contributory material producing neurobehavioral and incapacitating effects, toxicity, and death.

Any material containing carbon and nitrogen will liberate HCN under appropriate combustion conditions. Also, various cyanogens may be thermally generated, such as acrylonitrile, acetonitrile, benzonitrile, and propionitrile (Woolley, 1982); and cyanogens have been detected in the blood of fire

victims (Anderson et al., 1979). Investigations into the generation of HCN by combustion, and its relative contribution to mortality and morbidity in fires has been undertaken using three major pathways:

(a) Chemical analysis of the effluent smoke and gases from materials subjected to heating or burning under various conditions.
(b) Exposure of laboratory animals to atmospheres produced from test materials under various generation conditions.
(c) Measurement of CN in body fluids and tissues removed from those who are occupationally exposed to fires and from casualties and fatalities from fires.

30.19.2 Sources of Hydrogen Cyanide

Any material containing carbon and nitrogen will generate HCN under appropriate combustion conditions, with polymeric materials being particularly notable sources of HCN. These include, for example, nylon, polyacrylonitrile (PAN), polyurethanes, urea-formaldehyde, melamine, silk, and wool (Ballantyne, 1987b). Although some investigations have shown that the evolution of HCN is proportional to the N content of the polymer (Morikawa, 1978) (Figure 30.7), this is not a universal finding. For example, Bertol et al. (1983) found that proportionately more HCN (1500 ppm) was evolved from PAN (19.0% elemental N) than from wool (200 ppm; 14.3% elemental N). Also, Urbas and Kullik (1977) using pyrolysis gas chromatography found that with pyrolysis temperatures in the range 625–925°C, the yield of HCN was inversely related to the N content of three fibers. The structure of a polymer within a given chemical class may significantly influence the evolution of HCN. Thus, Jellinek and Takada (1977) found that, under comparable conditions, the evolution of HCN from polyurethanes was segmented polymer > aliphatic polyurethanes > aromatic polyurethanes. In measuring the HCN content of fire gases it is important to determine the most appropriate method for particular circumstances. Thus, Tscuhiya and Sumi (1976) found that in HCN determinations, two common problems are concentration decrease by absorption and interfering reactions.

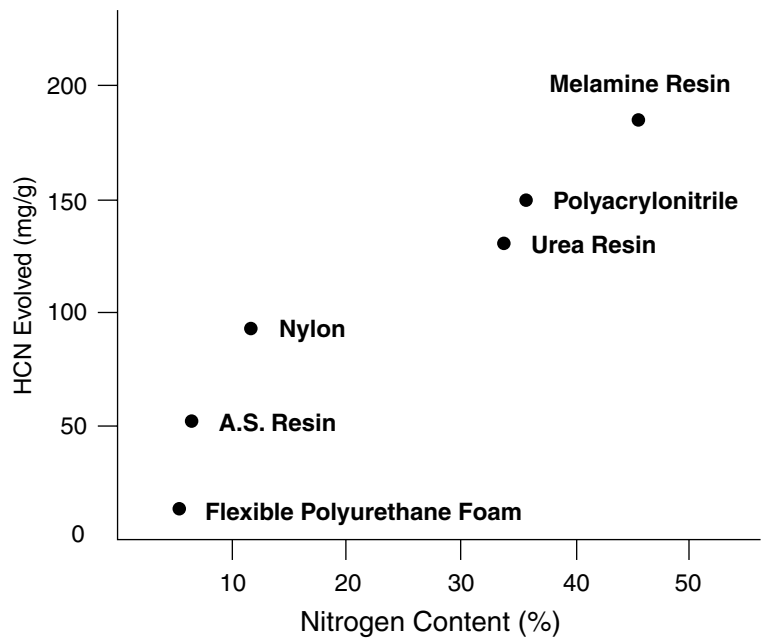


FIGURE 30.7 Relationship between the nitrogen content of polymers and the amount of HCN evolved under pyrolytic conditions at 900°C. A.S. Resin, acrylonitrile-styrene resin. (Data after Morikawa, 1978.)

Both temperature and O_2 availability in the heating zone are important factors that influence the yield of HCN from a N-containing material. The inter-related influence of O_2 and temperature is illustrated by studies with rigid polyurethane foam (PUF), which when heated to 600˚C under non-flaming oxidative conditions produced a significant decomposition of the methylene-di-*p*-phenylene *iso*cyanate component with relatively high HCN yield (Purser and Grimshaw, 1984), whereas under pyrolytic conditions, such fragmentation only occurred above 700˚C (Woolley et al., 1975). With flexible PUF it was determined that under oxidative conditions evolution began at 600˚C, whereas with pyrolysis HCN generation began at 770˚C (Woolley and Fardell, 1977). The influence of temperature on HCN yield from N-containing polymers is schematically represented in Figure 30.8. In general, under pyrolytic conditions high temperatures result in the generation of HCN, the yield of which increases with increasing temperature. With oxidizing atmospheres, HCN begins to be generated at lower temperatures, and as the temperatures increases so does HCN yield, up to a maximum, and then decreases with further increase in temperatures; a secondary rise in HCN yield may occur with even higher temperatures. The precise temperature at which evolution occurs varies with the chemical class of polymer with structural difference between polymers in a given class. Some examples of HCN generation from various polymers are as follows. Polyester and polyether flexible urethane foams decompose at low temperature (200–300ºC) in inert atmospheres to produce a yellow smoke which is stable up to 800ºC; however, over the range 800–1000ºC there is decomposition

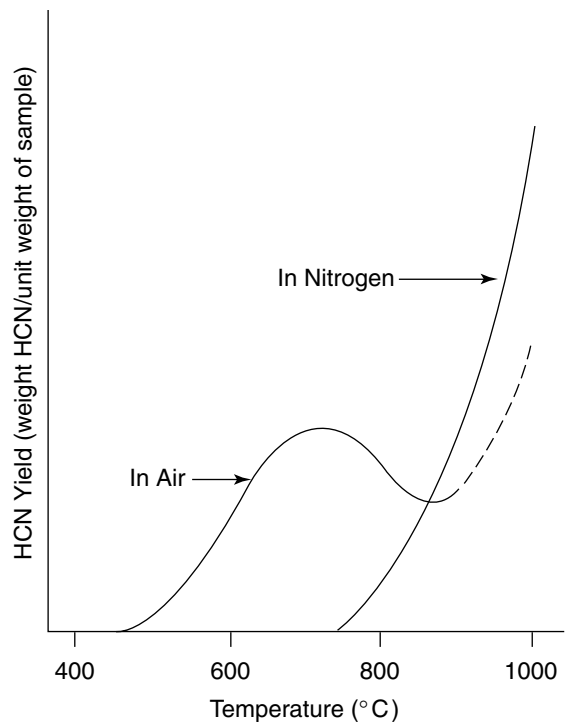


FIGURE 30.8 Graphical representation of typical yields of HCN from a N-containing polymer as a function of heating temperature in oxidative (air) and inert (N_2) atmospheres. In air there is an initial increase in HCN evolution, followed by a decrease; a secondary increase may then occur at higher temperatures. For pyrolysis conditions, the yield of HCN occurs at temperatures greater than those under oxidative conditions and, in general, increases with increasing temperature.

yielding HCN, acetonitrile, benzonitrile and pyridine as the major N-containing products (Wooley, 1972). Purser and Grimshaw (1984) found that when flexible PUF is pyrolyzed at 300–600º a dense yellow smoke is produced that contains little or no HCN. However, flexible PUF pyrolyzed at 900ºC, and rigid PUF subjected to non-flaming oxidative conditions at 600ºC, produce a clear atmosphere containing CO and HCN. The CO yields were 38.6% for flexible PUF and 75.7% for rigid PUF, and the corresponding values for HCN were 5.3 and 6.6%. Thermal degradation studies showed that there is cleavage of the urethane bond at temperatures in the region of 200–300ºC with the formation of *iso*cyanate and polyol (Chambers and Reese, 1976). With flexible foams, the *iso*cyanate ion reacts with itself to form carbodiimide structures, but also with amine (left over from the foaming reaction) to give volatile polymers. The subsequent decomposition of the yellow smoke (essentially a toluene di*iso*cyanate polymer) produces the low MW N-containing products. Thus higher temperatures are required to produce HCN, which may only be contributory to overall toxicity in domestic situations but can be more serious during fires that attain higher temperatures with industrial amounts of the foam (Woolley and Fardell, 1982). With rigid PUF, foaming is achieved using fluorocarbon-blowing agents, and the absence of amine promotes nonvolatile carbodiimide formation, such that the *iso*cyanate is retained with subsequent high temperature degradation. As with flexible foams, severe fire conditions are likely to be required to covert rigid PUF to HCN, with CO being the major species in overall toxicity (Woolley and Fardell, 1982). Morikawa (1978) found that with urea resin and melamine resin, and using a relative airflow rate of 2.5×10^{-2} l mg^{-1} min^{-1}, HCN evolution began round 400ºC reached a peak at 650ºC, and then decreased with further increase in temperature. However, when heating was conducted in an atmosphere of N_2, the rate of liberation from melamine resin increased progressively with rises in temperature. In this case the difference may be attributed to the fact that around 800ºC in air there was flaming, and the HCN was consumed through a flaming combustion by excess O_2. For wool, Urbas and Kullik (1977) showed that the evolution of HCN increased with pyrolysis temperature, but that the graph of HCN evolution versus temperature showed phasic variations in yield. In the presence of a flame retardant, the HCN yield varied with temperature. Thus in the presence of a chlorine-containing polymer the yield of HCN was reduced, but in the presence of a polymer containing both chlorine and antimony the evolution increased. With PAN, pyrolytic decomposition at 400ºC yielded a gas chromatographic pattern with 19 compounds, shown by mass spectrometry to include HCN, a variety of nitriles, and lower MW alkanes and alkenes. At 500–700ºC the complexity of the products increased, but at 700ºC and higher the chromatographic pattern simplified, with the emergence of a range of thermally stable products including low MW nitriles and aromatic species, rather than aliphatic hydrocarbons or complex organics (Woolley et al., 1979). HCN was the major toxic product at all temperatures. HCN yields, as weight % of polymer, were 1.03 at 600ºC, 6.28 at 700ºC, and 36.0 at 900ºC; these correspond to % conversion of available nitrogen of 2.0, 12.35, and 70.72%, respectively. The corresponding yields of nitriles were (for acetonitrile, acrylonitrile, and benzonitrile) 0.21, 0.1, and 0.0% at 400º; 4.58, 7.92, and 0.59% at 700ºC; and 3.90, 2.60, and 2.62% a 900ºC. In studies on the pathophysiological effects of pyrolysis products from PAN, Purser et al. (1984) found yields of HCN (as % of original polymer mass) of 1.7% at 300ºC, 4.5% at 600ºC, and 13.9% at 900ºC.

For any given situation, the rate of generation of CN may depend on the time from start of combustion. This will influence the rate of build-up of HCN concentrations, their duration, and therefore hazard. For example, Paabo et al. (1979) found that the rate of generation of HCN during non-flaming thermal degradation of urea-formaldehyde foam and wool was much more rapid than for polyurethane. In a large-scale test involving flexible polyether-polyurethane foam, it was demonstrated that both CO and HCN were detected in potentially dangerous concentrations during the early stages of the fire, but HCN concentrations declined rapidly thereafter (Woolley, 1973). In any experimental or real-life fire the evolution of HCN will vary with temperature, O_2 availability, the nature of the N-containing material, and the burning time. Thus, the atmospheric HCN concentration at any time will depend on the existing actual fire conditions and the phase of the combustion process. However, various estimates and measurements give some indications of the likely practical

situation. Morika (1978), for example, determined experimentally that in general with N-containing polymers the maximum yield of HCN is greatest with high temperatures and low air supply and noted that if nylon is burned at 950ºC under restricted air conditions then only 1.5 g is required to raise the HCN vapor concentration to around 135 ppm in a 1-m^3 space. Bertol et al. (1983) extrapolating data from combustion with PAN, calculated that a toxic concentration of HCN vapor (1500 ppm) could be developed in an average sized living room by burning 2 kg PAN.

Morikawa (1978) investigated the evolution of HCN under pyrolytic conditions from various low MW materials. It was found that all N-containing materials examined, except nitro-compounds, produced HCN when heated. Substantial amounts of HCN were produced from lactonitrile at 300ºC, and HCN began to be evolved from dimethylglyoxime at 400ºC. With other materials HCN evolution began in the temperature range 500–800ºC, and yields increased rapidly with increasing temperature. At 1000ºC maximum yields were $>$100 mg CN g^{-1} with dimethylurea, acrylonitrile, pyridine, phthalonitrile, ethyl thiocyanate, acetonitrile, aniline, hexylamine, and glycine; yields $<$100 mg g^{-1} were obtained with acetamide, urea azobenzene. When HCN evolved at 1000ºC was expressed as a function to the N content of the parent molecule, the yield was proportional for dimethylurea, acrylonitrile, lactonitrile, pyridine, ethyl thiocyanate, aniline, hexylamine and phthalonitrile, but lower for azobenzene, glycine, acetamide, dimethylglyoxime and acetonitrile. The possible role of ammonia in cyanogenesis was investigated by Morikawa (1978). When ammonia was heated with ethanol, HCN evolution began at 700ºC and increased to 50 mg g^{-1} at 1000ºC; a higher yield was obtained with n-hexane at nearly 300 mg g^{-1} at 1000ºC. These findings indicate that materials producing ammonia in thermal decompositions can produce HCN through secondary reactions. Both N-containing polymeric and low MW materials evolve ammonia. The evolution of ammonia was found to increase with increasing temperature for melamine, melamine resin, acetamide and aniline; reached maxima and then decreased for nylon, hexylamine and 1,3-dimethylurea; and decreased with increasing temperature for urea resin, wool, PAN and PUF. Ammonia generation was relatively high with urea resins, wool, melamine, acetamide, aniline, hexylamine and 1,3-dimethylurea. Ammonia yield from nitriles (lacto-, acyrlo- and aceto-) was low. The kinetics of the gas-phase decomposition of methyl *iso*cyanate has been investigated in the temperature range of 427–548ºC at pressures from 55–300 torr, and two decomposition routes were found (Blake and Ljadi-Maghsoodi, 1982). The predominant one was a radical chain process giving CO, hydrogen and HCN as major products, and the minor route was the bimolecular formation of *N,N′*-dimethylcarbodiimide and CO_2.

30.19.3 Assessment of the Contribution of HCN to Toxicity by Experimental Exposure of Animals to the Products of Combustion

Many studies have shown that when laboratory animals are exposed to combustion products, principally from N-containing polymers, then HCN may be generated in amounts sufficient to produce physical incapacitation or lethality. Some representative examples are given below. Yamamoto (1975) studied the acute toxicity of combustion products from various fibers and found that in rats exposed for up to 30 min signs of incapacitation developed most rapidly after exposure to PAN ($<$10 min). Blood removed after signs developed showed COHb concentrations were about 10% with PAN and wool, and in the range 20–40% for silk; blood CN concentrations ranged from 1.5 to 3.0μg ml^{-1} for silk, 1.5 to 2.0 μg ml^{-1} for PAN, and 0.5 μg ml^{-1} for wool. Thus, under the conditions of the study HCN from the combustion products of PAN and silk was the major cause of incapacitation, and blood concentrations of lethal significance developed. In contrast, with wool combustion products, neither CO nor HCN could be implicated as causes of incapacitation or death. Purser et al. (1984) exposed primates to the products of pyrolysis of PAN and compared the findings with the results from primates exposed to comparable concentrations of HCN vapor alone; range of HCN concentrations produced from PAN at various pyrolysis temperatures was 166–196 ppm (900˚C), 120–174 ppm (600˚C), and 87–170 ppm (300˚C). The pathophysiological effects produced by the

PAN-pyrolysis atmosphere and those by exposure to pure HCN vapor were identical and consisted of hyperventilation, increased respiratory minute volume, loss of consciousness, bradycardia, cardiac arrythmias, and T-wave abnormalities. After these effects, the breathing rate slowed and respiratory minute volume was markedly decreased. For both the PAN pyrolysis products and pure HCN vapor exposure groups there was a relationship between chamber HCN concentration and the time to occurrence of both hyperventilation and subsequent incapacitation that was approximately linear over the range studied. Regression analyses of the time to incapacitation versus HCN concentration were for PAN pyrolysis studies ($r = 0.89$, $p < 0.01$) and for HCN vapor alone ($r = 0.96$, $p < 0.02$). The difference between the two slopes was not significant, but the means times to incapacitation were shorter with the HCN-alone group at 13.2 min than with the PAN pyrolysis atmosphere at 16.2 min. The slope of the regression line of HCN concentration versus time to incapacitation was steep; thus, doubling the HCN concentration from 100 ppm to 200 ppm decreased the time to incapacitation from 25 min to 2 min. Thus, a short exposure time to HCN vapor can result in rapid incapacitation. Blood CN concentrations in the PAN pyrolysis groups were comparable to those of the HCN group. Purser and Grimshaw (1984) exposed primates for 30 min to the pyrolysis products of flexible PUF generated at 900˚C or oxidative thermal decomposition of rigid PUF at 600˚C. Signs included hyperventilation followed by loss of muscle tone and limb reflexes, then loss of consciousness. Cardiac rate decreased, cardiac arrythmias developed, the ECG showed T-wave abnormalities, and there was a massive increase in δ-wave activity in the EEG. Venous blood COHb concentrations at the end of the exposure period ranged from 17 to 28%, and whole-blood CN ranged from 1.9 to 2.3 μg ml^{-1}. They noted that the CNS-incapacitating effects could be extreme because of the cytotoxic hypoxia, circulatory failure, and hyperventilation that may produce cerebral arterioconstriction due to hypercapnia. There was a clear relationship between the HCN concentration in the exposure atmosphere and time to incapacitation ($r = 0.85$). Thomas and O'Flaherty (1979) pyrolyzed rigid PUF at 500˚C and exposed rats to the diluted smoke products. Three exposure groups were used: (1) 5-min exposure, followed by immediate sacrifice (i.p. sodium pentobarbital), measurement of blood COHb and CN, measurement of brain and heart cytochrome *c* oxidase; (2) a 5-min exposure and postexposure sacrifices at 0, 5, 10, 15, and 30 min for measurement of liver cytochrome oxidase activity; (3) exposure until death and then measurement of hepatic cytochrome oxidase activity. Brain and myocardial cytochrome oxidase activity was inhibited in proportion to the blood CN concentration. The blood CN concentration corresponding to a 50% inhibition of brain cytochrome oxidase activity was 0.26 μg ml^{-1}. This is close to the measured *in vitro* I_{50} of 6.38 μ*M* CN (0.16 μg ml^{-1}) for rabbit brain cytochrome oxidase (Ballantyne, 1977a). For the 5-min exposures, hepatic cytochrome oxidase activity was only slightly inhibited; however, rats receiving lethal exposures (8-min) had more marked inhibition of hepatic cytochrome oxidase activity. The significantly lesser inhibition of hepatic cytochrome oxidase activity accords with lower amounts of CN available to the liver following inhalation exposure (Ballantyne, 1984a). The studies above indicate that exposure of experimental animals to the products of combustion from various N-containing polymers results in toxicologically significant doses of CN being absorbed by the animals, as indicated by a variety of biological monitors.

30.19.4 Evidence for Exposure of Humans to CN from the Combustion Products in a Fire and the Contribution to Morbidity and Mortality

Exposure to and absorption of CN produced in a fire have been investigated mainly by analysis of blood samples taken from firefighters and nonlethal casualties and from analysis of tissues from lethal victims. The first detailed description of CN in the blood of fire victims was given by Wetherell (1966), who found CN in the blood of 39 of 53 individuals dying in fires; the average blood CN concentration was 0.65 μg ml^{-1} (range, 0.17–2.2 μg ml^{-1}). Other representative studies are as follows. Levine and Radford (1978) measured serum SCN concentrations in 479 firefighters

and found them to be increased above nonexposed controls, independent of smoking habits of the firefighters. Thus, mean ± SD SCN concentrations (as $\mu g\ ml^{-1}$) were the following: all controls, 4.36 ± 1.80; all firefighters, 5.69 ± 2.62 ($p < 0.001$); nonsmoking controls, 3.21 ± 0.71; nonsmoking firemen, 3.75 ± 1.50 ($p = 0.008$); light smoker controls, 4.60 ± 1.53; light smoking firefighters, 6.74 ± 2.39 ($p < 0.001$); heavy smoking controls, 6.53 ±1.45; heavy smoking firefighters, 7.78 ± 2.23 ($p = 0.02$). Clark et al. (1981) measured blood CN and COHb concentrations in 53 fire survivors. For 17 of 53 there was no evidence of smoke inhalation, and the blood CN and COHb concentrations were in the normal range; CN average (range) was 0.14 (0.02–0.35) $\mu g\ ml^{-1}$, COHb 3.8 (0.9–9.6)%. In the 36 survivors with evidence of smoke inhalation the average (and range) of blood CN was 0.70 (0.05–3.40) $\mu g\ ml^{-1}$, which was higher than for those without smoke inhalation. For a study in Maryland, blood CN was measured in fire deaths from January 1975 to December 1977 (Birky et al., 1979; Caplan, 1982; Copeland, 1985). Range of normal blood CN was taken as 0.0–0.25 $\mu g\ ml^{-1}$, based on random autopsy of impact airplane crash victims. In the fire fatalities, CN concentrations were normal in 31%, in the range of 0.26–1.00 $\mu g\ ml^{-1}$ for 35%, 1.01–2.00 $\mu g\ ml^{-1}$ for 24%, and >2.01 $\mu g\ ml^{-1}$ for 10%. Silcock et al. (1978) reported blood CN analyses on 15 victims of four fires. No CN could be detected in 2 victims, and in the other 13 the average blood CN was 0.94 $\mu g\ ml^{-1}$ (range, 0.14–1.80 $\mu g\ ml^{-1}$); in 7, the blood CN concentration was 1.0 $\mu g\ ml^{-1}$ or higher. All those with measurable CN had increased COHb. Hart et al. (1985) reported five patients with smoke inhalation from house fires who were comatose and had metabolic acidosis on hospitalization. Blood CN concentrations ranged from 0.35 to 3.90 $\mu g\ ml^{-1}$ (average, 1.62 $\mu g\ ml^{-1}$). Four of the patients survived with antidotal treatment for acute CN poisoning, but the subject with the highest blood CN concentration (3.9 $\mu g\ ml^{-1}$) died 4 days after hospitalization. In the context of postcrash airplane fires, Mohler (1975) reported blood CN concentrations in the range 0.01–3.9 $\mu g\ ml^{-1}$, and for one airplane fire in the United Kingdom (August 1985) involving 54 deaths CN was detected in blood of all victims and ranged from 5.3 to 8.4 $\mu g\ ml^{-1}$ (Mayes, 1991). Matsubara et al. (1990) noted that in fire victims CN concentrations were markedly higher in blood taken from the left heart compared with blood taken from the right heart.

The representative studies above indicate that survivors and victims of fires, and those who may be occupationally exposed, may absorb significant amounts of CN. The concentrations measured are variable; in some cases concentrations are high enough to be a factor in morbidity and in other cases mortality. In the series by Wetherell et al. (1966) there were 2 cases in 39 with blood CN concentrations compatible with death from acute CN poisoning, where COHb concentrations were <50%. In some situations blood CN concentrations are measured that are clearly compatible with HCN being the prime cause of death. For example, Tscuhiya (1977) cited a report of 2 victims found dead from a small fire involving a polyurethane mattress; respective blood CN concentrations were 7.2 and 23.0 $\mu g\ ml^{-1}$. Caplan et al. (1977) described an automobile fire casualty having a blood CN level of 3.13 $\mu g\ ml^{-1}$ and a COHb of 18%.

In assessing the contribution of CN to death in fire victims, a very careful analysis of all the information is required. Whole-blood CN concentrations in fire victims vary markedly, ranging from very low and unrelated to death to high and clearly compatible with death because of acute CN poisoning. When CN has been detected in a body discovered at the scene of a fire, and there is a suspicion that the fire had been started to distract from a homicide, then very detailed forensic considerations need to be taken into account. A consideration of the materials burned and the position of the body with respect to the area of combustion may assist in determining whether high blood CN concentrations would be possible from inhaling the products of combustion. Particular attention needs to be given to whether the products of combustion have been inhaled; this requires an examination for local respiratory tract pathology and a determination if inhaled airborne materials, other than HCN, have been absorbed into the systemic circulation. Local respiratory tract evidence that the victim was alive and breathing, at least during the early stages of the fire, will be provided by the presence of soot particulates in the respiratory tract. Also, if sufficient concentrations of chemically irritant materials or hot particles have been inhaled, acute inflammatory effects will be seen, the

extent of which depends on the nature of the inhaled materials, duration of exposure, and concentration (Crappo, 1981). When whole-blood CN concentrations are increased because of the inhalation of HCN as a combustion product, then the concentration of COHb will also be elevated. However, in the context of forensic investigations, the following need particular attention:

(a) COHb concentrations may vary markedly with duration of exposure, environmental CO concentration, and ventilation–perfusion relationships.
(b) COHb is present in normal subjects, being lowest in rural nonsmokers and highest with urban smokers (Stewart et al., 1973; Wallace et al., 1974; World Health Organization, 1979). High concentrations of COHb resulting from heavy smoking could give a false indication of the degree of exposure resulting from environmental CO in a fire. Clearly, in the context of forensic investigations, information on the smoking habits of the deceased should be obtained. Measurement of urine cotinine may help in this respect.
(c) In some cases where individuals have been in proximity to a fire, COHb may not be detected (Schwerd and Schulz, 1978). This could possibly be due to laryngospasm or to reflex arrest of breathing following the inhalation of hot gases. The presence of blood CN concentrations greater than normal in such cases may be grounds for suspicion.
(d) When high concentrations of CN are present in blood due to the inhalation of HCN, CN is not present in the stomach contents.

Exposure to HCN in a fire can lead to muscle weakness, difficulty in coordination, physical incapacitation, a confusional state, and partial or complete loss of consciousness. Low concentrations of HCN vapor in the range 100–200 ppm could be hazardous in a fire because of the incapacitation caused (Purser et al., 1984). With respect to the possibility of altered CNS function from exposure to a fire atmosphere containing both HCN and CO, the experimental findings of Pitt et al. (1979) are relevant. They found that although CO and HCN produced an additive effect on increasing cerebral blood flow, there was an associated decrease in cerebral O_2 consumption, and this was synergistic at lower concentrations of HCN and CO. In addition to the incapacitating and lethal effects of HCN in a fire, high concentrations could result in organ-specific effects. These could include cardiotoxicity and neurotoxicity. That HCN myocardial toxicity could be more severe in the hypoxia of a fire environment is suggested by the observation of Ganote et al. (1976) that hearts perfused with KCN in the presence of O_2 had release of creatine phosphokinase that was 30 min later than in the presence of N_2.

30.19.5 Practical Implications of HCN Generation in Fires

In view of the liberation of potentially toxicologically significant amounts of HCN from N-containing materials resulting from combustion processes in a fire, it is necessary that such materials which are used in construction, home and office furnishings, and car and airplane interiors, should be subjected to laboratory investigations to determine the extent to which such CN liberation can occur and under what conditions. When it found that a practically significant potential for HCN release exists for materials with in-use applications, then prohibitive or restrictive measures may need to be introduced in the use of those materials. As new information becomes available, remedial measures may be required for existing structures and fabrics. For example, Mohler (1975) recommended the retrofitting of in-service passenger aircraft with cabin interiors that have a low potential for liberating HCN in fires. Also, chemical modification and/or fire retardation of materials to inhibit HCN production may have to be considered. However, when flame resistance treatment is applied, there is a clear need to ensure that the potential for HCN toxicity has in fact been reduced and that no additional hazard is introduced from the retardant itself. For example, Urbas and Kullik (1978) investigated the influence of various flame retardants on HCN release from the pyrolysis of wools. Flame resistance treatment with a polymer containing chlorine decreased HCN production in the range 600–700°C, but treatment with a polymer containing chlorine and antimony increased HCN evolution over this temperature

range, and at 800°C more HCN was evolved in both cases. With a phosphorus-containing polymer, HCN evolution was higher up to 800°C, and then evolution was the same as for untreated wool.

Exposure to HCN released during a fire can produce muscle weakness, coordination difficulties, physical incapacitation, confusional state, and partial or complete loss of consciousness. The initial hyperventilation, due to chemoreceptor stimulation, may lead to an increase in the absorption of HCN and other airborne toxic materials. As a result of the CNS neurobehavioral effects, there will be hindrance to escape from the fire environment, increasing the potential for physical injury and continued exposure to toxic materials. There is thus a clear need to monitor firefighters and victims of fires for absorbed CN. Serum SCN measurements may be a guide for occupational or survival exposure (providing the smoking history is known), but whole-blood CN measurements are to be preferred for most situations. For on-site use at a fire it may be possible to use gas detectors of the Draeger-type to detect any HCN exhaled by the victim.

A plasma lactate concentration of 10 mmol l^{-1} or greater in fire victims without severe burns is a sensitive indicator of CN poisoning (Mégarbane and Baud, 2003). However, such measurements cannot be made at the scene of a fire. Because of the evidence for HCN being a factor in smoke inhalation systemic toxicity, it has been proposed that the use of CN antidotes should be considered in the treatment of smoke inhalation victims, especially if clinically or analytically confirmed (Daunderer, 1979; Hart et al., 1985). Clark et al. (1981) stated that since the majority of effective CN antidotes are not free from side effects, they should not be given empirically to all fire victims. Because of the relative ease to measure COHb concentrations, and because patients with high CN concentrations will also have elevated COHb, COHb may be used as a guide for therapy. In their series Clark et al. (1981) considered that had CN antidotes been administered to patients with COHb >15%, then only in one case would therapy not have been necessary, and no patient with possible CN toxicity would have been missed. Daunderer (1979) recommended that the breath of fire victims should be monitored for HCN, or if respiratory arrest has occurred then a blood sample should be obtained, acidified, and monitored for any released HCN with a Draeger detector. He described three fire victims who were successfully treated by CN antidotes; he recommended that patients should be given 50–100 ml 50% sodium thiosulfate i.v. immediately. It was suggested that unconscious victims be given 4-DMAP (3 mg kg^{-1}, i.v.); when cardiac arrest has occurred, 4-DMAP should be given by intracardiac injection. Hart *et al.* (1985) advised that CN antidote be administered when patients from closed-space fires present with findings of metabolic acidosis (base deficit >-10mmol l^{-1}, pH < 7.3), elevated COHb, and decreased Pao_2 saturation. Currently, and with respect to on-scene use, it is considered that suitable antidotes for CN poisoning resulting from smoke inhalation are hydroxocobalamin (Baud et al., 1991; Editorial, 1991; Houeto et al., 1996; Mégarbane and Baud, 2003) and sodium thiosulfate (Kulling, 1992), principally because their intrinsic low toxicity would permit use at the scene of a fire by medical personnel.

One important issue that needs to be considered in the forensic investigation of acute CN poisoning in fire situations is the possible effect of ethanol. Drinking alcohol has been a factor in the accidental onset of some fires, and the presence of ethanol in blood may cause drowsiness and difficulty in locomotion and impede escape from a fire. In one series of fire deaths in the United Kingdom, mainly in dwellings, ethanol was present in the blood of most victims, and 59% of adults had blood ethanol concentrations greater than 80 mg dl^{-1}, or urine ethanol concentrations in excess of 107 mg dl^{-1}; the average blood ethanol concentration was 276 mg dl^{-1} (Harland and Wooley, 1979). In a study of fire deaths in Maryland, blood ethanol concentrations in excess of 150 mg dl^{-1} were found in 35% of victims (Radford et al., 1976). The possible influence of ethanol on acute CN intoxication was studied by Yamamoto et al. (1983), who compared the effects of exposure of a group of rats to 500 ppm HCN alone with another group given an incapacitating dose of ethanol (3 g kg^{-1}, i.p.) before exposure to HCN. Ethanol-treated rats (blood ethanol, 2.83 ± 0.19 SD mg ml^{-1}) exposed to HCN vapor had a survival time of 10.6 ± 2.7 min, compared with 9.7 ± 3.2 min for rats exposed to HCN alone; the difference was not statistically significant. Blood CN concentrations were about 20% higher in the ethanol + HCN group (3.44 ± 0.44 μg ml^{-1}) than in the HCN-alone

group (2.80 ± 0.32 μg ml^{-1}), but the differences were not statistically significant. However, the blood lactate concentrations were significantly lower in the ethanol + HCN group (36.7 ± 5.0 mg dl^{-1}) than in the HCN-alone group (47.4 ± 5.4 mg dl^{-1}). The slightly longer survival of the ethanol + HCN groups was attributed to depressed pulmonary ventilation, and the lower blood lactate to decreased muscle activity, since convulsions were seen less than in the HCN-alone group.

The potential for the attainment of behavioral, toxic, and lethal concentrations of HCN in some fire atmospheres due to the burning of N-containing polymers emphasizes the requirement for firefighters to wear respirators of the pressure-demand type (Ballantyne, 1981). Other personnel should not be allowed to enter an area after a fire has been extinguished until measurements have shown that no HCN is being released from, for example, residual smoldering upholstered furniture (Daunderer, 1979).

30.20 Forensic Aspects of Acute Cyanide Poisoning

30.20.1 Causes of Acute Cyanide Poisoning

Mortalities from acute cyanide poisoning result from a variety of circumstances, many of which are a cause of general interest and most involve, for various reasons, a forensic interest. They may, in general, be considered under the following groups.

30.20.1.1 Accidental Exposure

Accidental acute CN poisoning may be caused by occupational exposure, by exposure to the products of combustion, and by accidental misuse or exposure in the home. Occupationally related acute CN poisoning may occur where cyanides or cyanogens are used in industrial operations; during industrial, agricultural, or horticultural fumigation operations; or in research and teaching laboratories. Such exposures can be minimized or prevented by careful industrial hygiene practices. Laxity in this respect may result in a forensic involvement, and possibly with subsequent criminal charges and litigation. In one notorious case of bad industrial practices that caused the death of a worker, imprisonment and fines resulted for the management personnel (Annotation, 1983; Burnett, 1985; Danbury Times, 1985a, 1985b). Accidental domestic acute CN poisoning usually results from the misuse of CN-containing products, often by swallowing in children and, to a lesser extent, by adults. Sometimes overexposure to vapor has been reported (Ballantyne, 1987a). Ingestion of cyanogens may occur, such as amygdalin in fruit pits (Lasch and Shawa, 1981) or laetrile (Humbert et al., 1977; Sadoff et al., 1978). HCN may be a major component of the products of combustion, and CN poisoning can be a factor in smoke inhalation poisoning, as discussed above. Thus, acute CN intoxication may result from exposure to the products of combustion in domestic, automobile, and aircraft fires (Borron and Baud, 1996; Mayes, 1991; Noguchi et al., 1988), and is a potential occupational hazard of fire fighting.

30.20.1.2 Intentional Exposure

Intentional exposure covers, in the main, mortalities resulting from suicide, homicide, mass murder, deliberate judicial and political killings, and covert activities. Clearly, the majority of these will have major direct or indirect forensic involvement. For suicidal purposes, CN is usually swallowed. Contrary to popular opinion the use of CN is an uncommon method of suicide (Ballantyne, 1987a). For example, in Thailand over the period 1960–1969, of 339 suicidal poisoning only 4 (1.1%) were due to CN (Eungprabhanth, 1975). In a survey in Allegheny County (Pennsylvania), population 1.8 million, over the period 1970–1975, of 700 suicides only 6 used CN (Winek et al., 1975). Although not a common cause for suicide, CN may account for a high proportion of deaths. For example, the Medico-Legal Society of Japan determined that for 245 cases of CN poisoning, the incidence for suicide was 210 (85.7%) and for murder was 18 (7.4%) (Matsukuva and Fukui, 1968). Also, the use of CN may be higher in certain occupational groups than for the general population; for example, in a review by the American Chemical Society it was found that for 3637 deaths over 1948–1967,

119 were due to suicide (3.3%), of which 29 were due to CN (Li, 1969). In a subsequently reported series, Walrath et al. (1985) examined the cause for the death of 347 female members of the American Chemical Society over 925–1979; there were 36 (10.4%) with CN, accounting for 39%.

The use of CN for homicidal purposes has attracted considerable morbid interest. In many cases this has been accomplished by adulterating drinks. Another method has involved the repacking of medicinal capsules (Curry, 1963; Dunea, 1983; Holland, 1983). More extreme methods have been by intravaginal instillation (Holzer, 1939; Drasch et al., 1982), or pouring into the mouth following the induction of general anesthesia (Weiler and Kloppel, 1984). Intramuscular injection was a possibility in one case (Ballantyne et al., 1972). Mass murders with CN have included genocidal programs by Nazis during the Second World War, cult killings such as the Jonestown tragedy in which over 900 people were encouraged to drink CN (Thompson et al., 1987), and substituting CN for medication in Sudafed capsules on shop shelves (Brahams, 1991; Centers for Disease Control, 1991). Politically approved killings with CN include judicial execution of criminals with HCN vapor, chemical warfare operations, and covert activities. The latter have included spraying CN in the face, incorporation in cigarettes, and administration by injection (Gee, 1987; Harris and Paxman, 1982; Seagrave, 1982).

The diagnosis and confirmation of acute CN poisoning requires consideration of the circumstances leading to death, premortem history, autopsy features, and CN analyses of scene evidence and of victim body fluids and tissues, and the findings from special investigations. These are briefly reviewed below.

30.20.2 Circumstances of Poisoning

Occupational poisoning is generally recognized because of the scene and the circumstances of the incident. However, when nonemployees are symptomatically exposed, then the presenting features may not be totally clear. For example, if HCN vapor diffuses from a fumigation site into a nearby building or dwelling, or when children accidentally pick up CN traps laid to destroy vermin in fields or on farms (Fiori and Marigo, 1963). With suicide there may be clues at the scene of the incident. However, when there is a deliberate intent to commit suicide then traces of evidence may have been obscured. Likewise, with homicide there will have been attempts to remove all indications of the cause of death, or the presentation may be bizarre.

30.20.3 Antemortem Presentation

As noted above, the clinical features of CN poisoning may be variable in their nature, time to onset, order of appearance and severity; this may clearly confuse the diagnosis. Major determinants of presentation include the nature of the exposure (cyanide or cyanogen), route and magnitude (concentration/dose/duration) of exposure, and the possible influence of any first aid, support, or antidotal treatment measures. In some cases the signs and symptoms before death may be atypical or insufficient to allow a clinical diagnosis of cause for death, particularly in the absence of any circumstantial evidence. When death occurs with convulsions and tachypnea, without cyanosis, and in the presence of lactate acidosis with normoglycemia, then acute CN poisoning should be suspected.

Time to death may be variable. By high concentration vapor (inhalation) exposure it is common for death to occur during the exposure, or within a few minutes of being removed from the contaminated area. Because of the rapidity of CN detoxification, when vapor inhalation exposure ceases the blood and tissue CN concentrations decrease, and recovery without relapse is common. In contrast, when CN is swallowed the times to onset of signs and death may be very variable and will depend primarily on the nature and amount of CN or cyanogen swallowed. Thus, swallowing a large volume of a concentrated solution of CN is likely to produce a rapid onset of severe signs and symptoms of intoxication, with few intervening signs between swallowing and the onset of convulsions, coma, and death. When smaller amounts of CN or a slow-releasing cyanogen are swallowed, then there may be a slower onset of symptoms and signs, and possibly the gradual progression of classical symptoms and signs, with death delayed for several hours. Also, it has been reported that following successful

immediate antidotal treatment of acute CN poisoning, relapse may result from the absorption of CN from collections in the gastrointestinal tract which have not been removed during support measures such as induced emesis, gastric lavage, or the use of activated charcoal (Ballantyne, 1987a). By cutaneous contact there may be a delay of several hours between contamination of the skin and the attainment of clinically toxic blood and tissue CN concentrations. In a proportion of cases it is not possible to determine the time between ingestion of CN and death. For example, in a series of CN deaths in Japan, out of 245 cases it was not possible to ascertain the time to death for 97 (40%) of the cases (Matsukura and Fukui, 1968).

30.20.4 Necropsy Features

In experimental acute CN poisoning by various routes it is not possible to define any gross pathological or histopathological features that can be regarded as specific or uniquely characteristic (Ballantyne, 1970, 1974; Ballantyne et al., 1972). In human acute lethal CN poisoning the features are also nonspecific. They include congestion of the viscera, cerebral and/or pulmonary edema, and the presence of petechiae in brain, meninges, pleura, lungs, heart, and pericardium (Gettler and St George, 1934; Pryce and Ross, 1963; Jetter, 1966; Ballantyne, 1974;). The disseminated petechiae have been ascribed to terminal convulsions, hypoxia, and visceral congestion (DuBois and Geiling, 1959; Robbins and Cotran, 1979). Although CN produces a cytotoxic hypoxia and it is therefore anticipated that blood would be bright red, this is not consistently found in either experimental situations (Ballantyne, 1970) or fatal human cases of acute CN poisoning (Pryce and Ross, 1963). In cases of swallowed CN, the stomach appearances may be suggestive of CN poisoning. There may be congestion of the gastric mucosa, and in the fundus over the greater curvature there may be early necrotic change (Buchanan et al., 1976). In 3 of 4 fatal cases of swallowing CN there was a marked hemorrhagic appearance of the gastric mucosa, in one case associated with gross edema of the gastric rugae (Fernando et al., 1991). Odor confusion may present a problem in confirming a diagnosis for several reasons. First, when putrefactive changes are occurring, the resultant odor may mask that due to CN. Second, when the death is intentional there may have been a deliberate masking of the CN odor by the presence of another odiferous material (Camps et al., 1976). Finally, and as noted in detail above, a proportion of the population may not be able to detect the characteristic odor of HCN in the atmosphere. This CN anosmia may also be relevant to possible hazard during the conduct of an autopsy on a subject suspected of having died from acute CN poisoning (see section 30.16).

30.20.5 Cyanide Concentrations in Tissues and Body Fluids in Acute Lethal Cyanide Poisoning and the Influence of Variables

The concentration of CN measured in body fluids and tissues depends on a variety of factors, the most important of which include the route of exposure, the magnitude of exposure (amount and duration of exposure), nature of the causative material (free CN or cyanogen, and the composition of a formulation), time to death, antemortem support and treatment measures, time to autopsy, and the interval between tissue/fluid sampling and the time to analysis. In a particular case many of these factors will be interrelated, and thus the measured value will be the result of several influences.

30.20.5.1 Influence of the Route of Exposure

The differential distribution of CN, as reflected in measurements of tissue and body fluid CN concentrations, can be significantly influenced by the route of exposure. This was experimentally investigated by Ballantyne (1983a, 1984a) who demonstrated he following:

1. Significant concentrations of CN were measured in blood by all routes of dosing (i.m., i.p., p.o., p.c., transocular, and inhalation). The lowest concentrations were generally measured from animals dosed by inhalation, transocular, and p.c. routes.

2. Liver concentrations were high following i.p. and p.o. dosing, intermediate by i.m., and lowest following p.c. or transocular dosing. CN was not usually detected in liver samples following inhalation exposure.
3. Moderately high and relatively consistent CN concentrations were measured in lung, brain, and myocardium
4. Kidney and spinal cord concentrations were low and variable.
5. For any given tissue, the lowest CN concentrations were usually measured following inhalation exposure.

Similar experimental results were obtained by (Yamamato et al., 1979, 1982, 1984). The difference in hepatic CN concentration between the p.o. and inhalation routes of exposure relates to the high concentration of CN in portal venous blood after p.o. dosing, compared with the low proportion of blood going to the liver after pulmonary absorption of CN. However, postmortem diffusion of CN from the stomach to surrounding tissues may be a minor contributory factor. This was confirmed by measurements of CN concentrations in animals having had a gastrectomy after death from acute CN poisoning (Rusiecki and Dynakowski, 1963). The experimental studies indicate that because of regional variations in CN concentrations measured with differing routes of exposure, to ensure a diagnosis of CN poisoning it is necessary to remove multiple tissues for analysis. Also, the pattern of differential distribution of CN concentrations in various tissues can confirm poisoning by a particular route.

Concentrations of CN measured in tissues from human cases of acute CN poisoning allow the following conclusions:

(a) In contrast with experimental findings, in human cases the concentrations of CN in spleen are high. The species discrepancy is probably related to differences in blood flow rates and perfusion volumes in the human spleen compared with those in common laboratory mammals.
(b) The highest blood and tissue concentrations of CN are found following death by swallowing CN.
(c) Liver CN concentrations are high following p.o. poisoning and confirm the experimental findings that liver CN concentrations may aid in defining the route of exposure.
(d) Following death due to the inhalation of HCN vapor, blood and tissue CN concentrations are generally low, particularly in the liver.

Considerations on experimental and human cases of acute CN poisoning indicate that: (1) the differential distribution of CN concentrations in various tissues may allow a definition of the route of exposure, and (2) the most appropriate tissues to remove for CN analysis are blood, stomach contents, lung, liver, kidney, brain, myocardium, and spleen.

Whole-blood CN concentrations are significantly higher than concentrations in blood plasma or serum (Ballantyne, 1976; Ballantyne et al., 1970). This reflects the high affinity of erythrocytes for CN (Barr, 1966) and also explains the high concentration of splenic CN in cases of human acute CN poisoning resulting from erythrocyte sequestration. For diagnostic purposes it is therefore most appropriate to take blood samples for analysis. However, since plasma CN is in equilibrium with tissue fluid CN, it is more meaningful for functional investigational and clinical studies to measure plasma CN concentrations (Ballantyne, 1983c; McMillan and Svoboda, 1982). Interpretation of the results for blood CN concentration measurements should be made against the background of values for normal individuals. The results from normal individuals, even heavy smokers, show that the CN concentrations are very significantly less than those from CN-poisoned individuals (Ballantyne, 1987c). When blood from normal individuals is kept at deep-freeze temperatures then the increase in CN content, even with smokers, is no more than 0.2 μg ml^{-1}. For normal human

tissues, cyanide cannot be analytically detected in brain, liver, kidney, and lung, if removed and analyzed immediately after death (Ballantyne, 1983a). In attempting to define what may constitute a "lethal" blood CN concentration it is important to differentiate between concentrations measured in blood samples removed in the living poisoned patient and those measured in blood samples removed at autopsy. Thus, when intensive medical support and clinical toxicology facilities are available, and as a result of treatment, patients may survive if measured CN concentrations are equal to, or greater than, those measured in autopsy specimens from individuals dying before they reached an appropriate medical facility or received any support treatment (Bismuth et al., 1984; Pontal et al., 1982). With respect to autopsy specimens it is more appropriate to refer to "blood CN concentrations which are compatible with death from acute CN poisoning," and avoid the expression "lethal blood CN concentrations." Within these constraints, a review of the published data indicates that the lowest blood CN concentration compatible with death from acute CN poisoning is of the order of 1–2 $\mu g\ ml^{-1}$ (Ballantyne, 1987c).

30.20.5.2 Influence of Time to Autopsy

Experimental studies have shown that marked transformation of CN may occur in tissues left in the body following death from acute CN poisoning. This emphasizes the need for both prompt autopsy and removal of tissues in suspected cases of CN poisoning. With acute CN poisoning as a consequence of swallowing, there may be an initial increase in liver and lung CN concentrations during the first day, probably due to local diffusion of CN from the stomach. Ballantyne et al. (1974) found that when animals were given lethal doses of CN and autopsy delayed, there was a rapid decrease in measurable CN concentrations in blood and tissues. Thus, CN was not measurable in liver and kidney by 3 days after death, and in lung and brain by 14 days, and only trace amounts were present in whole blood at 3 weeks. Yamamoto et al. (1984) measured changes in CN and SCN in liver, lung and brain of rats killed by peroral CN or inhalation of HCN. Animals were kept for up to 3 days after death at room temperature. By the first day after death by p.o. CN, liver and lung CN concentrations had slightly increased, possibly by diffusion from the stomach. At this time following death from inhaled HCN, liver and brain concentrations were slightly reduced. By 3 days, liver concentrations were reduced to very low levels in both the p,o, and inhalation groups, and decreases in lung concentrations were noted. Transformation rates vary with tissues, and this clearly influences the effect of sampling time. For example, Terblanche et al. (1964) found experimentally that whereas liver CN concentrations were approximately three times those of skeletal muscle, the rate of transformation of liver CN was about twice that in skeletal muscle. CN could not be detected in liver after 12 h and in skeletal muscle after 28 h.

That transformation of CN occurs in cases of human CN poisoning is confirmed by the observation that blood samples taken at or near the time of death have higher measurable CN concentrations than ones subsequently removed. For example, Curry (1963) found, in a case of poisoning due to inhaling HCN vapor, that a sample taken at the moment of death the CN concentration was 3.5 $\mu g\ ml^{-1}$, whereas sample taken at autopsy the following day had concentrations of 1.0 $\mu g\ ml^{-1}$ (femoral) and 0.5 $\mu g\ ml^{-1}$ (carotid).

Postmortem transformation of CN may be due to various causes, of which the following have been suggested (Ballantyne, 1974; Guatelli, 1964):

1. Simple evaporation. Acidification resulting from the putrifactive process may accelerate the effect.
2. SCN formation, by enzymatic (initially) and nonenzymatic mechanisms.
3. Kiliani's reaction with aldehydes and ketones.
4. Hydrolysis and production of ammonium formate.
5. Polymerization and transformation to aminomalononitrile.

It is also relevant to note that CN in tissues is rapidly lost when they are embalmed in formaldehyde, with the CN content being reduced to about 1% of the original amount within 3 days (Gettler and Baine, 1933; Svirbely and Roth, 1953, 1954).

30.20.5.3 Influence of Time to Analysis on Measured Cyanide

It is of great importance that samples be kept in tightly stoppered containers to prevent immediate loss of CN. For example, if CN-containing blood is left in open containers for up to 10 min at ambient temperature this may result in appreciable amounts of CN being lost (Bright et al., 1990). *In vitro* studies using blood or serum spiked with CN have shown that that there is a rapid decrease in measurable CN concentrations. When CN was added to serum, only about 31–44% could be analytically recovered by 1 h (Ballantyne et al., 1973). Under similar conditions, the rate of decrease of measurable CN in whole blood was much less marked than in serum, with 67–83% of the added CN being analytically detectable at the end of 1 h, and between 62 and 74% of the initial concentration being measurable at 24 h. This differential recovery between serum and whole blood can be attributed, in part, to the high affinity of the erythrocyte for CN (Barr, 1966; Ballantyne et al., 1973). A more detailed *in vitro* study of changes in whole-blood CN as functions of storage time and temperature was conducted by Ballantyne (1976). CN was added to blood to produce a range of concentrations typical of those found in cases of human acute CN poisoning, and CN concentrations measured periodically over a period of 3 months for samples stored at temperatures of 20°C, 4°C, and −20°C. During the first week, concentrations decreased at all storage temperatures, but after this time fluctuations in concentrations varied with temperature. Changes were least marked at −20°C, and maximum in specimens stored at 4°C. However, CN concentrations did not fall to values that were "not compatible with death due to CN poisoning." These findings confirm the value of whole blood for analysis in suspect cases of acute CN poisoning, since the values are not reduced to nondiagnostic levels even at 4°C. However, they also emphasize the need to analyze samples for CN as soon as they are removed from the body. Additionally, heparinization of blood is reported to result in better recovery of CN from blood. When autopsy blood is obtained it should be homogenized as thoroughly as possible prior to analysis; e.g., using ultrasonic homogenization (Goenechia, 1982).

For tissues, it has been demonstrated experimentally that there may be rapid transformation of CN after their removal from the body. Thus, Ballantyne et al. (1974) showed that CN could not be detected within about 3 days for kidney and liver, and 7 days with brain, and 14 days with lung. However, and in contrast with values obtained where there is a delay to autopsy, significant concentrations of CN could still be measured in blood at 3 weeks. The difference in rates of decrease in CN for removed blood and tissues is because tissues still have a significant transforming capacity, whereas blood does not. However, when there is a delay to removing blood from the body, then transformation mechanisms and diffusion processes are readily available to blood.

30.20.5.4 Postmortem Formation of Cyanide

Several investigators have described the postmortem formation of cyanide, which could result in a false-positive result in the context of diagnosing extrinsic acute CN poisoning. However, this is not considered a significant event by other investigators. CN production has been described in blood, brain, liver, kidney, uterus, and gastric contents, with measured concentrations that could be interpreted as being of toxicological significance (Curry et al., 1967; Curry, 1976). Bernt et al. (1961) also found CN production in human blood and stomach contents, with a maximum formation at 7–14 days and a slow disappearance thereafter. Optimum conditions for this effect were 4°C in an alkaline medium. According to Sunshine and Finkle (1964), CN formation occurs more readily in refrigerated samples than in ones kept at room temperature.

Several authors have implicated contamination of tissues with cyanogenic bacteria as the cause for CN generation in tissues. CN production has been demonstrated with several micro-organisms including *Pseudomonas aeruginosa* and *fluorescens* (Castric, 1975; Askeland and Morrison, 1983), *Chromobacterium violaceum* (Knowles, 1970; Collins et al., 1980), and a wide range of fungi including the psychrophilic basidiomycete of snow mold disease (Ward and Thorn, 1966; Bunch and Knowles, 1980), and several strains of *Marsmius, Pholiota, Polypuras,* and *Tricholoma* (Bach, 1956; Knowles, 1970). There is some evidence that bacterial cyanogenesis is a feature of secondary metabolism (Castric, 1975), and it is influenced by a variety of factors including phosphate concentration (Meganathan and Castric, 1977) and iron concentration (Askeland and Morrison, 1983). Maximum cyanogenesis has been observed during the transition from the exponential to the stationary growth phase (Castric, 1975; Castric et al., 1979; Askeland and Morrison, 1983). Other effects that can modify cyanogenesis include pH, temperature, and amino acid incorporation (Wissing, 1968; Knowles, 1970; Castric, 1975; Castric et al., 1979; Collins et al., 1980; Askeland and Morrison, 1983). Cyanide C appears to originate from the 2-C of glycine for *Pseudomonas* sp. (Michaels et al., 1965; Brysk et al., 1969; Askeland and Morrison, 1983), although Castric (1977) also implicated the 1-C of glycine. The production of HCN from glycine in *P. aeruginosa* is under control of an enzyme, HCN synthase (Castric et al., 1981). Solubilization and some characterization of the enzyme have been reported by Wissing and Anderson (1981). The radiochemical yield of CN (free and bound as β-cyanalamine) from glycine has been determined as 32% (Brysk et al., 1969). In contrast, with *Chromobacterium violaceum* the C and N originate from the methylene and amino groups of glycine, respectively (Ward et al., 1977). The metabolism of CN by bacteria has been reviewed by Castric (1981).

The potential for postmortem cyanogenesis appears to be variable, and several workers have failed to demonstrate this either experimentally or in human tissues, or if it occurs then it is in amounts that are not of toxicological significance. For example, Gettler and Baine (1933) found that when human liver and brain were kept for 7−28 days, the maximum concentration of CN produced during putrefaction was 0.3 $\mu g\ g^{-1}$, which is approximately 10% of the lowest concentrations of CN found in these organs in cases of acute lethal CN poisoning that they investigated. Berndt et al. (1961) failed to detect CN formation in brain, liver, kidney and myocardium at 4°C and room temperature with storage up to 3 months. A detailed study of the potential to produce CN in normal human blood was conducted by Ballantyne (1977b). Blood from normal human smokers and nonsmokers was stored for up to 3 months at deep-freeze (−20°C), refrigerator (4°C), and room (20°C) temperatures, and analyzed periodically for CN concentration. Initial mean (± SE) concentrations of CN in whole blood were 1.6 ± 0.2 $\mu g\ dl^{-1}$ (0.016 ± 0.002 $\mu g\ ml^{-1}$) for nonsmokers and 5.9 ± 0.9 $\mu g\ dl^{-1}$ (0.059 ± 0.009 $\mu g\ ml^{-1}$) for smokers ($p < 0.005$). At room temperature there was a slow transformation of CN, but at deep-freeze temperature there was formation of CN, which apparently occurred mainly during the first few days of storage. However, peak concentrations were never greater than 20 $\mu g\ dl^{-1}$ (0.2 $\mu g\ ml^{-1}$). The amount of CN formed under deep-freeze conditions appeared to be a function of both the initial SCN concentration and the freezing and/or thawing of blood. The formation of CN at −20°C is probably an artifact due to the conversion of SCN to CN by the hemoglobin which is released following mechanical hemolysis. Blood stored at 4°C had the least fluctuation in CN concentration over the 3-month storage period. The maximum concentration of CN formed, under deep-freeze conditions, should not lead to any problem in the differential diagnosis of acute CN poisoning provided that autopsy has been carried out shortly after death. However, this formation of CN could be a significant artifact in studies concerned with measuring sublethal exposures to CN. Egekeze and Oehme (1980) also found the least *in vitro* change in CN concentration occurred during storage at 4°C. One probable source of postmortem CN is the hydrolysis of cyanogens in the gastrointestinal tract, with the possibility of forensically significant concentrations being measured in the gut contents. Berndt et al. (1961) found that at 37°C no CN was formed by 7 or 14 days; however, CN production occurred in 13 of 20 samples at 4°C. In cases where CN is detected in the gastrointestinal tract, a careful analysis of multiple tissues is necessary to confirm, or exclude, the presence or absence of systemic CN.

30.20.5.5 Influence of Supportive and Antidotal Procedures

In general, CN antidotes fall into the following four classes with respect to their possible effects on analytically measured CN concentrations (Ballantyne, 1987b):

(a) Antidotes that hasten endogenous detoxification of CN (e.g., thiosulfates) and cause a real fall in CN concentrations.
(b) Antidotes that bind CN directly (e.g., cobalt compounds) or indirectly (e.g., methemoglobin generators) and may, or may not, result in a spuriously low CN concentration depending on the particular antidote, the degree of binding, and the method used.
(c) Antidotes that themselves, or through conversion products, interfere with the method, and give falsely high or low values.
(d) Antidotes whose activity is directed toward the relief of signs and/or symptoms, rather than being specifically antidotal (e.g., anticonvulsants) will generally not interfere with CN methodologies.

It is probable that all the commonly used CN antidotes affect CN concentrations in biological fluids. Thus it would be anticipated that administration of thiosulfate, which increases the rate of transulfuration, will lower the CN concentration and higher the SCN concentration. With methemoglobin-producing antidotes, such as sodium nitrite, 4-DMAP, and *p*-aminopropiophenone, CN is complexed as cyanMetHb. This Hb derivative is not easy to measure in the presence of other hemoglobin derivatives, and in experimental CN treatment most workers have measured whole-blood or erythrocyte CN and plasma CN. The methods of Epstein (1947) and Feldstein and Klenshoj (1954) give satisfactory results in such circumstances. It is anticipated that in CN poisoning treated with sodium nitrite or 4-DMAP measured whole-blood or erythrocyte CN will be very high in comparison with the plasma concentration (Christel et al., 1977; Marrs et al. (1982, 1985). The results probably represent the true *in vivo* situation in that the antidotal effect of metHb-producing CN antidotes depends on their ability to sequester CN inside the erythrocyte, and thus lowering the plasma concentration. A more difficult problem occurs with the cobalt salts that bind CN. It has been shown by Marrs and Bright (unpublished; personal communication) that the Epstein (1947) and Feldstein and Klendshoj (1954) methods underestimate CN in dog blood spiked with dicobalt edetate, when a sufficient amount of that antidote was added to produce a blood concentration equivalent to a human antidotal dose. Sodium thiosulfate can interfere with some of the colorimetric, fluorimetric, and potentiometric methods for measurement of CN (Morgan and Way, 1977; Morgan et al., 1979; Sylvester et al., 1982). With colorimetric methods, on acidification the thiosulfate undergoes a series of complex reactions, and among the products of which are polythionic acids, of empirical formula $H_2S_nO_6$, which are unstable at room temperature. One degradation product is SO_2, which at low pH, will diffuse with HCN and be trapped as sulfite, and which is known to interfere with the colorimetric estimation of CN (Epstein, 1947). To overcome this problem, the colorimetric method can be modified by an approach based on the difference in pK_a values of HCN (9.2; Feeney et al., 1973) and the various polythionic acids (about 2.0; Skoog and West, 1969) formed upon acidification of the thiosulfate ion. Interference can be circumvented by using pH 5.2 buffered solution rather than H_2SO_4 as an acidification agent (Morgan and Way, 1977; Morgan et al., 1979). The fluorimetric method involves the conversion of pyridoxal to 4-pyridoxylacetone, and sodium thiosulfate interferes with the chemical conversion of this photophore. It is possible to circumvent this interference by the use of pH 5.2 buffer acetate buffer as acidifying agent (Way, 1984). With potentiometric methods for CN analysis, sodium thiosulfate produces an interference that leads to falsely high CN concentrations, particularly in the presence of blood. This artifact is ascribed to enhanced biotransformation of thiosulfate, and in this case the contaminant producing the apparent elevation in CN concentration is believed to be the sulfide ion. The latter ion can be removed by oxidation with hydrogen peroxide, and the excess peroxide subsequently eliminated

with sodium sulfate (Sylvester et al., 1982). It is important to carefully remove the excess hydrogen peroxide, otherwise it will inactivate the ion-selective electrode (Way, 1984).

30.20.5.6 Possible Influence of Ethanol and Other Drugs

When CN has been used for suicide, or possible homicide, then ethanol may have been taken before the ingestion of CN. Also, in fires there may have been prior consumption of alcohol; for example, it has been shown that ethanol levels are high in a proportion of fire victims (Radford et al., 1976; Birkby et al., 1979a; Harland and Wooley, 1979; Anderson and Harland, 1982; Hart et al., 1985). Yamamoto et al. (1983) studied experimentally in rats the effects of ethanol (3 g kg^{-1}, i.p.) on exposure to 500 ppm HCN vapor. They found that blood CN concentrations were 20% higher in the ethanol + HCN group (3.44 ± 0.44 μg ml^{-1}) than in HCN-alone controls (2.80 ± 0.32 μg ml^{-1}), but the differences were not statistically significant. However, the blood lactate concentrations were significantly lower in the ethanol + HCN group (367 ± 50 μg ml^{-1}) than in the HCN controls (474 ± 54 μg ml^{-1}). The decreased lactate concentrations in the ethanol group were attributed to decreased muscle activity, because convulsions were seen less frequently than in the HCN-alone group.

The presence of other drugs could influence CN measurements, but many have not been studied in detail. One possible interfering drug, which may be relevant in the context of chemical warfare operations, is pralidoxime mesylate (P2S) which is of use in the treatment of, or a prophylaxis for, poisoning by oxime-sensitive organophosphate anticholinesterases (Sundwall, 1960; Sidell et al., 1972; Holland and Parkes, 1976). Cyanogenesis does not appear to be a significant factor in the lethal toxicity of P2S to experimental animals; for example, little SCN was excreted in the urine of rats receiving sublethal doses of P2S (Enander et al., 1961), and CN could not be detected in the blood of animals immediately following death from a lethal i.m. injection of P2S (Ballantyne et al., 1975). However, Ballantyne (1984b) demonstrated that P2S interferes with the colorimetric estimation of CN in solution and produces an apparent increase in CN concentration that is most marked at extreme pH values. The most marked increases in CN occurred at pH 12.5 and were probably due to cyanogenesis by P2S; thus the effect was time dependent and was proportional to the P2S concentration. At pH 4.5, the apparent increases in CN concentration were not dependent on time. Also, CN could not be detected after acidification and aeration of solutions containing P2S alone. These observations suggest that at alkaline pH, the apparent increase in CN is due to a direct interference by the P2S molecule. The effect is minimal at pH 7.4.

30.20.6 Thiocyanate Concentrations in Acute Cyanide Poisoning

In view of some problems in the interpretation of blood CN concentrations in establishing a firm diagnosis in acute CN poisoning, other biochemical factors have been examined as possible support measures for diagnostic purposes. One candidate was SCN, but this was of very limited usefulness. Thus, plasma and urinary SCN concentrations are variable, even in normal subjects. Part of this variability is due to difference is dietary cyanogens (Montgomery, 1969; Baumeister et al., 1975; Narty, 1980; Caplan, 1982), and another factor is cigarette smoking; some typical values for blood, plasma, and serum SCN concentrations in smokers and nonsmokers are given by Ballantyne (1987b). Yacoub et al. (1970) showed that smoking increased the urinary SCN by a factor of about 3 and that there was a correlation between urinary SCN and the number of cigarettes smoked. Although there is good analytical recovery of SCN from serum and plasma, the recovery from whole blood is poor. Thus, Ballantyne (1977c) found that the whole-blood SCN concentration from smoking and nonsmoking human subjects were between 3.4 and 5.2 times less than the corresponding plasma SCN concentrations The SCN is lost to analysis by reaction with, and absorption to, precipitated hemoglobin (Goldstein, 1950; Vesey, 1979). It follows that whole-blood SCN measurements are not a direct indicator of CN exposure. Also, whole-blood SCN measurements are not reliable because of the inadequate time for measurable amounts of transulfuration to occur. Both the time to death and the availability of sulfur donors will influence the degree of transulfuration. In acute

CN poisoning the survival is often insufficiently long enough for substantial transulfuration to occur (Drawbaugh and Marrs, 1987). When there is a moderate delay to death, then some transulfuration may occur and give supporting evidence for CN poisoning. One particular example of inadequacy of transulfuration before death is seen in victims of fire with smoke inhalation poisoning who have markedly increased blood CN concentrations, but whose blood SCN concentrations are significantly less than in survivors having increased blood CN concentrations (Symington et al., 1978; Anderson and Harland, 1982).

SCN concentration measurements, particularly in urine, may be more valuable in poisoning by cyanogens, as opposed to free cyanides, but in such cases it is important to have reference ranges from a population with similar eating and smoking habits to the poisoned patients. SCN in serum or, in particular, in timed urine specimens is more useful to assess prolonged or repeated exposure to nonlethally toxic concentrations of CN, such as in smoking, passive smoking (Poulton et al., 1984), or industrial exposure (Chandra et al., 1980).

30.20.7 Measurement of Cytochrome Oxidase Activity in the Confirmation of Acute Cyanide Poisoning

Measurement of cytochrome *c* oxidase activity in tissues, and its inhibition, has been proposed as a mechanistic approach to confirming death from acute CN poisoning (Ballantyne, 1974), and has been studied experimentally. Biochemical studies have shown that when tissues are removed promptly after death from acute CN poisoning, significant decreases in cytochrome *c* oxidase activity can be measured in myocardium, brain, spinal cord, kidney, and liver (Ballantyne, 1977a). The most suitable tissues regarding the reproducibility of results and assessment of enzyme activity are myocardium and brain (Camerino and King, 1966; Ballantyne, 1977a; Ballantyne and Bright, 1979a, 1979b). Although, on the basis of such results, it would appear that measurement of the inhibition tissue cytochrome *c* oxidase activity is a reasonable approach for the confirmation of acute CN poisoning, if removal of tissues is delayed for more than a few days then the enzyme activities measured are the same as those in normal tissues from non-CN-poisoned animals removed at similar times postmortem (Ballantyne, 1977a). Conventional histochemistry, in place of biochemical methods, is of little use in the diagnosis of acute CN poisoning for the following reason. When cryostat sections of tissues removed immediately or shortly after death from acute CN poisoning are incubated in a histochemical medium for cytochrome *c* oxidase, then there are no differences in either the distribution or qualitatively assessed intensity of the reaction product between such sections and those of normal (non-CN) sections. Also, if the reaction product is assessed quantitatively by microspectrophotometry on such conventionally prepared histochemical sections there are only minor differences in extinction values between tissues from control and cyanide groups (Ballantyne, 1977a). This is due to the rapid spontaneous reactivation of the enzyme–inhibitor complex during the incubation time in the histochemical medium, resulting in an apparent normal distribution and intensity of reaction product (Ballantyne, 1977a). To avoid spontaneous reactivation, and also to provide a reliable quantitative basis to measure cytochrome *c* oxidase, a technique was developed using a substrate-gel film coupled with measurement by kinetic microdensitometry (Ballantyne and Bright, 1979a, 1979b). In this method, fresh-frozen cryostat sections of tissue are laid on gel films containing phenylamine substrates which allow an autochromic histochemical reaction. The rate of utilization of substrate is measured continuously by kinetic microdensitometry in which the initial substrate utilization rate is measured as the rate of change in extinction (ΔE_{550} min^{-1}) due to dye product. The use of substrate gel films allows microspectrophotometric measurements immediately after the gel-tissue complex is placed in the light path, and thus before any significant reactivation has occurred. Optimum conditions were defined as follows: E_{max} 550 nm; section thickness, 12 μm; pH 7.4; section thickness, 2–4 mm. Relative cytochrome *c* oxidase activity was similar in normal tissues by biochemistry and kinetic microdensitometry (Table 30.13). Also, inhibition of cytochrome *c* oxidase activity in myocardium and brain was similar by the two techniques (compare Tables 30.8

TABLE 30.13 Comparison of Cytochrome Oxidase Activities in Normal Rabbit Tissues Assessed by Biochemical and Quantitative Enzyme Histochemical Methods

Method (units)	Activity (as mean ± SE)		
	Myocardium	Cerebral Cortex	Ratio (M/C)[a]
Biochemistry[b] (ΔE_{510} min^{-1} g^{-1})	4.95 ± 0.08	1.31 ± .027	3.78
Kinetic microdensitometry[c] (ΔE_{550} min^{-1})	0.063 ± 0.001	0.016 ± 0.001	3.88

[a] Ratio of cytochrome oxidase activities (myocardium/cortex [M/C]).

[b] Method of Pearl et al. (1963).

[c] Method of Ballantyne and Bright (1979a).

and 30.9). Thus, kinetic microdensitometry is a reliable method for measuring cytochrome *c* oxidase activity in normal tissues, and for the *in vivo* assessment of enzyme inhibition following acute CN poisoning.

30.20.8 General Considerations and Conclusions on Measurement of Cyanide Concentrations in Biological Materials and of Confirmatory Analytical Methods

The most appropriate tissues for CN analysis are blood, brain, myocardium, liver, lung, spleen, kidney, and gastric contents. The CN concentrations attained in body fluids and tissues depend on numerous factors, the principal of which are route of exposure, dose received (magnitude and duration of exposure), whether exposure is to free cyanide or cyanogen, and time to death. A consideration of the absolute and relative concentrations CN in blood and tissues can assist in defining or confirming the route of exposure. Experimental and human studies indicate that the most informative tissues for analysis are blood, stomach contents, lung, spleen, brain, myocardium, and liver. The lowest concentrations of CN are most likely to occur with death from inhalation of HCN vapor. Important factors to be considered that may determine the CN concentrations measured in body fluids and tissues include any delay to autopsy which may result in transformation of CN and decrease in analytically measured concentrations of CN. Delays to analysis and the conditions and time of storage of biological samples may modify CN concentrations, either increasing or decreasing concentrations depending on particular conditions. For example, tissues stored at room temperature show a rapid transformation of CN, and none may be detected in days to weeks. In contrast, although there are fluctuations in CN concentrations in whole blood stored at differing temperatures, over a period of many weeks the values do not decrease to values below those compatible with a diagnosis of acute CN poisoning. The considerations above confirm that autopsy and analysis of samples should be carried out at the earliest possible time after death. The analytical method to measure CN should be carefully chosen, particularly if there has been prior antidotal treatment. The potential for postmortem formation of CN in normal tissues is an important consideration in order to avoid a false-positive diagnosis. However, findings and opinions vary with different investigators, but, in general, if it occurs, then it is not in forensically significant amounts. Several authors have implicated contamination with micro-organisms as being a major causative factor. One situation where an increase in CN concentrations occurs is the storage of blood at deep-freeze temperature. Although these increases are not of forensic significance, they could be relevant in investigations on CN metabolism. The effect is probably a result of conversion of SCN to CN by Hb liberated during the mechanical hemolysis of erythrocytes. Another possible source for postmortem generation of

CN is from cyanogens in foodstuff or drugs in the gastrointestinal tract. Whole-blood SCN measurements are not a valuable or direct indicator of acute CN poisoning because SCN is lost to analysis by reaction with and absorption to precipitated Hb and because there is usually inadequate time for significant transulfuration to occur. SCN is more valuable for poisoning with cyanogens and nonlethal exposure to CN if measurements are in urine or plasma. Cytochrome oxidase measurements may be valuable for confirming acute CN poisoning if they are made biochemically or on substrate-gel films by kinetic microdensitometry using tissues removed and analyzed shortly after death.

REFERENCES

ACGIH (American Conference of Governmental Industrial Hygienists), 2003 TLVs® and BEIs®, American Conference of Governmental Industrial Hygienists, Cincinnati, OH, 2003, p. 35.

Acker, H. and Eyzaguirre, C., Light absorbance changes in the mouse carotid body during hypoxia and cyanide poisoning, *Brain Res.*, 409, 380–385, 1987.

Aharony, D., Smith, J.B., and Smith, M.J., Inhibition of human platelet lipoxygenase by cyanide, *Experientia*, 38, 1334–1335, 1982.

Aitken, P.G. and Braitman, J., The effect of cyanide on neural and synaptic function in hippocampal slices, *Neurotoxicology*, 10, 239–248, 1989s.

Alvarez-Buylla, R. and Alvarez-Buylla, E.R. de, Carotid sinus receptors participate in glucose homeostasis, *Respir. Physiol.*, 72, 47–60, 1988.

Avarez-Buylla, R. and Alvarez-Buylla, E.R. de, Changes in blood glucose concentration in the carotid-body sinus modify brain glucose retention, *Brain Res.*, 654, 167–170, 1994.

Alvarez-Buyalla, R., Alvarez-Buyalla, E., Mendoza, H., Montero, S.A., and Alvarez-Buyalla, A., Pituitary and adrenals are required for hyperglycemic reflex initiated by stimulation of CBR with cyanide, *Am. J. Physiol.*, 272, R392–R399, 1997.

American Cyanamid Company, Report on sodium cyanide: 30-day repeated feeding study to dogs, Report 59-14, Central Medical Department, American Cyanamid, Princeton, NJ, 1959.

Anderson, R.A. and Harland, WAY., Fire deaths in the Glasgow area. III. The role of hydrogen cyanide, *Med. Sci. Law*, 22, 25–40, 1982.

Anderson, R.A., Thomson, I. and Harland, W.A., The importance of cyanide and organic nitriles in fire fatalities, *Fire and Materials*, 3, 91–99, 1979.

Andrews, J.M., Sweeney, E.S., Grey, T.C., and Wetzel, T., The biohazard potential of cyanide poisoning during postmortem examination, *J. Forens. Sci.*, 34, 1280–1284, 1989.

Annotation, Cyanide sometimes not so sudden, *Emerg. Med.*, January, 229–233, 1978.

Annotation, Litigation. Murder, manslaughter indictments issued in work-related death of Chicago-area man, *Occup. Safety Health Rep.*, 13, 576, 1983.

Antonini, E.A., Brunori, M., Greenwood, C., Malstrom B.G., and Rotilio. C., The interaction of cyanide with cytochrome oxidase, *Eur. J. Biochem.*, 23, 396–400, 1971.

Ardelt, B.K., Borowitz, J.L., Maduh, E.U., Swai, S.L., and Isom, G.E., Cyanide-induced lipid peroxidation in different organs: subcellular distribution and hydroperoxide generation in neuronal cells, *Toxicology*, 89, 127–137, 1994.

Askeland, R.A. and Morrison, S.M., Cyanide production by *Pseudomonas fluorescens* and *Pseudomonas aeruginosa, Appl. Environ Microbiol.*, 45, 1802–1807, 1983.

ATSDR (Agency for Toxic Substances and Disease Registry), Toxicological profile for cyanide, ATSDR Toxicological Profiles on CD-ROM (Version 3:1; 2000), Agency for Toxic Substances and Disease Registry, U.S. Public Health Service, Chapman and Hall/CRC Press, Boca Raton, FL, 1997.

Autor, A.P. and Fridovich, I., The interactions of acetoacetate decarboxylase with carbonyl compounds, hydrogen cyanide, and an organic mercurial, *J. Biol. Chem.*, 245, 5214–5222, 1970.

Bach, E., The agaric *Pholiota steareothermophilus* grown in different media, *J. Appl. Bacteriol.*, 45, 1820–1807, 1956.

Baftis, H., Smolensk, M.H., His, B.P., Mahoney, S., and Kresse, H., Chronotoxicity of male BALB/cCr mice to potassium cyanide, in *International Symposium on Chronopharmacology and Cronotherapy*, 1981.

Ballantyne, B., Autopsy findings following death by intramuscular hydrogen cyanide: an experimental study, *Med. Sci. Law*, 10, 171–174, 1970.

Ballantyne, B., The forensic diagnosis of acute cyanide poisoning, in *Forensic Toxicology*, Ballantyne, B., Ed., Wright, Bristol, 1974, pp. 99–113.

Ballantyne, B., Blood, brain and cerebrospinal fluid cyanide concentrations in experimental acute cyanide poisoning, *J. Forens. Sci. Soc.*, 15, 51–56, 1975.

Ballantyne, B., Changes in blood cyanide as a function of storage time and temperature, *J. Forens. Sci. Soc.*, 16, 305–310, 1976.

Ballantyne, B., An experimental assessment of the diagnostic potential of histochemical and biochemical methods for cytochrome oxidase in acute cyanide poisoning, *Cell. Mol. Biol.*, 22, 109–123, 1977a.

Ballantyne, B., In vitro production of cyanide in normal human blood and the influence of thiocyanate and storage temperature, *Clin. Toxicol.*, 11, 173–193,1977b.

Ballantyne, B., Factors in the analysis of whole blood thiocyanate, *J. Toxicol. Clin. Toxicol.*, 11, 195–210, 1977c.

Ballantyne, B., Letter to the Editor, *Clin. Toxicol.*, 14, 311–312, 1979.

Ballantyne, B., Inhalation hazards of fires, in *Respiratory Protection. Principles and Applications*, Ballantyne, B. and Schwabe. P.H., Eds., Chapman and Hall, London, 1981, pp. 351–372.

Ballantyne, B., The influence of exposure route and species on the acute lethal toxicity and tissue concentrations of cyanide, in *Developments in the Science and Practice of Toxicology*, Hayes, A.W., Schnell, R.C., and Miya, T.S., Elsevier, Amsterdam, 1983a, pp. 583–586.

Ballantyne, B., Acute systemic toxicity of cyanides by topical application to the eye, *J. Toxicol., Cut. Ocular Toxicol.*, 2, 119–129,1983b.

Ballantyne, B., Artifacts in the definition of toxicity by cyanides and cyanogens, *Fundam. Appl. Toxicol.*, 3, 400–408, 1983c.

Ballantyne, B., Comparative acute toxicity of hydrogen cyanide and its salts, in *Proceedings of the Fourth Annual Chemical Defense Bioscience Review*, Lindstrom, R.E., Ed., U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD, 1984a.

Ballantyne, B., The influence of pralidoxime mesylate on the measurement of cyanide concentrations in solution, *Vet. Hum. Toxicol.*, 26, 23–27, 1984b.

Ballantyne, B., Hazard evaluation of cyanide fumigant powder formulations, *Vet. Hum. Toxicol.*, 28, 472, 1986.

Ballantyne, B., Toxicology of cyanides, in *Clinical and Experimental Toxicology of Cyanides*, Ballantyne, B. and Marrs, T.C., Wright, Bristol, 1987a, pp. 41–126.

Ballantyne, B., Post-mortem features and criteria for the diagnosis of acute lethal cyanide poisoning, in *Clinical and Experimental Toxicology of Cyanides*, Ballantyne, B. and Marrs, T.C., Wright, Bristol, 1987b, pp. 217–247.

Ballantyne, B., Hydrogen cyanide as a product of combustion and a factor in morbidity and mortality from fires, in *Clinical and Experimental Toxicology of Cyanides*, Ballantyne, B. and Marrs, T.C., Eds., Wright, Bristol, 1987c, pp. 248–291.

Ballantyne, B., Toxicology and hazard evaluation of cyanide fumigation powders, *Clin. Toxicol.*, 26, 325–335, 1988.

Ballantyne, B., Acute percutaneous systemic toxicity of cyanides, *J. Toxicol., Cut. Ocular Toxicol.*, 13, 249–262, 1994a.

Ballantyne, B., Acute inhalation toxicity of hydrogen cyanide vapor to the rat and rabbit, *Toxic Subst. J.*, 13, 263–282, 1994b.

Ballantyne, B., Peripheral sensory irritation: basic and applications, in *General and Applied Toxicology*, Vol. 2, 2nd ed., Ballantyne, B,. Marrs, T.C., and Syversen, T., Eds., Macmillan Reference Ltd., London, 1999, pp. 611–630.

Ballantyne, B. and Bright, J.E., Comparison of kinetic and end-point microdensitometry for the direct quantitative histochemical assessment of cytochrome oxidase activity, *Histochem. J.*, 11, 173–116, 1979a.

Ballantyne, B. and Bright, J.E., A kinetic histochemical method for the determination of cytochrome oxidase activity, *J. Physiol., Lond.*, 300, 1–2, 1979b.

Ballantyne, B., Bright, J., and Williams, P., Levels of cyanides in whole blood and serum following lethal intramuscular injections to experimental animals, *Med. Sci. Law*, 10, 225–229, 1970.

Ballantyne, B., Bright, J., Swanston, D.W., and Williams, P., Toxicity and distribution of free cyanides given intramuscularly, *Med. Sci. Law*, 12, 209–219, 1972.

Ballantyne, B., Bright, J.E., and Williams, P., An experimental assessment of decreases in measurable cyanide levels in biological fluids, *J.Forens. Sci. Soc.*, 13, 111–117, 1973.

Ballantyne, B., Bright, J.E., and Williams, P., The post-mortem rate of transformation of cyanide, *Forens. Sci.*, 3, 71–76, 1974.

Ballantyne, B., Gazzard, M.F., Robson, D.C., and Swanston, D.W., Concentrations of 2-hydroxyimino-methyl-N-methylpyridinium ion in plasma and aqueous humour as indices of the toxicity of pralidoxime mesylate for the rabbit, *Toxicol. Appl. Pharmacol.*, 33, 559–567, 1975.

Banerjee, K.K., Bishayee, A., and Marimuthu, P., Evaluation of cyanide exposure and its effect on thyroid function of workers in a cable industry, *J. Occup. Environ. Med.*, 39, 258–260, 1997.

Barcroft, J., The toxicity of atmospheres containing hydrocyanic gas, *J. Hyg.*, 31, 1–34, 1931.

Barr, S., The microdetermination of cyanide. Its application to the analysis of whole blood, *Analyst*, 91, 268–272, 1966.

Baskin, S.I., Wilkerson, G., Alexander, K., and Blistein, A.G., Cardiac effects of cyanide, in *Clinical and Experimental Toxicology of Cyanides*, Ballantyne, B. and Marrs, T.C., Eds., Wright, Bristol, 1987, pp. 138–155.

Bass, N.H., Pathogenesis of myelin lesions in experimental cyanide encephalopathy, *Neurology*, 18, 167–176, 1968.

Bastian, G. and Mercker, H., Zur Frage der Zweckmäβigkeit der Inhalation von Amylnitrit in der Behandlung de Cyanidvergiftung, *Arch. Exp. Pathol. Pharmakol.*, 237, 285–295, 1959.

Baud, F.J., Barriot, P., and Toffis, V., Elevated blood cyanide concentrations in victims of smoke inhalation, *N. Engl. J. Med.*, 325, 1761–1766, 1991.

Baumeister, R.G.H., Schreulbein, H., and Zick-Graft-Rüdel, G., Toxicological and clinical aspects of cyanide metabolism, *Arzeim.-Forsch.*, 25, 1056–1064, 1975.

Beasley, D.M.G. and Glass, W.I., Cyanide poisoning: pathophysiology and treatment recommendations, *Occup. Med.*, 48, 427–431, 1998.

Beasley, R.W.R., Blow, R.J., Lunau, F.W., and Taylor, B.M., Amyl nitrite and all that, *J. Soc. Occup. Med.*, 28, 142–143, 1978.

Berndt, A., Kerde, Ch., and Prokop, O., Zur Frage der Verwertbarkeit von Cyanidbefunden in Liechenmaterial, *Drsch. Z. Gericht. Med.*, 51, 522–534, 1961.

Bertol, E., Mari, F., Orzalesi, G., and Volpato, I., Combustion products from various kinds of fibers: toxicologic hazards from smoke exposure, *Forens. Sci. Int.*, 22, 111–116, 1983.

Bhattacharya, R., Kumar, P., and Sachan, A.S., Cyanide induced changes in dynamic pulmonary mechanics in rats, *Indian J. Physiol. Pharmacol.*, 38, 281–284, 1994.

Birkby, M.M., Halpin, B.M., Caplan, Y.H., Fisher, R.S., McAllister, J.M., and Dixon, A.M., Fire fatality study, *Fire Mater.*, 3, 211–217, 1979.

Bismuth, C., Cantineau, J.P., Pontal, P., Baud, F., Garnier, R., and Poulos, L., Cyanide poisoning: priority to the symptomatic treatment, *Presse Méd.*, 13, 2493–2497, 1984.

Blake, P.G. and Ijadi-Maghsoodi, S., Kinetics and mechanism of the thermal decomposition of methyl isocyanate, *J. Chem. Kinet.*, 14, 945–952, 1982.

Blanc, P., Hogan, M., Mallin, K., Hydorezuk, D., Hesse, S., and Bernard, B., Cyanide intoxication among silver-reclaiming workers, *J. Am. Med. Assoc.*, 253, 367–371, 1985.

Bondy, S.C. and Komulainen, H., Intracellular calcium as an index of neurotoxic damage, *Toxicology*, 49, 35–41, 1988.

Bonsall, J.L., Survival without sequelae following exposure to 500 mg m^{-3} of hydrogen cyanide, *Hum. Toxicol.*, 3, 57–60, 1984.

Borders, C.L., Jr. and Fridovich, I., A comparison of the effects of cyanide, hydrogen peroxide, and phenylglyoxal on eucaryotic and prokaryotic Cu, Zn superoxide dismutases, *Arch. Biochem. Biophys.*, 241, 472–476, 1985.

Borgohain, R., Singh, A.K., Radhakrishna, H., Rao, V.C., and Mohandas, S., Delayed onset generalized dystonia after cyanide poisoning, *Clin. Neurol. Neurosurg.*, 97, 213–215, 1955.

Borowitz, J.L., Kanthasamy, A., and Isom, G.E., Toxicodynamics of cyanide, in *Chemical Warfare Agents*, Somani, S.M., Ed., Academic Press, San Diego, 1992, pp. 209–236.

Borowitz, J.L., Rathinavelu, A., Kanthasamy, A., Wilsbacher, J., and Isom, G.E., Accumulation of labeled cyanide in neuronal tissue, *Toxicol. Appl. Pharmacol.*, 129, 80–85, 1994.

Borowitz, J.L., Isom, G.E., and Baskin, S.I., Acute and chronic cyanide toxicity, in *Chemical Warfare Agents: Toxicity at Low Levels*, Somani, S.S. and Romano, J.A., Jr., CRC Press, Boca Raton, FL, 2001, pp. 301–320.

Borron, S.W. and Baud, F.J., Acute cyanide poisoning: clinical spectrum, diagnosis, and treatment, *Arh. Hig. Toksikol.*, 47, 307–322, 1996.

Bosses Get 25 Years in Death, *Danbury News-Times*, Jul. 2, 1985.

Boswell, G.W., Brooks, D.E., Murray, A.J., Doye, A.A., Disselhorst, D.J., Chin, C.L., and Clifford, C.B., Exogenous methemoglobin as a cyanide antidote in rats, *Pharm. Res.*, 5, 749–752, 1988.

Bourdoux, P., DeLange, F., Gerard, M., Mafuta, M., Hansen, A., and Ermans, A.M., Evidence that cassava ingestion increases thiocyanate formation: a possible etiologic factor in endemic goiter, *J. Clin. Endocrinol. Metab.*, 46, 613–621, 1978.

Boxer, G.E. and Rickards, J.C., Chemical determination of vitamin B_{12}. IV. Assay of vitamin B_{12} in multivitamin preparations and biological materials, *Arch. Biochem.*, 30, 392–401, 1951.

Boxer, G.E. and Rickards, J.C., Studies on the metabolism of the carbon of cyanide and thiocyanate, *Arch. Biochem.*, 39, 7–26, 1952.

Brahams, D., 'Sudafed capsules' poisoned with cyanide, *Lancet*, 337, 968, 1991.

Brierley, J.B., Brown, A.W., and Calverly, J., Cyanide intoxication in the rat: physiological and neuropathological aspects, *J. Neurol. Neurosurg. Psychiatry*, 39, 129–140, 1976.

Brierley, J.B., Prior, P.F., Calverly, J., and Brown, A.W., Cyanide intoxication in *Macaca mullata.* Physiological and neuropathological aspects, *J. Neurol. Sci.*, 31, 133–157, 1977.

Bright, J.E., Inns, R.H., Tuckwell, N.J., and Marrs, T.C., The effect of storage upon cyanide in blood samples, *Hum. Exp. Toxicol.*, 9, 25–130, 1990.

Bright, J.E. and Marrs, T.C., Effect of p-aminopropiophenone (PAPP), a cyanide antidote, on cyanide given by intravenous infusion, *Hum. Toxicol.*, 6, 133–138, 1987.

Brink, N.G., Kuehl, F.A., and Folkers, K., Vitamin B_{12}: The identification of vitamin B_{12} as a cyano-cobalt coordination complex, *Science*, 112, 354, 1950.

Brivet, F., Delfraissy, J.F., Duche, M., Bertrand, P., and Dormont, J., Acute cyanide poisoning recovery with non-specific supportive therapy, *Intensive Care Med.*, 9, 33–35, 1983.

Brown, K.S. and Robinette, R.R., No simple pattern of inheritance in ability to smell solutions of cyanide, *Nature*, 215, 406–408, 1967.

Brown, W.E., Wood, C.D., and Smith, A.N., Sodium cyanide as a cancer chemotherapeutic agent, *Am. J. Obstet. Gynecol.*, 80, 907–918, 1960.

Brysk, M.M., Lauinger, C., and Ressler, C., Biosynthesis of cyanide from [2-$^{14}C^{15}N$] in *Chromobacterium violaceum, Biochim. Biophys. Acta*, **184**, 583–588, 1969.

Bryson, D.D., Cyanide poisoning, *Lancet*, i, 92, 1978.

Buchanan, I.S., Dhamee, M.S., Griffiths, F.E.D., and Yeoman, W.B., Abnormal fundal appearance in a case of poisoning by a cyanide capsule, *Med. Sci. Law*, 16, 29–32, 1976.

Buchwald, A., Cyanide toxicity, *Ann. Emerg. Med.*, 18, 1257, 1989.

Bunch, A.W. and Knowles, C.J., Cyanide production and degradation during growth of the snow mould fungus, *J. Gen. Microbiol.*, 116, 9–16, 1980.

Burnett, J., Corporate murder verdict may not become trend, say local experts, *Occup. Health Safety*, 54, 22–25, 1985.

Burrows, G.E. and Way, J.L., Cyanide intoxication in sheep: therapeutic value of oxygen or cobalt, *Am. J. Vet. Res.*, 38, 223–227, 1977.

Camerino, P.W. and King, T.E., Studies on cytochrome oxidase. 3. Reaction of cyanide with cytochrome oxidase in soluble and particulate forms, *J. Biol. Chem.*, 241, 970–979, 1966.

Camps, F.E., Robinson, A.E., and Lucas, B.G.B., *Gradwohl's Legal Medicine*, 3rd ed., Wright, Bristol, 1976, p. 648.

Camu, F., Effect of imidazole and cyanide upon rat tissue lipolysis, *Arch. Int. Physiol. Biochem.*, 77, 663–669, 1969.

Caplan, Y.H., Pathology and pathophysiology of the systemic toxicants carbon monoxide and cyanide, in *Pathophysiology of Shock, Anoxia and Ischemia*, Cowley, R.A. and Trump, B.F., Eds., Williams and Wilkins, Baltimore, 1982, pp. 270–279.

Caplan, Y., Thomson, B., Dixon, A., Fisher, R.S., and Halpin, B., Toxicologic factors in fire fatalities—study of 259 cases in Maryland, paper presented at Twenty-ninth Annual Meeting of the American Academy of Forensic Sciences, San Diego, 1977.

Carella, F., Grassi, M.P., and Savoiardo, M., Dystonic-parkinsonian syndrome after cyanide poisoning: clinical and MRI findings. *J. Neurol. Neurosurg. Psychiatry*, **51**, 1345–1348, 1988.

Carmello, S., New contributions to the study of subacute-chronic hydrocyanic acid intoxication in man, *Rass. Med. Ind.*, 24, 254–271, 1955.

Carroll, J.L., Gozal, D., Rector, D.M., Aljadeff, G., and Harper, R.M., Ventral medullary neuronal responses to peripheral chemoreceptor stimulation, *Neuroscience*, 73, 989–998, 1996.

Cassel, G., Estimation of the convulsive effect of cyanide in rats, *Pharmacol. Toxicol.*, 77, 259–263, 1995.
Cassel, G. and Persson, S.A., Effect of acute cyanide intoxication on central catecholaminergic pathways, *Clin. Toxicol.*, 23, 461–462, 1985.
Cassel, G. and Perrson, S.A., Effects of acute lethal cyanide intoxication on central dopaminergic pathways, *Pharmacol. Toxicol.*, 70, 148–151, 1992.
Cassel, G., Karlsson, L., and Sellstrom, A., On the inhibition of glutamic acid decarboxylase and gamma-amino butyric acid transaminase by sodium cyanide, *Pharmacol. Toxicol.*, 69, 238–241, 1991.
Castric, P.A., Hydrogen cyanide, a secondary metabolite of *Pseudomonas aeruginosa, Can. J. Microiol.*, 21, 613–618, 1975.
Castric, P.A., Glycine metabolism by *Pseudomonas aeruginosa*: hydrogen cyanide biosynthesis, *J. Bacteriol.*, 130, 826–831, 1977.
Castric, P.A., The metabolism of hydrogen cyanide by bacteria, in *Cyanide in Biology*, Vennesland, B., Conn, E.E., Knowles, C.J., Westley, J., and Wissing, F., Academic Press, London, 1981, pp. 231–261.
Castric, P.A., Castric, K.F., and Meganathan, R., Factors influencing the termination of cyanogenesis in *Pseudomonas aeruginosa*, in *Cyanide in Biology*, Vennesland, B., Conn, E.E., Knowles, C.J., Westley, J., and Wissing, F., Academic Press, London, 1981, pp. 263–274.
Castric, P.A., Ebert, R.F., and Castric, A.F., The relationship between growth phase and cyanogenesis in *Pseudomonas aeruginosa, Curr. Microbiol.*, 2, 287–292, 1979.
Centers for Disease Control, Cyanide poisonings associated with over-the-counter medication—Washington State, 1991, *J. Am. Med. Assoc.*, 265, 1806–1807, 1991.
Chambers, J. and Reese, C.B., The thermal decomposition of some polyurethane foams, *Br. Polymer J.*, 8, 48–53, 1976.
Chance, B., Spectra and related kinetics of respiratory pigments of homogenized and intact cells, *Nature*, 16, 215–221, 1952.
Chandra, H., Gupta, B.N., Bhargava, S.K., Clerk, S.H., and Mahendra, P.N., Chronic cyanide exposure—a biological and industrial hygiene study, *J. Anal. Toxicol.*, 4, 161–165, 1980.
Chaturvedi, A.K., Endecot, R., Ritter, R.M., and Sanders, D.C., Variations in Time-to-Incapacitation and Blood Cyanide Values for Rats Exposed to Two Hydrogen Cyanide Concentrations, Federal Aviation Administration Report DOT/FAA/AM-93–8, National Technical Information Service, Springfield, VA, 1993.
Chin, R.G. and Calderon, Y., Acute cyanide poisoning: a case report, *J. Emerg. Med.*, 18, 441–445, 2000.
Chisholm, I.A., Bronte-Stewart, J., and Foulds, W.S., Hydroxocobalamin versus cyanocobalamin in the treatment of tobacco amblyopia, *Lancet*, 2, 450–451, 1967.
Christel, D., Eyer, P., Hegemann, P., Kiese, M., Lörcher, W., and Weger, N., Pharmacokinetics of cyanide poisoning of dogs and the effect of 4-dimethylaminophenol or thiosulfate, *Arch. Toxicol.*, 38, 177–189, 1977.
Chung, J. and Wood, J.L., Oxidation of thiocyanate to cyanide catalysed by hemoglobin, *J. Biol. Chem.*, 246, 555–560, 1971.
Clark, C.J., Campbell, D., and Reid, W.H., Blood carboxyhaemoglobin and cyanide levels in fire survivors, *Lancet*, 1, 1332–1335, 1981.
Cliff, J., Lundquist, P., Rosling, H., Sorbo, B., and Wide, L., Thyroid function in cassava-eating population affected by epidemic spastic paraparesis, *Acta Endocrinol.*, 113, 523–528, 1986.
Coleman, D.L., Smoke inhalation, *West. J. Med.*, 135, 300–309, 1981.
Collins, P.A., Rodgers, P.B., and Knowles, C.J., The effect of growth conditions on cyanogenesis by *Chromobacterium Violaceum, J. Gen. Microbiol.*, 117, 73–80, 1980.
Comroe, J.M., *Physiology of Respiration*, Year Book Publishers, Chicago, 1974, pp. 33–54.
Copeland, A.R., Accidental fire deaths. The 5-year Metropolitan Dade County experience from 1979 until 1983, *Z. Rechtsmed.*, 94, 71–79, 1985.
Corporation on Trial in Workplace Death, *Danbury News-Times*, Apr. 15, 1985.
Coughlan, M.P., Johnson, J.L., and Rajagopalan, K.V., Mechanisms of inactivation of molybdoenzymes by cyanide, *J. Biol. Chem.*, 255, 2694–2699, 1980.
Coughlan, M.P., Rajagopalan, K.V., and Handler, P., The role of molybdenum in xanthine oxidase and related enzymes, *J. Biol. Chem.*, 244, 2650–2663, 1969.
Crampton, R.F., Gaunt, I.F., Harris, R., Knowles, J.F., Langman, M.J., Linnel, J.C., Matthews, D.M., Molli, D.I., Pettigrew, A.R., Smith, R.T., Waters, A.H., Wilson, J., and Wise, I.J., Effects of low cobalamin diet and chronic cyanide toxicity in baboons, *Toxicology*, 12, 221–234, 1979.

Crappo, R.O., Smoke-inhalation injuries, *J. Am. Med. Assoc.*, 246, 1694–1696, 1981.
Curry, A.S., Cyanide poisoning, *Acta Pharmacol. Toxicol.*, 20, 291–294, 1963.
Curry, A.S., *Poison Detection in Human Organs*, 3rd ed., Thomas, Springfield, 1976, pp. 97–98.
Curry, A.S., Price, D.E., and Rutter, E.R., The production of cyanide in postmortem material, *Acta Pharmacol. Toxicol.*, 25, 339–334, 1967.
Curry, S.C. and Patrick, H.C., Lack of evidence for a percent saturation gap in cyanide poisoning, *Ann. Emerg. Med.*, 60, 523–528, 1991.
Dahl, A.R., The cyanide-metabolizing enzyme rhodanese in rat nasal respiratory and olfactory mucosa, *Toxicol. Lett.*, 45, 199–203, 1989.
Dally, S. and Gaultier, M., Choc anaphylactique dû à l'hydroxocobalamine, *Nouv. Press. Med.*, 5, 1917, 1976.
Daly, M. DeB. and Scott, M.J., The cardiovascular effects of hypoxia in the dog with special reference to the contribution of the carotid body chemoreceptors, *J. Physiol. (Lond.)*, 173, 201–204, 1964.
Daunderer, M., Fatal smoke inhalation of hydrogen cyanide from smouldering fires, *Fortsch. Med.*, 97, 1401–1405, 1979.
DeBush, R.F. and Seidl, L.G., Attempted suicide by cyanide, *California Med.*, 110, 394–396, 1969.
DeFlora, S., Study of 106 organic and inorganic compounds in the Salmonella/microsome test, *Carcinogenesis*, 2, 283–298, 1981.
DeLorenzo, R.A., Cyanide. The deadly terror weapon that every EMS provider must know about, *J. Emerg. Med. Serv.*, 24, 54–58, 1999.
D'Mello, G.D., Neuropathological and behavioral sequelae of acute cyanide toxicosis in animal species, in *Clinical and Experimental Toxicology of Cyanides*, Ballantyne, B. and Marrs, T.C., Eds., Wright, Bristol, 1987, pp. 127–137.
Doherty, P.A., Ferm V., and Smith, R.P., Congenital malformations induced by infusion of sodium cyanide in the Golden hamster, *Toxicol. Appl. Pharmacol.*, 64, 456–464, 1982.
Donnelly, D.F., Response to cyanide of two types of glomoid cells in mature rat carotid body, *Brain Res.*, 630, 157–168, 1993.
Drasch, G., Meyer, L.V., Kauert, G.U., and Weger, N., Ein Fall einer vaginalen Cyanid-Beibringung, *Arch. J. Krim.*, 170, 21–28, 1982.
Drawbaugh, R.B. and Marrs, T.C., Interspecies differences in rhodanese (thiosulphate sulphur-transferase, E.C. 2.8.1.1) activity in liver, kidney and plasma, *Comp. Biochem. Physiol.*, 86B, 307–310, 1987.
Drinker, P., Hydrocyanic gas poisoning by absorption through the skin, *J. Ind. Hyg.*, 14, 1–2, 1932.
DuBois, K.P. and Geiling, E.M.K., *Textbook of Toxicology,* Oxford University Press, New York, 1959, p. 68.
Dudley, H.C., Sweeney, T.R., and Miller, J.W., Toxicology of acrylonitrile (vinyl cyanide). II. Studies of effects of daily inhalation, *J. Ind. Hyg. Toxicol.*, 24, 255–258, 1942.
Dugard, P.H., The absorption of cyanide through human skin *in vitro* from solutions of sodium cyanide and gaseous HCN, in *Clinical and Experimental Toxicology of Cyanides*, Ballantyne, B. and Marrs, T.C., Eds., Wright, Bristol, 1987, pp. 127–137.
Dunea, G., Death over the counter, *Br. Med. J.*, 286, 211–212, 1983.
Edwards, A.C. and Thomas, I.D., Cyanide poisoning, *Lancet*, i, 92–93, 1978.
Editorial, Cyanide antidotes and fire toxicology, *New Engl. J. Med.*, 325, 1801–1802, 1991.
Egekeze, J.O. and Oehme, F.W., Cyanides and their toxicity; a literature review, *Vet. Q.*, 2, 104–114, 1980.
El Ghawabi, S.H., Gaffar, M.A., E-Sabarti, A.A., Almed, S.H., Malash, K.K., and Faves, R., Chronic cyanide exposure: a clinical, radioisotope and laboratory study, *Br. J. Ind. Med.*, 32, 215–219, 1975.
Enander, I., Sundwall, A., and Sorbo, B., Metabolic studies on N-methylpyridinium-2-aldoxime. 1. The conversion to thiocyanate, *Biochem. Pharmacol.*, 7, 226–231, 1961.
EPA (Environmental Protection Agency), Health Effects Assessment for Cyanide, U.S. Environmental Protection Agency, Publication PB86-134228, Washington, DC, 1984.
Epstein, J., Estimation of microquantities of cyanide, *Anal. Chem.*, 19, 272–274, 1947.
Ermans, A.M., Delange, F., and Van der Velden, M., Possible role of cyanide and thiocyanate in the etiology of endemic cretinism, *Adv. Exp. Med. Biol.*, 30, 455–486, 1972.
Estler, C.J., Metabolic alterations in the brain during the course of non-lethal potassium cyanide poisoning, and the influence of cyanide antagonists, *Naunyn-Schmeidebergs Arch Pharmacol.*, 251, 413–432, 1965.
Eungprabhanth, V., Suicide in Thailand, *Forens. Sci.*, 5, 43–51, 1975.
Fairley, A., Linton, E.C., and Wild, F.E., The absorption of hydrocyanic vapour through the skin, *J. Hyg.*, 34, 283–294, 1934.

Feeney, J., Burgen, A.S.V., and Greel, F., Cyanide binding to carbonic anhydrase, a ^{13}C nuclear magnetic resonance study, *Eur. J. Biochem.*, 34, 107–111, 1973.

Feldman, J.M. and Feldman, D.D., Sequelae of attempted suicide by cyanide ingestion; a case report, *Int. J. Psychiat. Med.*, 20, 173–179, 1990.

Feldstein, M.A. and Klendshoj, W.J., The determination of cyanide in biologic fluids by microdiffusion analysis, *J. Lab. Clin. Med.*, 44, 166–170, 1954.

Fenney, J. and Brugen, A.S.V., Cyanide binding to carbonic anhydrase, *Eur. J. Biochem.*, 34, 107–111, 1973.

Fernando, G.C.A. and Busuttil, A., Cyanide ingestion—case studies of four suicides, *Am. J. Forens. Med. Pathol.*, 12, 241–246, 1991.

Finelli, P.F., Changes in the basal ganglia following cyanide poisoning, *J. Comput. Assist. Tomog.*, 5, 755–756, 1981.

Fiori, A. and Marigo, M., Fatal cyanide poisoning in a child, *Arch. Toxicol.*, 20, 21–27, 1963.

Fitzgerald, L.R., Effect of injected sodium cyanide on newborn and adult mice, *Am. J. Physiol.*, 17, 60–62, 1954.

Fligner, C.L., Luthi, R., Linkaiyte-Weiss, E., Stimac, G.K., Riasys, V.A., and Reay, D.T., Cyanide poisoning from capsule tampering: clinical, pathologic and toxicological aspects, in Proceedings, 39th Annual Meeting, American Academy of Forensic Sciences, 1987, p. 106.

Forrest, A.R.W., Galloway, J.H., and Slater, D.N., The cyanide poisoning necropsy: an appraisal of risk factors, *J. Clin. Pathol.*, 45, 544–545, 1992.

Frankenberg, L. and Sörbo, B., Effect of cyanide antidotes on the metabolic conversion of cyanide to thiocyanate, *Arch. Toxicol.*, 3, 81–89, 1975.

Friedberg, K.D. and Schwarzkopf, H.A., Blausäureexhalation bei der Canidvergiftung, *Arch. Toxicol.*, 24, 235–248, 1969.

Fukayama, H., Nasu, M., and Murakami, S., Examination of antithyroid effects of smoking products in cultured thyroid follicles: only thiocyanate is a potent antithyroid agent, *Acta Endocrinol.*, 127, 520–525, 1992.

Fukumoto, Y., Nakajimo, H., Uetake, M., Matsuyama, A., and Yoshida, T., A study on the sense of smell with respect to potassium cyanide solution and its hereditary transmission, *Jpn. J. Hum. Genet.*, 2, 7–16, 1957.

Funata, N., Song, S-Y., Funata, M., and Hagishino, F., A study of experimental cyanide encephalopathy in the acute phase—physiological and neuropathological correlation, *Acta Neuropathol.*, 64, 99–107, 1984.

Ganote, C.E., Worstell, J., and Kaltenlach, J.P., Oxygen-induced enzyme release after irreversible myocardial injury, *Am. J. Pathol.*, 84, 270–350, 1976.

Garberg, P., Äkerblom, E.-L., and Bolcsfoldi, G., Evaluation of a genotoxicity test measuring DNA-strand breaks in mouse lymphoma cells by alkaline unwinding and hydroxyapatite elution, *Mutat. Res.*, 203, 155–176, 1988.

Gee, D.J., Cyanides in murder, suicide and accident, in *Clinical and Experimental Toxicology of Cyanides*, Ballantyne, B. and Marrs, T.C., Eds., Wright, Bristol, 1987, pp. 209–216.

Gettler, A.O. and Baines, J.O., The toxicology of cyanide, *Am. J. Med. Sci.*, 195, 182–198, 1933.

Gettler, A.O. and St. George, A.V., Cyanide poisoning, *Am. J. Clin. Pathol.*, 4, 429, 1934.

Goenechea, S., Cyanide losses in samples of stored blood, *Z. Rechtsmed.*, 88, 9–101, 1982.

Goldstein, F., The colorimetric determination of thiocyanate in whole blood, *J. Biol. Chem.*, 187, 523–527, 1950.

Goldstein, F. and Rieders, F., Formation of cyanide in dog and man following administration of thiocyanate, *Am. J. Physiol.,* 167, 47–50, 1951.

Goldstein, F. and Rieders, F., Conversion of thiocyanate to cyanide by an erythrocyte enzyme, *Am. J. Physiol.*, 173, 287–290, 1953.

Gonzalez, E.R., Cyanide evades some noses, overpowers others, *J. Am. Med. Assoc.*, 248, 2211, 1982.

Goodhart, G.L., Patient treated with antidote kit and hyperbaric oxygen survives cyanide poisoning, *South. Med. J.*, 87, 814–816, 1994.

Gorman, D.F., Problems and pitfalls in the use of hyperbaric oxygen for the treatment of poisoned patients, *Med. Toxicol. Adverse Drug Exp.*, 4, 393–399, 1989.

Graham, D.L., Laman, D., Theodore, J., and Robin, E.D., Acute cyanide poisoning complicated by lactic acidosis and pulmonary edema, *Arch. Int. Med.*, 137, 1051–1655, 1977.

Grandas, F., Artieda, J., and Obeso, J.A., Clinical and CT scan findings in a case of cyanide intoxication, *Mov. Disorders*, 4, 188–193, 1989.

Green, V. and Li, H., Synthetic nitrogen products, in *Riegel's Handbook of Industrial Chemistry*, 8th ed., Kent, J.A., Ed., Von Nostrand Reinhold, New York, 1983, pp. 143–211.

Guatelli, M.A., The toxicology of cyanides, in *Methods of Forensic Cyanides*, Vol. 3, Curry, A.S., Ed., Academic Press, New York, 1964, pp. 233–265.

Gunaseker, P.G., Ramesh, G.T., Borowitz, J.L., and Isom, G.E., Role of cytochrome c in cyanide-induced aptotic cell death, *Toxicologist*, 54, 116, 2000.

Gunasekar, P.G., Sun, P.W., Kanthasamy, A.G., Borowitz, J.L., and Isom, G.E., Cyanide-induced neurotoxicity involves nitric oxide and reactive oxygen species generation after N-methyl-D-aspartate receptor activation, *J. Pharmacol. Exp. Ther.*, 277, 150–155, 1996.

Gunaseker, P.G., Yan, S., Borowitz, J.L. and Isom, G.E., Involvement of caspase-3-like activity in cyanide-induced neuronal aptosis, *Toxicologist*, 48, 87, 1999.

Haber, F., *Funf Vortrage aus den Jahren 1920–1923*, Springer-Verlag, Berlin, 1924.

Hall, A.H. and Rumack, B.H., Clinical toxicology of cyanide, *Ann. Emerg. Med.*, 15, 1067–1074, 1986.

Hansen, B.A. and Dekker, E.E., Inactivation of bovine liver 2-keto-4-hydroxyglutarate aldolase by cyanide in the presence of aldehydes, *Biochemistry*, 15, 2912–2917, 1976.

Harland, W.H. and Woolley, W.D., Fire fatality study—University of Glasgow, Building Research Establishment Information Paper 18/79, Fire Research Station, Borehamwood, U.K., 1979.

Harris, R. and Paxman, J., *A Higher Form of Killing*, Chatto and Windus, London, 1982, p. 199.

Hart, G.B., Strauss, M.B., Lennon, P.A., and Whitcraft, D.D., Treatment of smoke inhalation by hyperbaric oxygen, *J. Emerg. Med.*, 3, 211–215, 1985.

Hartung, R., Cyanides and nitriles, in *Patty's Industrial Hygiene and Toxicology*, Vol. IIC, 3rd ed., John Wiley and Sons, New York, 1982, pp. 4845–4900.

Hathaway, G.J., Proctor, N.H., and Hughes, J.P., *Chemical Hazards of the Workplace*, 4th ed., Van Nostrand Reinhold, New York, 1996, pp. 346–347.

Haxhiu, M.A., Erokwu, B., Van Lunteren, E., Cherniack, N.S., and Strohl, K.P., Central and spinal effects of sodium cyanide on respiratory activity, *J. Appl. Physiol.*, 74, 574–579, 1993.

Hayase, K., Yomekawa, G., and Yoshida, A., Effects of thyroid hormone on turnover of tissue proteins in rats, *Nutr. Rep. Int.*, 35, 393–414, 1987.

Hayes, W.T., The 90-dose LD_{50} and a chronicity factor as measures of toxicity, *Toxicol. Appl. Pharmacol.*, 11, 327–335, 1967.

Haymaker, W., Ginzler, A.M., and Ferguson, R.L., Residual neuropathological effects of cyanide poisoning, *Mil. Surgeon*, 111, 231–246, 1952.

Hébert, C.D., NTP Technical Report on Toxicity Studies of Sodium Cyanide (CAS 143-33-9). Administered in drinking water to F344/N rats and B6C3F1 mice. NIH Publication 94–3386, Nov. 1993, National Institutes of Health, National Toxicology Program, Research Triangle Park, NC, 1993.

Herbert, V., Drugs effective in megaloblastic anemias, in *The Pharmacological Basis Therapeutics*, 5th ed., Goodman, L.S. and Gilman, A., McMillan, New York, 1975, p. 1332.

Herrting, G., Kraupp, O., Schnetz, H., and Wieketich, S., Untersuchungen über die Folgen einer chronischen Verabreichung akut toxischer Dosen von Natriumcyanid an Huden, *Acta Pharmcol. Toxicol.*, 17, 27–42, 1960.

Higgins, E.A., Floea, V., Thomas, A.A., and Davis, H.V., Acute toxicity of brief exposures to HF, HCl, NO_2 and HCN with and without CO, *Fire Technol.*, 2, 120–130, 1972.

Hilado, C.J. and Cumming, H.J., A review of available LC50 data, *J. Combust. Toxicol.*, 4, 415–424, 1977.

Hilmann, B., Bardham, K.D., and Bain, J.T.B., The use of dicobalt edetate (Kelocyanor) in cyanide poisoning, *Postgrad. Med. J.*, 50, 171–174, 1974.

Himwich, W.A. and Saunders, J.P., Enzymic conversion of cyanide to thiocyanate, *Am. J. Physiol.*, 153, 348–354, 1948.

Hirano, A., Levine, S., and Zimmerman, H.M., Experimental cyanide encephalopathy. Electron microscopic observations of early lesions of white matter, *J. Neuropathol. Exp. Neurol.*, 26, 200–213, 1967.

Holland, D.J., Cyanide poisoning: an uncommon encounter, *J. Emerg. Nurs.*, 9, 138–140, 1983.

Holland, M.A. and Kozlowski, I.M., Clinical features and management of cyanide poisoning, *Clin. Pharm.*, 5, 737–741, 1986.

Holland, P. and Parkes, D.C., Plasma concentrations of the oxime Pralidoxime Mesylate (P2S) after repeated oral and intramuscular administration, *Br. J. Ind. Med.*, 33, 43–46, 1976.

Holzer, J.F., Mord durch Einspritzung von Blausäure in die Weiblichen Geschlechtsteile, *Dtsch. Z. Gericht. Med.*, 23, 245–249, 1939.

Homan, E.R., Reactions, processes and materials with potential for cyanide exposure, in *Clinical and Experimental Toxicology of Cyanides*, Ballantyne, B. and Marrs, T.C., Eds., Wright, Bristol, 1987, pp. 1–21.

Houeto, P., Borron, S.W., Sandouk, P., Imbert, M., Levillain, P., and Baud, F.J., Pharmacokinetics of hydroxocobalamin in smoke inhalation victims, *Clin. Toxicol.*, 34, 397–404, 1996.

Howard, J.W. and Hanzal, R.F., Chronic toxicity for rats of food treated with hydrogen cyanide, *J. Agric. Food Chem.*, 3, 325–329, 1955.

Howlett, W.P., Brubakar, G.R., Mlingi, N., and Rosling, H., Konzo, an epidemic upper motor disease studied in Tanzania, *Brain,* 113, 223–236, 1990.

Hoyer, S., The effect of naftidroturyl on cyanide induced hypoxic damage to glucose and energy metabolism in brain cortex of rats, *Arzneim.-Forsch.*, 34, 412–416, 1984.

Humbert, J.R., Tress, J.H., and Braico, K.T., Fatal cyanide poisoning from accidental ingestion of amygdalin, *J. Am. Med. Assoc.*, 238, 482, 1977.

Hume, A.S., Mozingo, J.R., McIntyre, B., and Ing, K.H., Antidotal efficacy of alpha-ketoglutaric acid and sodium thiosulfate in cyanide poisoning, *Clin.Toxicol.*, 33, 721–724, 1995.

Huser, H.J., Moor-Jankowski, J.K., Truog, G., and Geiger, M., Clinical, genetic and coagulation-physiologic aspects of Hemophilia B in the bleeders of Tenna, with a contribution on the genetics of the coagulation factors, *Acta Genet.*, 8, 209–234, 1958.

Ibebunjo, C., Kamalu, B.P., and Ihemelandu, E.C., Comparison of the effects of cassava (*Manihot esculenta* Cratntz) organic cyanide and inorganic cyanide on muscle and bone development in a Nigerian breed of dog, *Br. J. Nutr.*, 68, 483–491, 1992.

Ifshin, S., Smoke control during fires in high-rise buildings, in *Physiologic and Toxicologic Aspects of Combustion Products*, National Research Council, National Academy of Science, Washington, DC, 1977, pp. 1–17.

Isom, G.E., Liu, D.H.W., and Way, J.L., Effects of sublethal doses of cyanide on glucose catabolism, *Biochem. Pharmacol.*, 24, 871–875, 1975.

Itskovitz, J. and Rudolph, A.M., Cardiorespiratory response to cyanide of arterial chemoreceptors in fetal lambs, *Am. J. Physiol.*, 252, H916–H922, 1987.

Jakob, A. and Diem, S., Activation of glycogenolysis in perfused rat livers by glucagons and metabolic inhibitors, *Biochim. Biophys. Acta*, 362, 469–470, 1974.

Jellinek, H.H.G. and Takada, K., Toxic gas evolution from polymers: evolution of hydrogen cyanide from polyurethanes, *J. Polym. Sci.*, 15, 2269–2288, 1977.

Jenks, W.R., Cyanides (HCN), in *Kirk-Othmer Encyclopedia of Chemical Technology*, Vol. 7, 3rd ed., Wiley, New York, 1983a, pp. 307–319.

Jenks, W.R., Cyanides (alkali metals), in *Kirk-Othmer Encyclopedia of Chemical Technology*, Vol. 7, 3rd ed., Wiley, New York, 1983b, pp. 320–331.

Jetter, W.W., In *Pathology*, Vol. 1. Anderson, W.A.D., Ed., Mosby, St. Louis, 1966, p. 148.

Jiang, S., Lin, Z., and Zhuang, X., Effect of procaine hydrochloride on cyanide intoxication and its effect on neuronal calcium in mice, *Toxicol. Appl. Pharmacol.*, 10, 32–36, 1998.

Johnson, J.D. and Isom, G.E., The oxidative disposition of potassium cyanide in mice, *Toxicology*, 37, 215–224, 1985.

Johnson, J.D., Meisenheimer, T.L., and Isom, G.E., Cyanide-induced neurotoxicity: role of neuronal calcium, *Toxicol. Appl. Pharmacol.*, 4, 64–46, 1986.

Johnson, J.D., Conroy, W.G., Burris, K.D., and Isom, G.D., Peroxidation of brain lipids following cyanide intoxication in mice, *Toxicology*, 46, 21–28, 1987.

Johnson, R.P. and Mellors, J.W., Arteriolization of venous blood gases: a clue to the diagnosis of cyanide poisoning, *J. Emerg. Med.*, **6**, 401–404, 1988.

Jones, M.G., Bickar, D., Wilson, M.T., Brunori, M., Colosimi, A., and Sarti, P., A re-examination of the reactions of cyanide with cytochrome c oxidase, *Biochem. J.*, 220, 57–66, 1984.

Kahler, R.L., Goldblatt, A., and Braunwald, E., The effects of acute hypoxia on the systemic venous and arterial systems and on myocardial contractile force, *J. Clin. Investig.*, 41, 1553–1563, 1962.

Kales, S.N., Dinklage, D., Dickey, J., and Golman, R.H., Paranoid psychosis after exposure to cyanide, *Arch. Environ. Health*, 52, 245–246, 1997.

Kamalu, B.P. and Agharanya, J.C., The effect of a nutritionally-balanced cassava (*Manihot esculenta Crantz*) diet on endocrine function using the dog as a model. 2. Thyroid, *Br. J. Nutr.*, 65, 373–379, 1991.

Kanthasamy, A.G., Borowitz, J.L., and Isom, G.E., Cyanide-induced increases in plasma catecholamines: relationship to acute toxicity, *Neurotoxicology*, 12, 777–784, 1991.

Kanthasamy, A.G., Borowitz, J.L., Pavlakovic, G., and Isom, G.E., Dopaminergic neurotoxicity of cyanide: neurochemical, histological, and behavioral characterization, *Toxicol. Appl. Pharmacol.*, 126, 156–163, 1994a.

Kanthasamy, A.G., Rathinavelli, A., Borowitz, J.L., and Isom, G.E., Interaction of cyanide with a dopamine metabolite: formation of a cyanohydrin adduct and its implications for cyanide-induced neurotoxicity, *Neurotoxicology*, 15, 887–896, 1994b.

Kaplan, H.L., Grand, A.F., Rogers, W.R., Switzer, W.G., and Hartzell, G.E., A Research Study of the Assessment of Escape Impairment By Irritant Combustion Gases in Postcrash Aircraft Fires, Technical Report DOT/FAA/CT-84/16, U.S. Department of Transportation, Federal Aviation Administration, Washington, DC, 1984.

Katsuma, Y., Sato, K., Oya, M., Susuki, O., and Yoshino, M. (1980). Kinetic analysis of the shift of aerobic metabolism in rats during acute cyanide poisoning, *Life Sci.*, 27, 1509–1512.

Kiese, M., *Methemoglobinemia: A Comprehensive Treatise*, CRC Press, Boca Raton, FL, 1974, p. 80.

Kirk, M.A., Gerace, R., and Kulig, K.W., Cyanide and methemoglobin kinetic in smoke inhalation victims treated with the cyanide antidote kit, *Ann. Emerg. Med.*, 22, 1413–1418, 1993.

Kirk, R.L. and Stenhouse, N.S., Ability to smell solutions of potassium cyanide, *Nature*, 171, 698, 1953.

Kiuchi, Y., Inagaki, M., Izumi, J., Matsumoto, M., Yamazai, Y., and Oguchi, K., Effects of local cyanide perfusion on rat striatal extracellular dopamine and its metabolites as studied by in vivo brain microdialysis, *Neurosci. Lett.*, 147, 193–196, 1992.

Klaasen, C.D., Nonmetallic environmental toxicants: Air pollutants, solvents and vapors, and pesticides, in *The Pharmacological Basis of Therapeutics*, 6th ed., Gilman, A.G., Goodman, L.S., and Gilman, A., Macmillan Publishing Company, Inc., New York, 1980, pp. 1651–1652.

Klimmek, R., Fladerer, M., and Weger, N., Circulation, respiration, and blood homeostasis of dogs during slow cyanide poisoning and after treatment with 4-dimethylaminophenol or cobalt compounds, *Arch. Toxicol.*, 43, 121–133, 1979.

Knowles, C.J., Micro-organisms and cyanide, *Bacteriol. Rev.*, 40, 652–680, 1970.

Korner, P.L., Circulatory adaptations in hypoxia, *Physiol. Rev.*, 39, 687–730, 1959.

Krasney, J.A., Efficient components of the cardioaccelerator responses to oxygen lack and cyanide, *Am. J. Physiol.*, 213, 1475–1479, 1967.

Krasney, J.A., Effects of sino-aortic denervation on regional circulatory responses to cyanide, *Am. J. Physiol.*, 218, 56–63, 1970.

Krasney, J.A., Cardiovascular responses to cyanide in awake sino-aortic denervated dogs, *Am. J. Physiol.*, 220, 1361–1366, 1971.

Krasney, J.A., Hogan, P.M., Lowe, R.F., and Youmans, W.B., Comparison of the effects of hypoxia and cyanide on cardiac rate in spinal dogs, *Fed. Proc.*, 25, 2, 1966.

Kremer, M.L., Inhibition of catalase by cyanide, *Israel J. Chem.*, 8, 79–87, 1970.

Kulig, W.K. and Ballantyne, B., Cyanide toxicity, *Am. Fam. Phys.*, 48, 107–114, 1993.

Kulling, P., Hospital treatment of victims exposed to combustion products, *Toxicol Lett.*, 64/65, 283–289, 1992.

Kumar, P., Das, M., and Kumar, A., Health status of workers engaged in heat treatment (case hardening) plant and electroplating at cyanide bath, *Ind. J. Environ. Protect.*, 12, 179–183, 1992.

Kushi, A., Matsumoto, T., and Youmans, W.B., Mutagen from the gaseous phase of protein hydrolysate, *Agric. Biol. Chem.*, 47, 1979–1982, 1983.

Lafferty, M.A. and Garrett, R.H., Purification and properties of the neurospora crassa assimilatory nitrite reductase, *J. Biol. Chem.*, 249, 7555–5757, 1974.

Lafin, S.M., Lucchesi, M., Fedak, M., and Barone, J.A., A need to revise the cyanide antidote package instructions, *J.Emerg. Med.*, 10, 623, 1992.

Lambert, S.W., Poisoning by hydrocyanic acid, with special reference to its effects on the brain, *Neurol. Bull.*, 2, 93–105, 1919.

Landhal, M.D. and Herrmann, R.G., Retention of vapours and gases in the human nose and lung, *Arch. Ind. Hyg. Occup. Med.*, 1, 36–45, 1950.

Lang, K., Di Rhodnbildung im Tierkörper, *Biochem. Z.*, 259, 243–256, 1933.

Lasch, E.E. and Shawa, R.E., Multiple cases of cyanide poisoning by apricot kernels in children from Gaza, *Pediatrics*, 68, 5–7, 1981.

Lee-Jones, M., Bennett, M.A., and Sherwell, J.M., Cyanide self-poisoning, *Br. Med. J.*, 2, 780–781, 1970.

Leimdorfer, A., About anoxia of the heart produced by intravenous sodium cyanide injections, *Arch. Int. Pharmacodyn.*, 84, 181–188, 1950.

Lemberg, M.R., Cytochrome oxidase, *Physiol. Rev.*, 49, 48–111, 1996.

Lendle, L., Wirkungsbedingungen von Blausäure und Schwefelwasserstoff und Möglichkelten der Vergiftungsbehandlung, *Jpn. J. Pharmacol.*, 14, 215–224, 1964.

Lessell, S., Experimental cyanide optic neuropathy, *Arch. Ophthalmol.*, 86, 194–204, 1971.

Leung, P., Cannon, E.P., Petrikovics, I., Hawkins, A., and Way, J.L. (1991). *In vivo* studies on rhodanese encapsulation in mouse carrier erythrocytes. *Toxicol. Appl. Pharmacol.*, 110, 268–274.

Leuschner, J., Winkler, A., and Leuschner, F., Toxicokinetic aspects of chronic cyanide exposure in the rat, *Toxicol. Lett.*, 57, 195–201, 1991.

Levin, B.C., Gurman, J.L., Paabo, M., Barer M., and Holt, T., Toxicological aspects of pure and mixed fire gases for various exposure times, *Toxicologist*, 7, 201, 1987.

Levine, M.S. and Radford, E.P., Occupational exposures to cyanide in Baltimore fire fighters, *J. Occup. Med.*, 20, 53–56, 1978.

Levine, S., Experimental cyanide encephalopathy. Gradients of susceptibility in the corpus callosum, *J. Neuropathol. Exp. Neurol.*, 26, 214–222, 1967.

Levine, S., Nonperipheral chemoreceptor stimulation of ventilation by cyanide, *Toxicologist*, 7, 201, 1975.

Levine, S. and Stypulkowski, W., Experimental cyanide encephalopathy, *Arch. Pathol.*, 6, 306–323, 1959.

Levine, S. and Wenk, E.J., Cyanide encephalopathy produced by intravenous route, *J. Nerv. Mental Dis.*, 129, 302–305, 1959.

Lewis, R.J., Sr., *Hawley's Condensed Chemical Dictionary*, 12th ed., Van Nostrand Reinhold, New York, 1993, pp. 613–614.

Li, E.P., Suicide among chemists, *Arch. Environ. Health*, 19, 518–520, 1969.

Li, P.P. and White, T.D., Rapid effects of veratridine, tetrodotoxin, garamicidin D, valinomycin, and NaCN on the Na^+, K^+ and ATP contents of synaptosomes, *J. Neurochem.*, 28, 967–975, 1977.

Limmek, R., Fladerer, H., and Weger, N., Circulation, respiration, and blood homeostasis in cyanide-poisoned dogs after treatment with 4-dimethylaminophenol or cobalt compounds, *Arch. Toxicol.*, 43, 121–133, 1979.

Litovitz, T., The use of oxygen in the treatment of acute cyanide poisoning, in *Clinical and Experimental Toxicology of Cyanides*, Wright, Bristol, 1987, pp. 457–472.

Lovatt Evans, C., Cobalt compounds as antidotes for hydrocyanic acid, *Br. J. Pharmacol.*, 23, 455–475, 1964.

Lundquist, P., Roslig, H., and Sorbo, B., The origin of hydrogen cyanide in breath, *Arch. Toxicol.*, 61, 270–274, 1988.

Lundquist, P., Kagedal, B., Nilsson, L., and Rosling, H., Analysis of the cyanide metabolite 2-aminothazoline-4-carboxlic acid in urine by high-performance liquid chromatography, *Anal. Biochem.*, 22, 1–8, 1995.

MacMillan, V.H., Cerebral energy metabolism in cyanide encephalopathy, *J. Cerebral Blood Flow Metab.*, 9, 156–162, 1989.

Maduh, E.U., Porter, D.W., and Baskin, S.I., Calcium antagonists. A role in the management of cyanide poisoning, *Drug Saf.*, 9, 237–248, 1993.

Maduh, E.U., Nealley, E.W., Song, H., Wang, P.C., and Baskin, S.I., A protein kinase C inhibitor attenuates cyanide toxicity in vivo, *Toxicology*, 100, 129–137, 1995.

Marrs, T.C., The choice of cyanide antidotes, in *Clinical and Experimental Toxicology of Cyanides*, Ballantyne, B., and Marrs, T.C., Eds., Wright, Bristol, 1987, pp. 383–401.

Marrs, T.C., Antidotal treatment of acute cyanide poisoning, *Adverse Drug React. Acute Poisoning Rev.*, 4, 17–206, 1988.

Marrs, T.C. and Ballantyne, B., Clinical and experimental toxicology of cyanides: an overview, in *Clinical and Experimental Toxicology of Cyanides*, Ballantyne, B. and Marrs, T.C., Eds., Wright, Bristol, 1987, pp. 473–493.

Marrs, T.C., Bright, J.E., and Swanston, D.W., The effect of prior treatment with 4-dimethyaminophenol (DMAP) on animals experimentally poisoned with hydrogen cyanide, *Arch. Toxicol.*, 51, 247–253, 1982.

Marrs, T.C., Bright, J.E., and Swanston, D.W., 4-Dimethylaminophenol and dicobalt edetate (Kelocyanor) in the treatment of experimental cyanide poisoning, *Hum.Toxicol.*, 4, 591–400, 1985.

Marsho, T.V. and Kung, S.D., Oxygenase properties of crystallized fraction I protein from tobacco, *Arch. Biochem. Biophys.*, 173, 341–346, 1976.

Massey, V. and Edmondron, D., On the mechanism of inactivation of xanthine oxidase by cyanide, *J. Biol. Chem.*, 45, 6595–6598, 1970.

Mathangi, C. and Namasivayam, A., Effect of chronic cyanide intoxication on memory in albino rats, *Food Chem. Toxicol.*, 38, 51–55, 2000.

Matijak-Schaper, M. and Alarie, Y., Toxicity of carbon monoxide, hydrogen cyanide and low oxygen, *J. Combust. Toxicol.*, 9, 21–61, 1982.

Matsubara, K., Akane, A., Maseda, C., and Shiono, H., "First pass phenomenon" of inhaled gas in the fire victims, *Forens. Sci. Int.*, 46, 203–208, 1990.

Matsukura, T. and Fukui, M., Report on medicolegal data from the mass investigations performed by the Medico-Legal Society of Japan (IV) (1967) on the death caused by cyanide poisoning, *Jpn. J. Legal Med.*, 22, 219–222, 1968.

Mayes, R.W., The toxicological examination of the victims of the British Air Tours Boeing 737 accident at Manchester in 1985, *J. Forens. Sci.*, 36, 179–184, 1991.

McMillan, D.E. and Svoboda, A.C., The role of erythrocytes in cyanide detoxification, *J. Pharmacol. Exp. Ther.*, 21, 37–41, 1982.

McNamara, B.P., Estimation of the Toxicity of Hydrocyanic Acid Vapors in Man, Edgewood Arsenal Technical Report EB-TR-76023, U.S. Department of the Army, 1976.

Mégarbane, B. and Baud, F., Cyanide poisoning: diagnosis and antidote choice in an emergency situation, *J. Toxicol., Clin. Toxicol.*, 41, 438–439, 2003.

Meganathan, R. and Castric, P.A., The effect of inorganic phosphate on cyanogenesis by *Pseudomonas aeruginosa, Arch. Microbiol.*, 114, 51–54, 1977.

Meredith, T.J., Jacobsen, D., Haines, J.A., Berger, J-C., and van Heijst, A.N.P., *Antidotes for Cyanide Poisoning*, Vol. 2, International Program of Chemical Safety/Commission of the European Communities Evaluation of Antidote Series, Cambridge University Press, Cambridge, 1993.

Messing, B., Extrapyramidal disturbances after cyanide poisoning (first MRT-investigation of the brain), *J. Neural Transm.*, 33, 141–147, 1991.

Michaels, R., Haniges, H.V., and Corpe, W.A., Cyanide formation from glycine by non-proliferating cells of *Pseudomonas aeruginosa, Arch. Biochem.,* 111, 121–125, 1965.

Mitra, J., Dev, N.B., Trivedi, R., Amini, S., Ernsberger, P., and Cherniak, N.S., Intramedullary sodium cyanide injection on respiratory and vasomotor responses in cats, *Respir. Physiol.*, 93, 71–82, 1993.

Moertol, C.G., Femig, T.R., Rubin, J., Kvols, L.K., Sarna, G., Koch, R., Currie, V.L., Young, C.W., Jones, S.E., and Davignon, J.P., A clinical trial of amygdalin (laetrile) in the treatment of human cancer, *N. Engl. J. Med.*, 306, 201–206, 1982.

Mohler, S.R., Air crash survival: injuries and evaluation of toxic hazards, *Aviat. Environ. Space Med.*, January, 86–88, 1975.

Monekossa, G.L. and Annan, W.G.T., Clinical epidemiological observations on an ataxic syndrome in eastern Nigeria, *Trop. Geogr. Med.*, 4, 316–323, 1964.

Montgomery, R.D., Cyanogens, in *Toxic Constituents of Plant Foodstuffs*, Leiner, I.E., Ed., Academic Press, New York, 1969, pp. 143–157.

Moore, S. and Gates, N., (1946). Cited by McNamara (1976).

Moore, S.J., Norris, J.C., Ing, K.H., and Hume, A.S., The efficacy of alpha-ketoglutaric acid in the antagonism of cyanide intoxication, *Toxicol. Appl. Pharmacol.*, 81, 265–273, 1986.

Morgan, R.L., Isom, G.E., and Way, J.L., Reduction of thiosulfate intererence in cyanide determinations, *Toxicol. Appl. Pharmacol.*, 50, 323–328, 1979.

Morgan, R.L. and Way, J.L., Cyanide determination in biologic fluids during therapy, in *Abstracts, International Congress on Toxicology*, Toronto, March 1977, p. 37.

Morikowa, T., Evolution of hydrogen cyanide during combustion and pyrolysis, *J. Combust. Toxicol.*, 5, 315–330, 1978.

Nakatani, T., Kosugi, Y., Mori, A., Tajimi, K., and Kobayashi, K., Changes in the metabolism of oxygen metabolism in a clinical course recovering from potassium cyanide, *Am. J. Emerg. Med.*, 11, 213–217, 1993.

Narty, F., Toxicological aspects of cyanogens in tropical foodstuffs, in *Toxicology in the Tropics*, Smith, R.L. and Bababunumi, E.A., Eds., Taylor and Francis, London, 1980, pp. 53–73.

Naughton, M., Acute cyanide poisoning, *Anesth. Intensive Care*, 4, 351–356, 1974.

Nicholls, P., Van Burren, K.J.H., and Van Gelder, B.F., Biochemical and biophysical studies on the cytochrome aa_3. Binding of cyanide to cytochrome aa_3, *Biochim. Biophys. Acta,* 275, 279–287, 1972.

Nicholson, P.J. and Vincenti, G.E.P., A case of phobic anxiety related to the inability to smell cyanide, *Occup. Med.*, 4, 107–108, 1994.

Nieminen, A-L., Gores, G.J., Wray, B.E., Tanaka, Y., and Herman, B., Calcium dependence of bleb formation and cell death in hepatocytes, *Cell Calcium*, 9, 237–246, 1988.

Noguchi, T.T., Eng, J.J., and Klatt, E.C., Significance of cyanide in medicolegal investigations involving fires, *Am. J. Forens. Med. Pathol.*, 9, 304–309, 1988.

Nolte, K.B. and Dasgupta, A., Prevention of occupational cyanide exposure in autopsy prosectors, *J. Forens. Sci.*, 41, 146–148, 1996.

Norris, J.C. and Ballantyne, B., Toxicology and implications of the products of combustion, in *General and Applied Toxicology*, Vol. 3, 2nd ed., Ballantyne, B., Marrs, T.C., and Syversen, T., Eds., Macmillan Reference Ltd., London, 1999, pp. 1915–1933.

NRC (National Research Council), *Chemical and Biological Terrorism*, Institute of Medicine, National Research Council, National Academy Press, Washington, DC, 1999.

O'Flaherty, E.J. and Thomas, W.C., The cardiotoxicity of hydrogen cyanide as a component of polymer hydrolysis smokes, *Toxicol. Appl. Pharmacol.*, 63, 373–381, 1982.

Okoh, P.N., Excretion of ^{14}C-labelled cyanide in rats exposed to chronic intake of potassium cyanide, *Toxicol. Appl. Pharmacol.*, 70, 335–339, 1983.

Okoh, P.N. and Pitt, G.A.J., The metabolism in the rat of cyanide and gastrointestinal circulation of the resulting thiocyanate under conditions of chronic cyanide intake, *Can. J. Physiol. Pharmacol.*, 60, 381–386, 1982.

Okolie, N.P. and Osagie, A.U., Liver and kidney lesions and associated enzyme changes induced in rabbits by chronic cyanide exposure, *Food Chem. Toxicol.*, 37, 745–750, 1999.

Okolie, N.P. and Osagie, A.U., Differential effects of chronic cyanide intoxication on heart, lung and pancreatic tissues, *Food Chem. Toxicol.*, 38, 543–548, 2000.

Olsen, N.S. and Klein, J.R., Effects of cyanide on the concentration of lactate and phosphates in brain, *J. Biol. Chem.*, 167, 739–746, 1947.

Oluwole, O.S.A., Onobolo, A.O., and Cotgreave, I.A., Low prevalence of ataxic polyneuropathy in a community with high exposure to cyanide from cassava foods, *J. Neurol.*, 249, 1034–1040, 2002.

Oluwole, O.S.A., Onabolu, A.O., Colgreave, I.A., Rosling, H., Persson, A., and Link, H., Incidence of endemic ataxic polyneuropathy and its relation to exposure to cyanide in a Nigerian community, *J. Neurol. Neurosurg. Psychiatry*, 74, 1417–1422, 2003.

Onobolo, A.O., Bokango, M. and Rosling, H. Cassava processing in a Nigerian community affected by a neuropathy attributed to dietary cyanide exposure, *Trop. Sci.*, 39, 129–135, 1999.

Onobolo, A.O., Oluwole, O.S.A., and Bokango, M., Ecological variation of intake of cassava food and dietary cyanide load in Nigerian communities, *Public Health Nutr.*, 4, 871–876, 2001.

Oró, J. and Lazcano-Araujo, A., The role of HCN and its derivatives in prebiotic evolution, in, *Cyanide in Biology,* Vennesland, B., Conn, E.E., Knowles, C.J., Westley, J., and Wissing, F., Academic Press, London, 1981, pp. 17–541.

Osuntokun, B.O. Cassava diet, chronic cyanide intoxication and neuropathy in Nigerian Africans, *World Rev. Nutr. Diet.*, 36, 141–173, (1981).

Osuntokun, B.O., Moekosa, G., and Wilson, J., Relationship of a degenerative tropical neuropathy to diet: report of a field survey. *Br. Med. J.*, i, 547–550(1969).

Osuntokun, B.O., Aladetoyinbo, A., and Adeuja, A.O.G., Free-cyanide levels in tropical ataxic neuropathy, *Lancet*, 2, 372–73, 1970.

Owais, W.M., Janakat, S., and Hunaiti, A. Activation of sodium cyanide to a toxic but non-mutagenic metabolite in *Salmonella typhimurium, Mutat. Res.*, 144, 119–125, 1985.

Owasoya, J.O. and Iramain, C.A., Acetylcholinesterase activity in rat brain: effect of acute cyanide intoxication, *Toxicol. Lett.*, 6, 1–3, 1980.

Paabo, M., Birky, M.M., and Womble, S.E., Analysis of hydrogen cyanide in fire environments, *J. Combust. Toxicol.*, 6, 99–108, 1979.

Padmaja, G. and Panikkar, K.R., Pattern of enzyme changes in rabbits administered linamarin or potassium cyanide, *Indian J. Exp. Biol.*, 27, 551–555, 1989a.

Padmaja, G. and Pannikar, K.R., Intermediary metabolic changes in rabbits administered linamarin or potassium cyanide, *Indian J. Exp. Biol.*, 27, 635–639, 1989b.

Palmer, I.S. and Olson, O.E., Partial prevention by cyanide of selenium poisoning in rats. *Biochem. Biophys. Res. Commun.*, 90, 1379–1386, 1979.

Parmagianni, K., Cyanogen, hydrocyanic acid and cyanides, in *Encyclopedia of Occupational Health and Safety*, 3rd ed., International Labor Office, Geneva, 1983, pp. 574–577.

Patel, M.N., Yim, G.K.W., and Isom, G.E., N-Methyl-D-aspartate receptors mediate cyanide-induced cytotoxicity in hippocampal cultures, *Neurotoxicology*, 14, 35–40, 1992.

Paulet, G., On the relative importance of respiratory failure and cardiac failure in the lethal action of cyanides, *Arch. Int. Physiol. Biochem.*, 63, 28–339, 1955.

Paulet, G., Nouvelles perspective dans le traitement de l'intoxication cyanhydrique, *Arch. Mal. Prof.,* 22, 120–127, 1961.

Pearl, W., Cascarano, J., and Zweifach, B.W., Microdetermination of cytochrome oxidase by the oxidation of N-phenyl-*p*-phenylene diamine or ascorbic acid, *J. Histochem. Cytochem.*, 11, 102–107, 1963.

Peden, N.R., Taha, A., McSorley, P.D., Bryden, G.T., Murdoch, I.B., and Anderson, J.M., Industrial exposure to hydrogen cyanide: implications for treatment, *Br. Med, J.*, 293, 538, 1986.

Perry, I., The effect of prolonged cyanide treatment on body and tumor growth in rats, *Am. J. Cancer*, 25, 592–598, 1935.

Persson, S-Å., Cassel, G., and Sellström, Å., Acute cyanide intoxication and central transmitter systems, *Fundam. Appl. Toxicol.*, 5, S150–S159, 1985.

Peters, W.J., Inhalation injury caused by the products of combustion, *Can. Med. Assoc. J.*, 125, 249–252, 1981.

Pettersen, J.C. and Cohen, S.D., Antagonism of cyanide poisoning by chlorpromazine and sodium thiosulfate, *Toxicol. Appl. Pharmacol.*, 81, 265–273, 1985.

Pettersen, J.C. and Cohen, S.D., Comparison of the effects of cyanide (CN) on cytochrome oxidase (cyt ox) activity and respiration in mouse brain mitochondria (mito), *Toxicologist*, 6, 199, 1986.

Pettersen, J.C. and Cohen, S.D., The effects of cyanide on brain mitochondrial cytochrome oxidase and respiratory activities, *J. Appl. Toxicol.*, 13, 9–14, 1993.

Philbrick, D.J., Hopkins, J.B., Hill, D.C., Alexander, J.C., and Thomson, R.G., Effects of prolonged cyanide and thiocyanate feeding in rats, *J. Toxicol. Environ. Health*, 5, 579–592, 1979.

Piantadosi, C.A. and Silvia, A.L., Cerebral cytochrome a, a_3 inhibition by cyanide in bloodless rats, *Toxicology*, 33, 67–79, 1986.

Piantadosi, C.A., Sylvia, D.L., and Jöbsis, F.F., Cyanide-induced cytochrome a, a_3 oxidation-reduction responses in rat brain in vivo, *J. Clin. Invest.*, 72, 1224–1233, 1983.

Pines, K.L. and Crymble, M.M., In vitro conversion of thiocyanate to cyanide in the presence of erythrocytes, *Proc. Soc. Expt. Biol. Med.*, 81, 160–163, 1952.

Pitt, B.R., Radford, E.P., Gurtner, G.L., and Traystman, R.J., Interaction of carbon monoxide and cyanide on cerebral circulation and metabolism, *Arch. Environ. Health*, 34, 354–359, 1979.

Pontal, P.G., Bismuth, C., Garnier, R., and Pronezuck de Garbino, J., Therapeutic attitudes in cyanide poisoning. Retrospective study of 34 non-lethal cases, *Vet. Hum. Toxicol.,* 24 (Suppl.), 90–93, 1982.

Ponte, J. and Sadler, C.I., Studies on the regenerated carotid sinus nerve of the rabbit, *J. Physiol.*, 410, 411–424, 1989.

Porter, D.J.T., Voet, J.G., and Bright, H.T., Reduction of D-amino oxidase by β-chloraniline: enhancement of the reduction rate by cyanide, *Biochem. Biophys. Res. Commun.*, 49, 257–263, 1972.

Poulton, J.E., Localization and catabolism of cyanogenic glycosides, in *Cyanide Compounds in Biology*, Ciba Foundation Symposium 40*,* John Wiley and Sons, Chichester, U.K., 1988, pp. 67–91.

Poulton, J., Rylance, G.W., Taylor, A.W.J., and Edwards, C., Serum thiocyanate levels as indicator of passive smoking in children, *Lancet*, December 15, 1405–1406, 1984.

Pritsos, C.A., Mitochondrial dysfunction and energy depletion from subchronic peroral exposure to cyanide using the Wistar rat as a mammalian model, *Toxic Sub. Mech.*, 15, 219–229, 1996.

Pronczuk de Garbino, J. and Bismuth, C., Propositions thérapeutiques actuelles en cas d'intoxification par les cyanures, *Toxicol. Eur. Res.*, III (2), 69–756, 1981.

Pryce, D.M. and Ross, C.F., *Ross's Post-mortem Appearances*, 6th ed., Oxford University Press, London, 1963, p. 40.

Purser, D., Behavioural impairment in smoke environments, *Toxicology*, 115, 25–40, 1996.

Purser, D.A. and Grimshaw, P., The incapacitating effects of exposure to thermal decomposition products of polyurethane foams, *Fire Mater.*, 8, 10–16, 1984.

Purser. D.A., Grimshaw, P., and Berrill, K.P., Intoxication by cyanide in fires: a study in monkeys using polyacrylonitrile, *Arch. Environ. Health*, 39, 394–400, 1984.

Radford, E.P., Pitt, B., Halpern, B., Caplan, Y., Fisher, R., and Schwedia, P., Study of fire deaths in Maryland, September 1971-January 1974, in *Physiologic and Toxicologic Aspects of Combustion Products*, National Research Council, National Academy of Sciences, Washington DC, 1976, pp. 26–35.

Radojicic, B., Determining thiocyanate in urine of workers exposed to cyanide, *Arch. Hig. Rada. Toksikol.*, 24, 227–232, 1973.

RETECS (Registry of Toxic Effects of Chemical Substances), XL2275000. Thiocyanic acid, sodium salt, *Registry of Toxic Effects of Chemical Substances*, Vol. 3, National Institute of Occupational Safety and Health, Cincinnati, OH, 1981–1982, p. 774.

Rickwood, D., Wilson, M.T., and Darley-Usmar, V.M., Isolation and characteristics of intact mitochondria, in *Mitochondria: A Practice Approach*, Darley-Usmar, V.M., Rickward, D., and Wilson, M.T., Eds., IRL Press, Oxford, 1987, pp. 1–16.

Rietveld, E.C., Delbressine, L.P.C., Waegemaekers, T.H.J.M., and Seutter-Berlage, F., 2-Chlorobenzylmercapturic acid, a metabolite of the riot control agent 2-chlorobenzylidene malononitrile (CS) in the rat, *Arch. Toxicol.*, 54, 139–144, 1983.

Robbins, S.L. and Cotran, R.S., *Pathologic Basis of Disease*, 2nd ed., W.B. Saunders, Philadelphia, 1979, pp. 535–536.

Robinson, C.P., Baskin, S.I., and Franz, D.R., The effects of cyanide on isolated aorta strips from the dog, rabbit and ferret, *Toxicologist*, 4, 103, 1984.

Robinson, C.P., Baskin, S.I., and Franz, D.R., The mechanisms of action of cyanide on the rabbit aorta, *J. Appl. Toxicol.*, 5, 372–377, 1985b.

Robinson, C.P., Baskin, S.I., Visnich, N., Jr., and Franz, D.R., The effect of cyanide and its interactions with norepinephrine on isolated aorta strips from the rabbit, dog and ferret, *Toxicology*, 35, 59–72, 1985a.

Rodkey, F.L., and Robertson, R.F., The solubility of hydrogen cyanide in water, *J. Combust. Toxicol.*, 6, 44–47, 1979.

Rosenberg, N.L., Myers, J.A., and Marin, W.R.W., Cyanide-induced Parkinsonism: Clinical, MRI and 6-fluorodopa Fd positron emission tomography pet studies, *Neurology*, 39, 142–144, 1989.

Rusiecki, W. and Dyanakowski, P., Distribution of cyanide in organs of rats after fatal poisoning, *Acta Polon. Pharmacol.*, 4, 61–71, 1963.

Russek, M., Fernandez, A., and Vegas, C., Increase in cerebral blood flow produced by low dosages of cyanide, *Am. J. Physiol.*, 204, 309, 1963.

Rutkowski, J.V., Roebuck, R.J., and Smith, R.P., Effects of protein-free diet and food deprivation on hepatic rhodanese activity, serum proteins and acute cyanide lethality in mice, *J. Nutr.*, 115, 132–136, 1985.

Rutkowski, J.V., Roebuck, R.J., and Smith, R.P., Liver damage does not increase the sensitivity of mice to cyanide given acutely, *Toxicology*, 38, 305–314, 1986.

Ruzo, L.O., Unai, T. and Casida, I.E., *J. Agric. Food Chem.*, 26, 916–925, 1978.

Sadoff, L., Fuchs, K. and Hollander, J., Rapid death associated with laetrile ingestion, *J. Am. Med. Assoc.*, 239, 1532, 1978.

Saia, B., DeRosa, E. and Calzigna, L., Remarks on the chronic poisoning from cyanide, *Med. Lav.*, 61, 580–586, 1970.

Salkowski, A.A. and Penney, D.G., Cyanide poisoning in animals and humans: a review, *Vet. Hum. Toxicol.*, 36, 455–466, 1994.

Salkowski, A.A. and Penney, D.G., Metabolic, cardiovascular, and neurologic aspects of acute cyanide poisoning in the rat, *Toxicol. Lett.*, 75, 19–27, 1995.

Sandberg, C.G., A case of chronic poisoning with potassium cyanide? *Acta Med. Scand.*, 181, 233–236, 1967.

Sayek, I., The incidence of the inability to smell solutions of potassium cyanide in the rural health center of Ortabereket, *Turk. J. Pediatr.*, 12, 72–75, 1970.

Schulz, V., Clinical pharmacokinetics of nitroprusside, cyanide, thiosulfate and thiocyanate, *Clin. Pharmacokinet.*, 9, 239–251, 1984.

Schulz, V., Bonn, R., and Kindler, J., Kinetics of elimination of thiocyanate in 7 healthy subjects and 8 subjects with renal failure, *Klin. Wochenschr.*, 57, 243–247, 1979.

Schulz, V., Loeffler, A., and Ggheorghui, Th., The absorption of hydrocyanic acid from linseed, *Leber Magen. Darm.*, 13, 10–14, 1983.

Schwerd, W. and Schulz, E., Carboxyhaemoglobin and methaemaglobin findings in burnt bodies, *Forens. Sci. Int.*, 12, 233–235, 1978.

Seagrave, S., *Yellow Rain*, Abacus, London, 1982, p. 150.

Sharpe, A.G., *The Chemistry of the Cyano Complexes of the Transition Metals*, Academic Press, New York, 1976.

Sheehy, M. and Way, J.L., Effect of oxygen on cyanide intoxication: III. Mithridate, *J. Pharmacol. Exp. Ther.*, 161, 163–168, 1968.

Sidell, F.R., Groff, W.A., and Kaminiskis, A., Pralidoxime methylsulphonate: plasma levels and pharmacokinetics after oral administration to man, *J. Pharmacol. Sci.*, 61, 1136–1140, 1972.

Silcock, A., Robindon, D., and Savage, N.P., Fires in Dwellings—an Investigation of Actual Fires. Part II. Hazards from Ground Floor Fires. Part III. Physiological Effects of Fires, Building Research Establishment Current Paper 70/78, dated December 1978, Building Research Establishment, Fire Research Station, Borehamwood, U.K., 1978.

Silver, E.H., Kutttab, S.H., Hasan, T., and Hassan, M., *Drug Metab. Disp.*, 10, 495, 1982.

Singh, J.D., The lethality and teratogenicity of potassium cyanide in the rat, *Teratology*, 25, 84A, 1982.

Singh, B.M., Coles, N., Lewis, P., Braithwaite, R.A., Nattras, M., and FitzGerald, M.G., The metabolic effects of fatal cyanide poisoning,. *Postgrad. Med. J.*, 65, 923–925, 1989.

Skoog, D.A. and West, D.M., *Fundamentals of Analytical Chemistry*, 3rd ed., Holt, Reinhart and Winston, New York, 1969, p. 817.

Smiley, R.A., Nitriles, in *Kirk-Other Encyclopedia of Chemical Technology*, Vol. 15, 3rd ed., Wiley, New York, 1983, pp. 888–909.

Smith, A.D.M., Duckett, S., and Waters, A.H., Neuropathological changes in chronic cyanide intoxication, *Nature*, 200, 179–181, 1963.

Smith, R.M. and Martell, A.E., *Critical Stability Constants*, Vol. 4, Plenum Press, New York, 1989.

Solomonson, L.P., Cyanide as a metabolic inhibitor, in *Cyanide in Biology*, Vennesland, B., Conn, E.E., Knowles, C.J., Westley, J., and Wissing, F., Academic Press, New York, 1982, pp. 11–28.

Sorbo, B., Thiosulfate sulfurtransferase and mercaptopyruvate sulfurtransferase, in *Metabolic Pathways*, Vol. 7. *Metabolism of Sulfur Compounds*, Greenberg, D.M., Ed., Academic Press, New York, 1975, pp. 433–456.

Soto-Bianco, B., Sousa, A.B., Manzano, J.L., Guerra, J.L., and Gorniak, L., Does prolonged cyanide exposure have a diabetogenic effect, *Vet. Hum. Toxicol.*, 43, 106–108, 2001.

Sousa, A.B., Soto-Blanco, B., Guerra, J.L., Kimura, E.T., and Górniak, S.L., Does prolonged oral exposure to cyanide promote hepatotoxicity and nephrotoxicity? *Toxicology*, 174, 87–95, 2002.

Steffens, W., Percutaneous hydrocyanic acid poisoning, *J. Toxicol., Clin. Toxicol.*, 41, 483, 2003.

Sternbach, G., Editor's comment, *J. Emerg. Med.*, 10, 623–624, 1992.

Stewart, R.D., Baretta, E.D., Platto, L.R., Stewart, E.B., Kalbfleisch, J.H., Van Yserloo, B., and Rimm, A.A., Carboxyhemoglobin concentrations in blood from donors in Chicago, Milwaukee, New York and Los Angles, *Science*, 182, 1362–1364, 1973.

Stewart, R.D., Hako, C.L., Wu, A., Stewart, T.A., and Kalbfleisch, J.H., Carboxyhemoglobin trend in Chicago blood donors, 1970–1974, *Arch. Environ. Health*, 31, 280–286, 1976.

Sundwall, A., Plasma concentration curves of N-methylpyridinium-2-aldoxime methane sulphonate (P2S) after intravenous, intramuscular and oral administration in man, *Biochem. Pharmacol.*, 5, 225–230, 1960.

Sunshine, I. and Finkle, B., The necessity for tissue studies in fatal cyanide poisoning, *Int. Arch. Gewerbepath. Gewerbehyg.*, 20, 558–561, 1964.

Suzuki, T., Ultrastructural changes of heart muscle in cyanide poisoning, *Tohoku J. Exp. Med.*, 95, 271–287, 1968.

Svirbely, W.J. and Roth, J.F., Carbonyl reactions. 1. The kinetics of cyanohydrin formation in aqueous solutions, *J. Am. Chem. Soc.*, 75, 3106, 1953.

Svirbely, W.J. and Roth, J.F., Analytical applications of the cyanohydrin reaction, *Appl. Chem.*, 2, 1377, 1954.

Sykes, A.H., Early studies on the toxicology of cyanide, in *Cyanide in Biology*, Vennesland, B., Conn, E.E., Knowles, C.J., Westley, J., and Wissing, F., Academic Press, London, 1981, pp. 1–9.

Sylvester, D.M., Holmes, R.K., Sander, C., and Way, J.L., Interference of thiosulfate with potentiometric analysis of cyanide in blood and its elimination, *Toxicol. Appl. Pharmacol.*, 65, 116–121, 1982.

Symington, J.S., Anderson, R.A., Oliver, J.S., Thomson, I., Harland, W.A., and Kerr, W.J., Cyanide exposure in fires, *Lancet*, July 8, 91–92, 1978.

Tanberger, G., The stimulating effect of cyanide on central nervous system, vagus and sympathetic activities and its importance on circulation, *Arch. Int. Pharmacodyn.*, 187, 61–66, 1970.

Terblanche, M., Minne, J.A., and Adelaar, T.F., Hydrocyanic acid poisoning. A note on the HCN content of animal tissue at various stages of decomposition, *J. S. African Vet. Med. Assoc.*, 35, 503–506, 1964.

Tewe, O.O., Effect of dietary cyanide on the performance, metabolism and pathology of the African rat (Cricetomys Gambianas Waterhouse1), *Nutr. Rep. Int.*, 26, 529–536, 1982.

Tewe, O.O. and Maner, J.H., Cyanide, protein and iodine interactions in the performance, metabolism and pathology of pigs, *Res. Vet. Sci.*, 29, 271–276, 1980.

Tewe, O.O. and Maner, J.H., Long-term and carry-over effect of dietary inorganic cyanide (KCN) in the life cycle performance of and metabolism of rats, *Toxicol. Appl. Pharmacol.*, 58, 1–7, 1981a.

Tewe, O.O. and Maner, J.H., Performance and pathophysiological changes in pregnant pigs fed cassava diets containing different levels of cyanide, *Res. Vet. Sci.*, 30, 147–151, 1981b.

Thomas, W.C. and O'Flaherty, E.J., The cardiotoxicity of hydrogen cyanide as a component of polymer pyrolysis smokes, *Toxicol. Appl. Pharmacol.*, 63, 373–381, 1982.

Thompson, R.L., Manders, W.W., and Cowan, W.R., Postmortem findings of the victims of the Jonestown tragedy, *J. Forens. Sci.*, 32, 433–443, 1987.

Thompson, S.J. and Bayer, M.J., Access to cyanide antidote kits, *Ann. Emerg. Med.*, 12, 515–516, 1983.

Tomaszewski, C.A. and Thom, S.R., Use of hyperbaric oxygen in toxicology, *Emerg. Med. Clin. N. Am.*, 12, 437–459, 1994.

Towill, L.E., Drury, J.S., Whitfield, B.L., Lewis, E.B., Galtan, E.L., and Hammons, A.S., *Review of the Environmental Effects of Pollutants: V. Cyanide*, Prepared for the Health Effects Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH, 1978.

Troup, C.M., and Ballantyne, B., Analysis of cyanide in biological fluids and tissues, in *Clinical and Experimental Toxicology of Cyanides*, Ballantyne, B., and Marrs, T.C., Eds., Wright, Bristol, 1987, pp. 22–40.

Tscuhiya, Y., Significance of HCN generation in fire gas toxicity, *J. Combust. Toxicol.*, 4, 271–282, 1977.

Tscuhiya, Y., and Sumi, K., Determination of hydrogen cyanide in fire gases, *J. Combust. Toxicol.*, 3, 363–370, 1976.

Turskey, T. and Sajter, V., The influence of potassium cyanide poisoning on the γ-aminobutyric acid level in rat brain, *J. Neurochem.*, 9, 519–523, 1962.

Tylleskar, T., Legue, F.D., and Peterson, S., Konzo in the Central African Republic, *Neurology*, 44, 959–961, 1994.

Tyrer, F.H., Treatment of cyanide poisoning, *J. Soc. Occup. Med.*, 31, 65–66, 1981.

Urbas, E. and Kullick, E., Pyrolysis gas chromatographic analysis of some toxic compounds from nitrogen-containing fibers, *J. Chromatogr.*, 137, 210–214, 1977.

Urbas, E. and Kullick, E., Pyrolysis gas chromatographic analysis of some untreated and flame proofed wools, *Fire Mater.*, 2, 25–26, 1978.

Utti, R.J., Rajput, A.H., Ashenhurst, E.M., and Rozdilsky, B., Cyanide-induced parkinsonism: a clinicopathologic report, *Neurology*, 35, 921–925, 1985.

Van Buuren, K.J.H., Nicholls, P., and Van Gelder, P.F., Biochemical and biophysical studies on cytochrome a, a_3. VI. Reaction of cyanide with oxidized and reduced enzyme, *Biochim. Biophys. Acta*, 256, 257–276, 1972.

Van der Heyden, G., Vereecka, J., and Carmliet, E., The effect of cyanide on the K-current in guinea pig ventricular myocytes, *Basic Res. Cardiol.*, 80 (Suppl. 1), 3–96, 1985.

VanderLaan, W.P. and Bissell, A., Effect of propylthiouracil and of potassium thiocyanate on the uptake of iodine by the thyroid of the rat, *Endocrinology*, 39, 157–160, 1946.

Varnell, R.M., Stimac, G.K., and Fligner, C.L., CT diagnosis of toxic brain injury in cyanide poisoning: considerations for forensic medicine, *Am. J. Neuroradiol.*, 8, 1063–1066, 1987.

Vennesland, B., Castric, P.A., Conn, P.E., Solomonson, L.P., Volini, M., and Westley, J., Cyanide metabolism, *Fed.Proc.*, 41, 2639–2648, 1982.

Verna, A., Roumy, M., and Leitner, L.M., Loss of chemoreceptive properties of the rabbit carotid body after destruction of the glomus cells, *Brain Res.*, 100, 13–23, 1975.

Vesey, C.J., Letter to the Editor, *Clin. Toxicol.*, 14, 307–309, 1979.

Vesey, C.J., Cole, P.V., and Simpson, P.J., Cyanide and thiocyanate concentrations following sodium nitroprusside infusion in man, *Br. J. Anaesthesiol.*, 48, 651–660, 1976.

Vesey, C.J. and Wilson, J., Red cell cyanide, *J. Pharm. Pharmacol.*, 30, 20–26, 1978.

Vick, J.A. and Froelich, H.L., Studies on cyanide poisoning, *Arch. Int. Pharmacodyn.*, 273, 314–322, 1985.

Wallace, N.D., Davis, G.L., Rutledge, R.B., and Kahn, A., Smoking and carboxyhemoglobin in the St. Louis metropolitan population. Theoretical and empirical considerations, *Arch. Environ. Health*, 29, 136–142, 1974.

Walroth, J., Le, F.P., Hoar, S.K., Mead, M.W., and Fraumeni, J.F., Jr., Causes of death among female chemists, *Am. J. Public Health*, 75, 883–884, 1985.

Ward, E.N.B. and Thorn, G.D., Evidence for the formation of HCN from glycine by a snow mould fungus, *Can. J. Botany*, 44, 95–104, 1966.

Ward, E.N.B., Starratt, A.W., and Robinson, J.R., Studies on the pathology of HCN formation from glycine in a psychrophilic basidiomycete, *Can. J. Botany*, 55, 2065–2069, 1977.

Way, J.L., Mechanism of cyanide intoxication and its antagonism, *Annu. Rev. Pharmacol. Toxicol.*, 24, 451–481, 1984.

Way, J.L. and Burrows, G.E., Cyanide intoxication: protection with chlorpromazine, *Toxicol. Appl. Pharmacol.*, 36, 1–5, 1976.

Weger, N.P., Treatment of cyanide poisoning with 4-dimethylaminophenol: experimental and clinical overview, *Fundam. Appl. Toxicol.*, 3, 387–396, 1983.

Weiler, G. and Kloppel, A., Ein ungewöhnlicher Cyanid-Mord, *Arch. Kriminol.*, 172, 16–20, 1984.

Westley, J., Cyanide and sulfane sulfur, in *Cyanide in Biology.*, Vennesland, B., Conn, E.E., Knowles, C.J., Westley, J., and Wissing, F., Academic Press, London, 1981, pp. 61–76.

Westley, J., Adler, H., Westley, I., and Nishda, C., The sulfurtransferases, *Fundam. Appl. Toxicol.*, 3, 377–382, 1983.

Wetherell, H.R., The occurrence of cyanide in the blood of fire victims, *J. Forens. Sci.*, 11, 167–172, 1966.

Wexler, J., Whittenberger, J.L., and Umke, P.R., The effect of cyanide on the electrocardiogram of man, *Am. Heart J.*, 34, 163–173, 1947.

Wilson, J., Leber's hereditary optic atrophy: a possible defect in cyanide metabolism, *Clin. Sci.*, 29, 505–515, 1965.

Wilson, J., Cyanide in human disease, in *Clinical and Experimental Toxicology of Cyanides*, Ballantyne, B. and Marrs, T.C., Eds., Wright, Bristol, 1987, pp. 292–311.

Winek, C.L., Fusia, E., Collom, W.D., and Shanor, S.P., Cyanide poisoning as a mode of suicide, *Forens. Sci.*, 11, 51–55, 1975.

Wisler, J.A., Dulaney, M.D., Pellicore, L.S., and Lenz, D.E., Transport of cyanide into guinea pig cardiac mitochondria, *Toxiciol. Lett.*, 56, 275–281, 1991.

Wissing, F., Growth curves and pH optima for cyanide producing bacteria, *Physiol. Plant*, 21, 589–593, 1968.

Wissing, F. and Anderson, K.S., The enzymology of cyanide production from glycine by a Pseudomonas species. Solubilization of the enzyme, in *Cyanide in Biology*, Vennesland, B., Conn, E.E., Knowles, C.J., Westley, J., and Wissing, F., Eds., Academic Press, New York, 1981, pp. 275–287.

Wood, J.L. and Cooley, S.L., Detoxification of cyanide by cystine, *J. Biol. Chem.*, 218, 449–457, 1956.

Woolley, W.D., Nitrogen-containing products from the thermal decomposition of flexible polyurethane foams, *Br. Polymer J.*, 4, 27–43, 1972.

Woolley, W.D., Toxic products from plastic materials in fires, *Plastics Polymers,* 41, 280–286, 1973.

Woolley, W.D., Smoke and toxic gas production from burning polymers, *J. Macromolecr. Sci-Chem.*, A-17, 1–33, 1982.

Woolley, W.D. and Fardell, P.J., The production of combustion products, *Fire Res.*, 1, 11–21, 1977.

Woolley, W.D. and Fardell, P.J., Basic aspects of combustion toxicology, *Fire Saf. J.*, 5, 29–48, 1982.

Woolley, W.D., Fardell, P.J., and Buckland, I.F., The Thermal Decomposition Products of Rigid Polyurethane Foam under Laboratory Conditions, Fire Research Station Note 1039, Building Research Establishment, Fire Research Station, Borehanwood, U.K., 1975.

Woolley, W.D., Ames, S.A., and Fardell, P.J., Chemical aspects of combustion toxicology fires, *Fire Mater.*, 3, 110–120, 1979.

Worksafe Australia, Cyanide Poisoning, National Occupational Health and Safety Commission, Publication WAP 89/032, Australian Government Publishing Service, Canberra, 1989.

World Health Organization, *Environmental Health Criteria No. 13, Carbon Monoxide*, World Health Organization, Geneva, 1979.

Yacoub, F., Faure, J., Mallion, J.M., and Cau, G., La thiocyanaturie du fumeur, *Méd. Lég. Dom. Corp.*, 3, 266–270, 1970.

Yamamoto, Y., Acute toxicity of the combustion products of from various kinds of fire, *Z. Rechtsmed.*, 76, 11–26, 1975.

Yamamoto, A., Amino acids (survey), in *Kirk-Othmer Encyclopedia of Chemical Technology*, Vol. 1, 3rd ed., Wiley, London, 1983, pp. 376–410.

Yamamoto, H.-a., Hyperammonemia, increased brain neutral and aromatic amino acid levels, and encephalopathy induced by cyanide in mice, *Toxicol. Appl. Pharmacol.*, 99, 415–420, 1989.

Yamamoto, H.-a., Protection against cyanide-induced convulsions with α-ketoglutarate, *Toxicology*, 61, 221–228, 1990.

Yamamoto, H.-a., A possible mechanism for the increase in brain tyrosine levels induced by cyanide in mice, *Food Chem. Toxicol.*, 11, 973–977, 1992.

Yamamoto, H.-a., Protective effect of calmodulin inhibitors against acute cyanide-induced lethality and convulsions in mice, *Toxicol. Lett.*, 66, 73–79, 1993a.

Yamamoto, H.-a., Relationship among cyanide-induced encephalopathy, blood ammonia levels, and brain aromatic amino acid levels in rats, *Bull. Environ. Contamin. Toxicol.*, 50, 240–281, 1993b.

Yamamoto, H.-a., A hypothesis for cyanide induced and toxic seizures with supporting evidence, *Toxicology*, 95, 19–26, 1995.

Yamamoto, H.-a. and Tang, H.-W., Effect of carbetapentane or melatonin on cyanide-induced neurotoxicity in mice, *Jpn. J. Toxicol. Environ. Health*, 6, 4–491, 1996a.

Yamamoto, H.-a. and Tang, H.-W., Antagonistic effect of melatonin against cyanide-induced seizures and acute lethality in mice, *Toxicol. Lett.*, 7, 19–24, 1996b.

Yamamoto, H.-a. and Tang, H.-W., Effects of 2-amino-7-phosphonoheptanoic acid, melatonin or N^G-nitro-L-arginine on cyanide or *N*-methyl-D-aspartate-induced neurotoxicity in rat cortical cells, *Toxicol. Lett.*, 94, 13–18, 1998.

Yamamoto, K. and Yamamoto, Y., The blood acid-base changes in acute cyanide poisoning in the rat in comparison with those in acute anoxic anoxia. With special references to the relation with time to death, *Z. Rechtsmed.*, 79, 125–135, 1977.

Yamamoto, K., Yamamoto, A., and Kuwahara, C., A blood cyanide distribution study in the rabbit intoxicated by the oral route and by inhalation, *Z. Rechtsmed.*, 83, 313–317, 1979.

Yamamoto, K., Yamamoto, Y., Hattori, H. and Samori, T., Effects of route of administration in the cyanide concentration distribution in various organs of cyanide-intoxicated rats, *Tohuku J. Exp. Med.*, 137, 73–78, 1982.

Yamamoto, K., Yamamoto, Y. and Hattori, H., Combined effects of ethanol and cyanide. An experimental study on rats, *Blutakohol*, 20, 513–518, 1983.

Yamamoto, K., Yamamoto, Y. and Hattori, H., Postmortem changes in concentrations of cyanide and thiocyanate, *Jpn. J. Legal Med.*, 38, 289–293, 1984.

Yeh, M.M., Becker, C.E. and Arieff, A.I., Is measurement of venous oxygen saturation useful in the diagnosis of cyanide poisoning, *Am. J. Med.*, 93, 582–583, 1992.

Yonetani, T. and Ray, G.S., Studies on cytochrome oxidase. VI. Kinetics of the aerobic oxidation of ferrocytochrome by cytochrome oxidase, *J. Biol. Chem.*, 243, 634–638, 1965.

Zanetti, G., Galante, Y.M., Arosio, P. and Carletti, P., Interactions of succinate dehydrogenase with cyanide, *Biochim. Biophys. Acta*, 321, 41–53, 1973.

31 The Pharmacology and Toxicology of Inhaled Dusts, Endotoxins, and Glucans

Samantha R. Lane, Paul J. Nicholls, and Robert D.E. Sewell

CONTENTS

31.1 INTRODUCTION

Public and occupational health is of growing concern, an important aspect being the increasing number of airborne pollutants to which our lungs are exposed as we simply breathe. To identify these agents and understand their potential for pulmonary toxicity is a priority. In general, individuals spend an increasing amount of time in the home and the "built" environment, in some cases as much as 80–90% of their time. The balance in exposure between indoor and outdoor environments could have a marked effect on the pollutant profile and concentration accessing the lung. It is likely that the indoor environment is the more important milieu, primarily because of human occupancy, coupled with its confined nature.

The generation of airborne dusts from man's large-scale exploitation of natural organic materials has been a long-standing problem for those exposed to such materials, particularly in respect to respiratory illness. In 1555, Olaus Magnus recorded his observations on everyday life in *Histira de Gentibus Septentrionalibus*. He noted, "when separating the grain from the chaff, care must be taken to choose a time when there is a suitable wind which will sweep away the grain dust, so that it will not damage the vital organs of the threshers." This historical view of environmental respiratory disorders

is no less relevant today despite high-technology air quality control. Modern industrial operations often pose a larger health risk, because they are responsible for the exposure of large workforces to high concentrations of atmospheric toxicants in maximum productivity environments.

Forty years ago, it was suggested that particular agents inherent within generated dusts were responsible for the respiratory damage noted for many years in industrial workers. Organic dusts contain a myriad of particles potentially damaging to the lungs. Of these, one of the first specific biological molecules to emerge was endotoxin, a component of the external membrane of gram-negative bacteria. Endotoxin is ubiquitous in nature because it is continually released by bacteria in small quantities and liberated completely when cells lyse. However, it is in the occupational environment where sufficiently high endotoxin concentrations with the potential to cause serious respiratory failure are attained.

Research into endotoxin toxicology has been extensive, clearly demonstrating its clinical pulmonary toxicity following inhalation. More recently, it has become clear that endotoxin is not unique in its capacity as a respiratory threat. Another group of toxicants, glucans, have been identified. (1→3)-β-D-Glucans are glucose polymers of fungal origin; they are also prolific contaminants of vegetable fibers and hence are present in the aerosols created during industrial processing of such materials. Glucans have been shown in animal and human models to elicit changes in respiratory function. Hence, they are also now being implicated as a cause of environmental lung disease, especially with respect to "sick building syndrome." They are also found in damp buildings where they are used to assess fungal biomass and air quality (Rylander and Lin, 2000). β-Glucans have also been shown to alter the response of cells to other agents, most importantly endotoxin, and this is currently an area of much investigation (Young et al., 2002).

31.2 INHALED DUSTS

Vegetable fibers from different sources have been exploited by humans for a variety of purposes over many years. These include textile manufacture and agricultural production. Airborne dust arising from such procedures has been shown to cause respiratory disorders on inhalation by exposed workers. Because of the high concentrations of dust present in certain industrial situations, these problems have been historically linked to particular work environments. Bernardo Ramazzini (1713), in his treatise *De Morbis Artificum*, described several occupational lung disorders, including those caused by textiles, grains, woods, and hays. Diseases resulting from the inhalation of these dusts include hypersensitivity pneumonitis, occupational asthma, and bronchitis.

Vegetable dusts are heterogeneous mixtures of biological and physical contaminants; the content of cotton dust is used as an example in Table 31.1 (Wakelyn et al., 1976). This heterogeneity yields considerable variability between individuals in their response to dust inhalation, which has frustrated identification of the responsible dust components. The main factors influencing the makeup of organic dusts relate to the (fiber) crop and include climate, soil, harvesting practice, storage conditions, and processing techniques. The effect of dusts on the lungs is dependent on the size of inhaled particles; this affects their ease of entry into the respiratory tract and governs the depth of penetration of this system. However, both total and respirable dust content of the environment must be taken into account when considering risk, along with the pattern of exposure to particular dusts (for example, farm workers have seasonal cycles of dust exposure). Variables directly relating to exposed individuals such as age, length and type of employment, atopy, and smoking history can also alter the exposure risk. In addition, the human respiratory tract may respond to inhaled dusts in a number of ways giving rise to several distinct diseases and the main conditions resulting from dust exposure are described below.

31.2.1 Hypersensitivity Pneumonitis

When an immunological reaction is observed in response to vegetable dust exposure, it is classified either as hypersensitivity pneumonitis (HP) or as occupational asthma (OA). Hypersensitivity

TABLE 31.1 The Composition of Cotton Dust

8–10% H_2O
up to 2% NO_3
10–20% inorganic compounds (sand etc.)
Carbohydrates
Cellulose
Hemicelluloses
Pectic compounds
Lignins
Condensed tannins
Hydrolyzable tannins
Phenolic pigments
Porphyrins
Lipids
Proteins and peptides
Glycoproteins and peptides
Free sugars, amino acids, aminosugars, amines
Miscellaneous compounds (from insecticides, etc.)

Source: Wakelyn (1976).

pneumonitis, also known as extrinsic allergic alveolitis, refers to a group of diseases characterized by inflammation of the peripheral airways and alveoli. This occurs when particles small enough to reach deep into the respiratory tract (<7 μm aerodynamic diameter) are inhaled, causing rhinitis, cough, fever, rigors, and breathlessness. The resulting reduction in airflow reaches its peak after about 8 h, although the symptom pattern varies between individuals and also with the causative agent.

HP may be acute, subacute, or chronic; the acute symptoms disappear following exposure cessation, although on repeated exposure they may eventually become permanent. Low-level prolonged dust exposure has a propensity toward the chronic condition, whereas a period of short exposure to high-level dust concentrations gives rise to the acute response. Progressive, intrapulmonary fibrosis often occurs via activation of alveolar macrophages, and advanced cases of the condition can be associated with signs of pulmonary heart disease and chronic heart failure. Examples of the dusts responsible for HP are shown in Table 31.2 and it is likely to be microbial contamination of the dusts by bacteria or fungi that are the ultimate common cause of the disease (Nicholls, 1992).

Not everyone exposed to these agents succumbs to disease, and only certain individuals appear to display sensitivity. Diagnosis of the disease requires clinical, radiological, and pathological investigation. The histological triad indicating HP includes bronchiolitis, interstitial lymphocytic infiltration, and sarcoid-type granulomas in the alveoli (Yi, 2002). The symptom patterns with this group of diseases show elements of both a nonimmunological response and a cell-mediated allergic response, which cannot readily be categorized by any of the four types of human allergic reaction. Farmer's lung is the best characterized of this group, whereby continued exposure to hay dust leads to the formation of an antigen–antibody type III reaction. Nevertheless, HP diseases indicate that both alveolar macrophages and T cells (Th-1 type) play a pivotal role in the immune responses and the formation of granulomas is more compatible with a type IV reaction, explained by a late-phase cell-mediated response (Rose, 1996; Yi, 2002).

TABLE 31.2 Types of Hypersensitivity Pneumonitis

Disease	Source of Dust
Bagassosis	Moldy sugar cane
Bird fanciers lung	Bird droppings
Farmers' lung	Moldy hay and straw
Humidifier fever	Contaminated water
Malt workers lung	Moldy barley
Maple bark disease	Moldy maple bark
Mushroom workers lung	Mushroom compost
Suberosis	Moldy cork bark

31.2.2 Occupational Asthma

Occupational asthma is the most common form of occupational disease in the developed world (Lombardo and Balmes, 2000). It is associated with diffuse, intermittent, reversible airway obstruction in response to the inhalation of a wide range of dusts such as wood dusts and grains. Unlike idiopathic asthma, OA is dependent on sensitization to the workplace antigen, though exposure may exacerbate symptoms in patients with preexisting asthmatic conditions. The disease is characterized by shortness of breath, chest tightness, wheezing, cough, and bronchoconstriction. Symptoms may appear immediately, commencing after 20 min, or late, emerging 18 months to 15 years after initial exposure, during which time sensitization occurs. It is a serious condition, which is potentially fatal when a massive dose causes a severe acute attack. A more chronic condition, however, may occur following prolonged exposure.

Over 250 agents have been identified as possible causes of OA, and putative mechanisms by which sensitization occurs can be arbitrarily defined by the molecular weight of the agent responsible. Most high-molecular-weight compounds induce OA by specific IgE antibody-dependent reactions. The disease mechanism may not be either a type I or a type III response as complex nonimmunological reactions have also been suggested with some agents.

OA may eventuate after exposure to the dust from a number of hardwoods such as the Western Red Cedar, where it is thought the dust allergens release histamine and other mediators by a nonimmune response. Wheat is the most common cause of OA in response to grain and flour dusts, inhalation of which causes rhinitis and asthma in a large number of workers. Rice is a major agricultural product in many parts of Asia where it is dried and milled, a process that creates large aerosol clouds. The dust causes irritation to the eyes, skin, and upper respiratory tract as well as allergic-type reactions such as asthma and eosinophilia, though nonspecific reactions also occur. Rice husks have high silica content, which could be a causative factor in OA. The husks also possess microscopic needle-like spines, which are capable of inducing corneal scarring. Husk fragments, which are inhaled may thus cause damage by this physical process. Acute and chronic diseases occur in a number of workers exposed to common tea dust. Tea-workers asthma is also described in those exposed to herbal tea dusts such as sage and chamomile although it has not been established whether the causal agent is derived from the plants themselves or contaminating fungi. Green and roasted coffee beans can cause asthma, rhinitis, conjunctivitis, and bronchitis. The resulting acute reduction in flow rate can be eased by cromoglycate, which implies that the reactions are IgE-mediated (Nicholls, 1992).

Prevalence of occupational asthma depends on the agent responsible, the level of exposure, and host susceptibility, which is influenced by atopy and smoking habits. Thus workers who are atopic have been shown to have a higher likelihood of developing occupational asthma and cigarette smoking increases the risk of IgE-mediated disease (Lombardo and Balmes, 2000).

31.2.3 Organic Dust Toxic Syndrome (ODTS)

ODTS involves febrile episodes that occur in the absence of other signs of alveolitis on *first contact* with agricultural dusts. Acute fever, chills, joint pain, and other usually mild, flulike symptoms develop in the afternoon or evening after exposure during the working day and subsequently last for 24–48 h. Tolerance to the dust develops and symptoms disappear completely with continued exposure, though they may return after a prolonged absence from the allergen or an episode of very heavy exposure. ODTS is not progressive and sensitization does not occur; hence, it is probably attributable to a toxic reaction rather than an immune response. It typically occurs with moldy hay and grains and is common in swine containment units. Synonymous with mill fever and factory fever, this condition is rare in modern mills because of improved ventilation (Schilling and Rylander, 1994). It is not known why some individuals are predisposed to this condition while others remain unaffected, but it has been suggested that those who develop mill fever may be more susceptible to byssinosis (described below) following prolonged exposure (Gill, 1947).

31.2.4 Byssinosis

Byssinosis (brown lung) is a disease specific to those employed in the textile-processing industry; it is a worldwide problem that has occurred in every country where these fibers are processed industrially. Onset of the disease normally occurs only after at least 5 years exposure to the mill atmosphere. It is characterized by a sensation of chest tightness and breathlessness, experienced on the first day back after the weekend break (the so-called Monday phenomenon) or a holiday period (Rylander et al., 1985). Acute byssinosis is associated with changes in lung function suggestive of reversible airway narrowing. The term is used to describe a whole range of Monday-related symptoms, including acute chest tightness, shortness of breath, coughing and wheezing, and nonspecific malaise (Rylander, 1992). These symptoms can spread to other days of the week with further exposure, until they are continuous throughout the week, resulting in the chronic state of the condition.

The textiles known to cause byssinosis include; cotton, flax, hemp, jute, sisal, and kapok. It is most often described in the context of cotton production because, here, the highest numbers of workers are at risk due to the sheer scale of the industry. Prevalence of byssinosis in cotton mills has been reported to be as high as 30–40% (Cloutier and Rohrbach, 1986), although this varies greatly according to the quantity and composition of the causative dust, which depends on many factors. Assessing disease prevalence also depends on correct recognition of symptoms. Consequently, accurate diagnosis of byssinosis may be complex, because it is also compounded by interindividual symptom variability. During onset, the disease is indistinguishable from nonoccupational chronic bronchitis. However, it is discernable as a separate disease because it is associated with a linear fall in indirect maximum breathing capacity (MMF) and an increase in airway resistance during the working day. There is also a decrease in forced expiratory volume in one second (FEV_1) and MMF during the working shift, and these functional changes are reversible by bronchodilator drugs.

There are well-defined stages of byssinosis, which were originally clinically graded by symptom profile (Schilling et al., 1955) (shown in Table 31.3). Few physical signs accompany this condition,

TABLE 31.3 The Schilling Clinical Grades of Byssinosis

Grade C	Symptoms
0	No symptoms of byssinosis
½	Occasional chest tightness on the first day of the working week
1	Chest tightness on every first day of the working week
2	Chest tightness on the first and other days of the working week
3	Grade 2 symptoms accompanied by evidence of permanent respiratory disability

the only common one being expiratory wheeze with grades C1/2–C2 and patients suffering from C3 byssinosis may also exhibit impaired breath sounds (Parkes, 1983). Therefore, diagnosis of C1/2–C2 relies on history of industrial exposure, history of clinical grades, and fall in FEV_1 or MMF, during the working day or week. Individuals with C1/2 and C1 invariably recover completely after leaving the industry, and this also applies to C2 workers, although there may be patients with grade C2 byssinosis, such as heavy smokers, who have persisting symptoms (Parkes, 1983).

Underlying mechanisms responsible for the symptoms of byssinosis have been proposed and include the release of physiologically active agents. Mediator release is accompanied by cell infiltration into the lungs; workers' acute airway changes are accompanied by an influx of neutrophils into the nasal mucosa, an increase in polymorphonuclear leukocytes in blood and a decrease in circulating platelets. Surveys performed on cotton mill operatives have shown relationships between airborne dust concentration and acute fall in FEV_1. Set procedures are now in place to monitor and control occupational cotton dust levels. Efficient industrial exhaust ventilation removes the majority of the hazardous dust. Surveillance of workers is also important so that employees are not exposed to unnecessary risks. All prospective employees are examined by a questionnaire in which a family history of allergy or asthma is taken, along with a physical examination. Workers have annual clinical examinations to monitor any progressive fall in FEV_1 and respirators are also worn to protect workers. By controlling airborne dust of respirable size, the problem of byssinosis in the U.K., USA, and other developed countries has been reduced to minor proportions.

Although the control of textile dust itself has been successful where it is efficiently carried out, correlations have been demonstrated between respiratory disorders and airborne gram-negative bacterial counts, more specifically to bacterial endotoxin concentration (Haglind and Rylander, 1984). Hence, research has more recently turned toward the monitoring and control of endotoxin levels in industrial situations (Simpson et al., 1996; Niven et al., 1998).

31.3 ENDOTOXIN—A WELL-RECOGNIZED TOXICANT

Endotoxin is an integral component of the outer membrane of all well-characterized gram-negative bacteria. It was first described in 1892 by Richard Pfeiffer. The molecule is heterogeneous in that it contains both lipid and polysaccharide moieties. For this reason, the term lipopolysaccharide (LPS) is used to refer to the chemically purified molecule obtained via several extraction steps. Endotoxin is a significant airborne hazard in many situations; it is shed in small amounts constantly into the environment by viable bacteria, and released completely from these cells when they lyse. Endotoxin is at least partly responsible for many occupational diseases, where the dusts contain the toxicant via bacterial contamination of organic fibers while they grow in the field. Inhalation occurs along with the aerosols created during the processing of these fibers in occupational environments. Moreover, endotoxin is also present in house dust in varying concentrations, although the extent of its activity in the domestic setting has emerged more recently.

31.3.1 Structure and Function

The structure of endotoxin has been well characterized. The purified LPS molecule consists of three different regions (see Figure 31.1):

- O-specific side chain (O-antigen)
- Core region (inner and outer core)
- Lipid region (Lipid A)

The O-specific side chain is a heteropolysaccharide consisting of repeating units, which contain up to eight sugar monomers. A large range of residues constitute these chains and include neutral sugars, amino sugars, deoxy sugars, sugar acids, and sugar phosphates (Holst et al., 1996). The

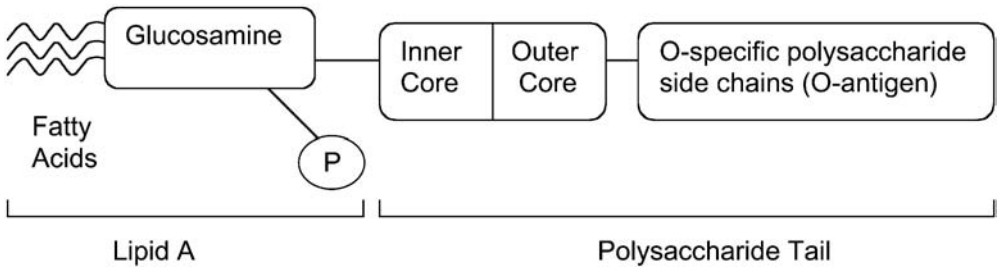


FIGURE 31.1 The basic structure of lipopolysaccharide.

O-specific side chain protrudes extracellularly and is the most structurally variable segment of the molecule. This diversity in chain structure is thought to help in evading the host immune system via stimulation of specific immune responses.

The core region is a small string of sugars, so named because comparison of this component from different species of bacteria displays limited variation (Holst et al., 1996). The core can be subdivided into the inner core (linked to lipid A) and the outer core. The effects on the human body of this section of the molecule are less profound, although mutant LPSs without an O-chain retain their ability to elicit an immune response. The inner segment contains 2-keto-3-deoxy-D-manno-octonic acid (KDO), an eight-carbon sugar that anchors the oligosaccharide molecule to lipid A.

The lipid A region is the major lipid component of the molecule and its structure is unique to gram-negative bacteria. It has specific roles in the assembly and function of outer membranes and secures the endotoxin molecule to the outer membrane of the bacteria. Lipid A consists of the sugar glucosamine, which is attached to a phosphate as well as long-chain fatty acids. Because of its complexity, the full structure of lipid A was not characterized until 1983 and this led to the realization that it is responsible for the biological activity of LPS (Raetz, 1990). Chemically synthesized and modified lipid A molecules have been used to elucidate the specific regions of the molecule responsible for its endotoxicity and it appears that major contributing factors are the number, length, and arrangement of the acyl chains and phosphate groups on this moiety (Erridge et al., 2002).

31.3.2 Pharmacology and Toxicology

The toxicology of endotoxin has been extensively studied, showing that the human body recognizes LPS as an indication of gram-negative bacterial infection. Accordingly, the respiratory system has efficient recognition and swift response systems in place to fight off the inflammatory insult. In humans, inhalation of LPS causes lung function responses characterized by a decrease in FEV_1 due to bronchoconstriction. Changes in the degree of nonspecific bronchial hyper-responsiveness and a reduction in alveolar-capillary diffusion are also seen (Michel, 2000).

Inhaled endotoxin initially stimulates a pulmonary inflammatory response, its primary target being alveolar macrophages whose role is the phagocytosis of particulate matter including that of microbial origin. Endotoxin-stimulated (activated) macrophages release chemotactic factors, which initiate an invasion mainly of neutrophils and eosinophils into the lung tissue a few hours after exposure, followed by entry into the airways 12–24 h after activation has occurred (Rylander, 1992). Endotoxin is a potent pyrogen, and tests have shown that macrophages are activated at concentrations of LPS as low as 1 ng/ml (Michel, 2000). The effects of endotoxin are widespread throughout body systems and a range of molecules, including cytokines and other peptides, lipids, and oxygen radicals, are released under its influence. This results in an extensive inflammatory mediator network, in which endotoxin not only actively participates but also initiates the production and release of mediators (Henson et al., 1994).

The roles of the main inflammatory mediators released in response to endotoxin are summarized in Table 31.4. Interleukin 1 (IL-1), IL-6, and IL-8 represent families of cytokines secreted

TABLE 31.4 The Main Inflammatory Agents Involved in Endotoxin-Induced Inflammation, Their Source and Action

Agent	Production	Action
	Cytokines (proteins)	
IL-1	In response to infection/injury from activated macrophage. Induced by TNF from mononuclear and endothelial cells	Regulates systemic inflammatory response, increase in blood neutrophils; causes fever. Induces other cytokines. Stimulates NO production, produces PLA_2 PAF, releases histamine
IL-6	Induced by IL-1 from epithelial cells	Pyrogen, activates stromal bone marrow to produce CSF
IL-8	Secreted by macrophages, endothelial cells, T cells and fibroblasts in response to LPS, IL-1, or TNF stimulation	Chemotactic for neutrophils and activates macrophages
TNF-α	Large amounts from endotoxin-stimulated macrophages	Induces arachidonic metabolites and synthesis of cytokines. Chemotactic for neutrophils and macrophages. Synergistic with IL-1. Causes fever
	Lipids (arachidonic acid metabolites)	
Prostaglandin (PG)E_2*	Enzymatic pathway from membrane phospholipid	A vasodilator, has pro and anti-inflammatory potential
Thromboxane (TX)A_2*	Enzymatic pathway from membrane phospholipid	Potent vasodilator, causes platelet sequestration
PAF	Epithelial cells via the arachidonic acid pathway, also neutrophils, basophils and platelets	Platelet aggregation. Recruits eosinophils, causes vasodilation, increases vascular permeability, degranulation of neutrophils. Spasmogenic on bronchial smooth muscle
	Reduced Oxygen Species (free radicals)	
OH, H_2O_2, super oxide anion	LPS stimulated macrophage/monocyte cells	Very reactive, responsible for lung tissue injury. Interacts with arachidonic acid pathway to increase levels of eicosanoids

[a] Also referred to as eicosanoids.

from various cells in response to endotoxin activation. The action of these cytokines leads to an acute inflammatory response. IL-1 also stimulates platelet aggregation factor (PAF) and arachidonic metabolites. PAF is produced directly from a number of cells, including neutrophils, platelets, and endothelial cells (Morrison and Ryan, 1987). Its range of effects includes degranulation of neutrophils and increased vascular permeability. Endotoxin-stimulated macrophages also produce and secrete tumor necrosis factor alpha (TNF-α) so rapidly that it can be detected in experimental animals within 1 h after initial exposure (Morrison and Ryan, 1987). TNF-α is a potent pyrogen and is able to induce further IL-1 production from mononuclear and endothelial cells. IL-8 is secreted from macrophages, endothelial cells, neutrophils, and T cells in response to direct and indirect (via IL-1 and TNF-α) LPS stimulation. IL-8 induces neutrophil chemotaxis causing large numbers of these cells to migrate across endothelial monolayers (Henson et al., 1994). There are also several lipids involved in the inflammation network, most significantly those arising from the arachidonic acid pathway. Arachidonic acid is a 20-carbon essential fatty acid that is released from membrane phospholipids by the enzyme phospholipase A_2 (PLA_2). This reaction produces a number of metabolites, some of which (i.e., prostaglandins, thromboxanes, and PAF) are involved in endotoxin-stimulated inflammation (Makhlouf et al., 1994).

Although many of the mediators involved in endotoxin inflammation have been identified and their general effects well characterized, each precise individual involvement in the cascade continued to evade complete resolution. Recognition of LPS by its primary targets such as the alveolar macrophage is the subject of current investigation and no unifying theory has so far been finally accepted, probably because the presence of multiple LPS receptors on a variety of cell types. It is well known that CD14, a 55-kDa glycoprotein expressed in monocytes, macrophages, and neutrophils, binds with high affinity to LPS. CD14, which can exist in membrane and soluble forms, is not only central to the recognition process but also crucial to the subsequent response to LPS. However, CD14-deficient human cells are still able to elicit an immune response to LPS, suggesting the involvement of additional factors (Erridge et al., 2002). Along with CD14, a second receptor was identified some time ago; lipopolysaccharide-binding protein (LBP) an acute-phase protein expressed on several host cell types, enhances the capacity of LPS to bind and activate macrophages and neutrophils, and binds specifically to the lipid A section of LPS (Kirikae et al., 1994). Along with LBP and CD14, the β_2 integrins, CD11b/CD18 (subunits of complement receptor CR3), are also involved in LPS recognition and subsequent signaling (Dobrovolskaia and Vogel, 2002).

Recently, Medzhitov et al. (1997) described the toll-like receptor TLR4, a type I transmembrane protein whose involvement in LPS recognition has become evident (Dobrovolskaia and Vogel, 2002). Furthermore, another molecule, the MD-2 protein, is required to associate with TLR-4 to allow it to interact with LPS (Akira, 2003). MD-2 also has a role in the intracellular distribution of TLR4 (Miyake, 2003). In light of these recent findings, a model has been proposed for LPS recognition in mammalian phagocytes, where the combined actions of the LPS-binding protein (LBP), the membrane-bound or -soluble forms of CD14, along with the recently identified TLR4-MD-2 complex, cofacilitate rapid molecular recognition (Heumann and Thierry, 2002). An intracellular signaling pathway is activated, resulting ultimately in the release of the inflammatory mediators described above. Evidence suggests that LPS complexes initially with LBP, this complex or LPS alone are then transferred from the bacteria cell wall to CD14, which interacts with TLR4 and MD-2. It has been recently reported that low concentrations of LPS require both CD14 and TLR4, whereas high concentrations elicit a CD14-independent response (Caroff et al., 2002; Dobrovolskaia and Vogel, 2002). This aspect of endotoxin pharmacology is advancing rapidly and the attainment of a widely accepted, detailed hypothesis cannot be far off.

In normal subjects, inhalation of endotoxin induces dose-related clinical symptoms, which invariably exhibit changes in lung function and bronchial inflammatory response. However, there is considerable interindividual variation with respect to the specific clinical and inflammatory effects of inhaled endotoxin. This variability could stem from polymorphisms in the genes encoding either endotoxin-released cytokines or the LPS receptor CD14 (Michel, 2000). Genetic evidence has also been recently reported showing that common mutations in TLR4 are linked to variations in human responses to LPS (Schwartz, 2001). Consequently, further characterization of additional genes involved in the deleterious effects of endotoxin inhalation is essential to any further conceptual understanding of this phenomenon.

31.3.3 Human Relevance and Risk Assessment

There is a wide range of occupational environments where endotoxin can be encountered and these are summarized in Table 31.5 (Jacobs, 1997). Endotoxin concentration in these environments varies considerably; hence, the exposure dose may or may not be high enough to elicit an inflammatory response. Discrepancy exists among reported threshold levels of endotoxin required to evoke clinical symptoms in healthy individuals. However, it is generally accepted that individuals with asthma have consistently lower thresholds than healthy subjects. Typically, inhalation of 30–40 μg of LPS is necessary to elicit lung function responses, although changes in blood neutrophil levels could be induced by a dose less than 0.5 μg (Thorn, 2001). In individuals with asthma, acute inhalation of 20 μg of endotoxin is reported to instigate a significant bronchoconstriction 30 min after challenge

TABLE 31.5 The Occupational Environments in Which Endotoxin Is Found

Agricultural environments
Animal confinement buildings
Dairy farming
Grain, field crops, and hay handling
Greenhouses
Industrial environments
Animal food production
Biotechnology (tissue culture, enzymes)
Fermentation industry
Paper production
Pharmaceutical
Vegetable fiber production
Wood processing
Waste processing
Composting
Recycling plants
Rubbish collection
Buildings
Those with contaminated ventilation ducts and humidiffers (schools, houses, offices)

(Michel et al., 1992). The concentrations of endotoxin in a range of industrial and domestic environments have been measured by using the *Limulus* amebocyte lysate (LAL) assay and many of these industrial organic dusts contain high enough endotoxin concentrations to supercede this threshold level of exposure; for example, the average endotoxin content of flax is reported as 92 μg/g of dust (Buick and Magee, 1999). There are no current safety limits for indoor environmental endotoxin levels in place anywhere in the world, although limits for general dust concentrations have been implemented. Recommendations made by the Dutch Expert Committee on Occupational Standards (DECOS) of the National Health Council have recommended a limit of 50 Endotoxin Units/m^3 (approximately 4.5 ng/m^3) (Heederik and Douwes, 1997).

In household dust, recorded levels of endotoxin vary between studies (Douwes et al., 2000; Heinrich et al., 2001). Several reasons for this could exist, because higher levels of endotoxin have been measured in homes linked to the presence of pets or vermin (Heinrich et al., 2001) and measurement protocols differ. The presence of significant concentrations of endotoxin in the home has a number of health-related implications. Low-level endotoxin exposure may have benefits in young children because it has protective effects against conditions such as atopic eczema in the first 6 months of life, although this may be coupled with increased incidence of nonspecific respiratory illness (Gehring et al., 2002). It has also been reported that inhalation of endotoxin may increase the severity of chronic conditions such as asthma (Fernandez-Caldas, 2002; Su et al., 2001; Thorn, 2001), especially in individuals sensitive to house dust mites. There are data suggesting that environmental endotoxin could be a synergistic factor on the magnitude of IgE-mediated response and this substantiates the multiplicity of risk factors linked to the development of asthma, especially in young children.

Endotoxin has also been recorded in tobacco (Hasday et al., 1999) where it has been measured at levels comparable to those reported in other agricultural products. Although temperatures at the tip of a smoked cigarette are high enough to inactivate endotoxin, tobacco upstream from the tip is lower in temperature, and it is thought that 1% of the toxin in unsmoked cigarettes survives as an active component of the smoke. This adds to the many toxins already present in smoking environments, especially in the household. Endotoxin is being increasingly implicated in domestic situations, particularly in damp housing and buildings with contaminated ventilation systems. It is constantly present in house dust, though specific sources of the toxin in the home and seasonal variations in concentration remain poorly understood. Home airborne endotoxin levels display a closer relationship with moisture than to temperature (Park et al., 2000) and other studies indicate that childhood respiratory illnesses are linked to dampness in the home, which could be due to the presence of molds harboring a number of toxic compounds, including glucans.

31.4 GLUCAN—AN EMERGING TOXICANT

Although endotoxin was the first significant component of industrial and domestic dust to be identified and investigated as a specific agent causing lung disease, this contaminant is not exclusively responsible for all environmental respiratory diseases and other candidates are known to exist. Thus, glucans (the polymers of glucose derived from the inner walls of various fungi, bacteria, and plant material) are now also implicated as a potential cause of respiratory disorders. The most significant glucans with respect to inhalation toxicology are fungal-derived glucans, because these are associated with indoor environmental mold. These toxins have been implicated as a cause of "sick building syndrome" where occupants complain of symptoms such as nasal and throat irritation. Glucans are also present in varying quantities in organic dusts due to fungal contamination of the parent fibers. The action mechanism(s) of glucans and endotoxin are thought to be different, although they appear to be synergistic when both toxicants are inhaled together.

31.4.1 Structure and Function

Glucans are glucose polymers consisting of glucopyranosyl subunits, which occur with α or β linkages; Figure 31.2 shows the basic structure. The most biologically active forms have a (1→3)-β-D backbone. (1→3)-β-D-Glucans (glucans/β-glucans) are important components in the cell walls of fungi, bacteria, and plants. They are also present in small amounts in fungal cytosol and as polymers secreted into the environment by certain bacteria. Glucans have known immune stimulatory effects and have now also been implicated in inhalation toxicity. In the fungal wall, glucans are linked to proteins, lipids, and other carbohydrates, forming a network of branches. The role of glucans in fungi is thought to be structural because they maintain the rigidity and integrity of the cell wall (Williams, 1996). Glucans can be branched or nonbranched and are able to exist as a single, helical,

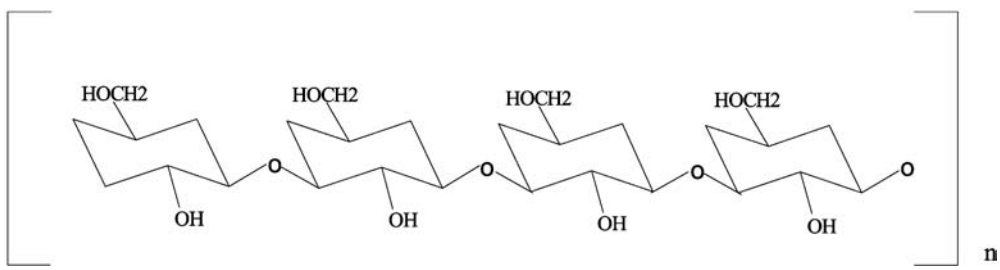


FIGURE 31.2 The basic structure of (1–3)-β-D-glucan.

polymer strand or as a complex of three polymer strands forming a triple helix. It is the triple helix that is most common in nature, probably because its stable structure caused by extensive hydrogen bonding (Williams, 1996). The biological activity of glucans is dependent on factors such as molecular weight, degree of branching, ultrastructure, and source (Young et al., 1998).

31.4.2 Pharmacology and Toxicology

The exact mode of action of this group of toxins has not yet been fully unraveled, though they are known to possess a wide range of biological activities. Some glucans have the ability to enhance the function of macrophages and neutrophils providing protection to the host against cancer, microbes, and radiation, and it is because of these properties that they are known pharmacologically as "biological response modifiers" (BRMs) (Bohn and BeMiller, 1995). Glucans however, are also well documented as potent immunotoxicants and zymosan, a β-glucan-containing particle prepared from the yeast *Saccharomyces cerevisiae* (baker's yeast), is a model for inducing acute inflammatory response. It has become apparent that soluble glucans are less biologically active, hence demonstrating less toxicity than particulate molecules (Young et al., 2003). Therefore, only soluble glucans have been clinically exploited; two of these, lentinan and sonifilan, have been approved for use as anticancer agents in Japan (Ohno et al., 1998).

Insoluble glucans (e.g., curdlan) exhibit several toxic effects, and in addition to causing inflammation and pain, they can also be involved in granuloma formation (Šandula et al., 1999). Zymosan has also been implicated in the formation of granulomas in the lung (Fogelmark et al., 1994). This is probably because vertebrates do not possess specific β-glucan hydrolases, so the molecule is broken down by slow oxidative degradation, allowing it to remain inside cells for weeks or months (Ohno et al., 1999). The insoluble and particulate glucans such as curdlan tend to have greater pharmacological effects, most importantly on macrophages, leading to severe inflammation.

The first cellular target of glucan is the macrophage, which is subsequently activated. There are thought to be two phases to this interaction: an initial binding phase followed by internalization (Williams et al., 1997). The macrophage mannose receptor (MR) and CR3 identified on neutrophils, mast cells, and natural killer cells (and also involved in LPS recognition) have been considered for some time as major macrophage lectins involved in the recognition of yeast-derived particles. However, other receptors have been discovered, and more recently, Dectin-1 was found to be a major β-glucan receptor on macrophages (Brown et al., 2002). A (1→3)-β-D-Glucan-specific receptor has also been described (Czop and Austen, 1985; Mueller, et al., 2000; Williams et al., 1999). This specificity may extend to differentiation between (1→)-β-D-glucans, as a wide range of receptor affinity has been described (Williams et al., 1999).

The binding of glucans activates macrophages, leading to the production and release of inflammatory mediators. *In vitro* studies have shown that the glucan grifolan stimulates the secretion of TNF-α and IL-6 from cultured macrophages (Thorn et al., 2001) as well as the production of chemotactic factors (Milanowski, 1997). This is further demonstrated *in vivo* by neutrophil migration (Nicholls et al., 2002). The stimulation of monocytes by β-glucan *in vitro* has also been found to cause IL-1 production (Abel and Czop, 1992). Although the release of these mediators implies a similar response to that elicited by endotoxin, inhalation of β-glucan does not cause an increase in neutrophils in the lungs. As neutrophil recruitment is characteristic of endotoxin inhalation, this indicates that glucans operate by a different mechanism (Thorn et al., 2001). Glucans have been shown to potentiate ovalbumin-induced infiltration of eosinophils into guinea pig airways on chronic exposure (Fogelmark et al., 2001). However, another study has reported that ovalbumin-induced eosinophilia is decreased by glucan (Rylander and Holt, 1998). These anomalies, which highlight the difficulties of studying such complex agents, may be due to the utilization of different glucan types, exposure regimes, and/or animal species.

Glucans are able to modify host responses to endotoxin, although data regarding the nature of modification is conflicting. There is evidence that glucan acts as an endotoxin adjuvant, yielding

an increase in the production of reactive oxygen metabolites. For example, baseline production of oxygen radicals on exposure to zymosan *in vitro* was increased in guinea pig lung lavage cells from endotoxin-exposed animals up to 48 h post endotoxin exposure (Hsieh et al., 1994). Increased inflammatory responses have also been demonstrated in animal experiments after simultaneous glucan/endotoxin exposure (Cook et al., 1980; Fogelmark et al., 1994). However, other studies have revealed that in some cases glucans can "protect" against endotoxin exposure by suppressing pro-inflammatory cytokines. Rylander (1994a) exposed guinea pigs to endotoxin and glucan simultaneously and found that the characteristic increase in neutrophils was smaller than with endotoxin exposure alone (a protective effect). However, if glucan exposure is carried out first, then followed 7 d later by endotoxin exposure, a larger increase in neutrophil influx is seen compared with endotoxin alone (an adjuvant effect). The authors suggested that the protective response seen after simultaneous endotoxin/β-D-glucan exposure is due to the glucan exerting toxic effects, which reduce macrophage function, as opposed to a protective effect. Hence, fewer neutrophil chemotactic factors are released, resulting in reduced numbers of macrophages in the airways. Sometime after exposure, macrophage adaptation may occur causing an increase in metabolic activity; this could account for the increased number of recruited neutrophils observed 7 d later (Rylander, 1994a).

A study investigating the secretion of TNF-α from macrophages in the presence of toxicants found that glucan concentrations of less than 500 μg/ml stimulated TNF-α release from macrophages. However, glucan concentrations greater than 500 μg/ml suppressed TNF-α release (Hoffman et al., 1993). High concentrations of glucan in this instance did not affect macrophage viability; hence, glucan suppression of TNF-α was unlikely to be due to any toxic effect exerted by glucan. Many other studies have found that particulate glucans, in particular those derived from baker's yeast, can exhibit potent cytotoxicity to macrophages at levels ranging from 100 μg/ml to 1000 μg/ml (Milanowski, 1997; Young et al., 2002). Differences in experimental protocol may well be responsible for conflicts in data and it has been suggested that soluble β-glucans demonstrate inhibitory effects towards endotoxin, whereas enhancement is usually associated with particulate (insoluble) glucans (Young et al., 2002). These findings emphasize the complexity of β-glucan activity on lung tissues and cells. They also suggest that the degree of toxicological risk is not simply defined by straightforward measurement of endotoxin and glucan alone; other influential factors must be considered.

31.4.3 Human Relevance and Risk Assessment

In a large-scale indoor study, a correlation was revealed between elevated concentrations of β-glucan and the proportion of individuals with nasal and throat irritation, dry cough, headache, and excessive tiredness (Rylander, 1996). In a similar investigation comparing two schools, one with elevated levels of mold (β-glucans = 15.3 ng/m^3) and one devoid of this problem (β-glucans = 2.9 ng/m^3), it was shown that the prevalence of respiratory symptoms was significantly higher in the school with β-glucan elevation (Rylander et al., 1998). This finding explains why glucans are now strongly associated with "sick building" syndrome, where particular buildings are linked with higher levels of staff absence due to illness.

Recorded information on β-glucan levels in occupational environments is not widely available, although values up to 370 ng/m^3 have been measured in occupational and general environments (Thorn et al., 2001). The threshold for β-glucan-induced biological effects to occur in humans is not known. However, it has been established that the threshold for animals is below 370 ng/m^3. Hence, the precise risk to workers is largely undefined but probably considerable. A day care center with a history of dampness had recorded glucan levels of 11.4 ng/m^3 (Rylander, 1997), indicating that reported values may be divergent. Glucan levels have also been measured in significant quantities in house dust, and these levels were found to correlate with endotoxin concentrations, a fact which has numerous implications for domestic air quality (Gehring et al., 2001).

As the efficient and reliable measurement of β-glucans can be an indicator of fungal biomass, they are relevant markers for the investigation of indoor air quality and for monitoring occupational

and environmental health (Beijer et al., 1998). Increased interest in molds and fungi has resulted in identification of β-glucan as a suspected agent causing airway dysfunction. Despite this focus, little has been done to identify levels of glucans in the indoor environment and their cellular effects. Future work should concentrate on further evaluation of this agent on the respiratory system in addition to determining the quantities distributed throughout both industrial and domestic environments.

31.5 HUMAN STUDIES

Human inhalation studies are difficult to carry out with toxicants such as endotoxin and glucans, and those that have been performed are restricted to short-term acute exposures in a limited number of subjects. An example is a study carried out by Herbert et al. (1992) on eight nonsmoking subjects who inhaled LPS suspended in distilled water at a concentration of 50 μg/ml aerosolized by a jet nebulizer. Effects of the LPS or control were assessed at intervals up to 24 h after exposure by measuring pulmonary function. Such studies have described the *in vivo* effect of endotoxin in humans, which include a decrease in FEV_1 and changes in blood leukocyte count. The results reveal the timescale on which these changes occur and the duration of response. However, our understanding of human responses is clouded by the innate differences in experimental procedure. The doses and type of endotoxin used in human studies are wide ranging and this may be a source of incomparability. There have only been approximately 40 human response studies carried out with respect to endotoxin, with the majority of investigations each involving less than 20 subjects (Thorn, 2001). There have been even fewer human studies carried out with glucans. One example is an investigation conducted to examine the effects after inhalation of glucan in healthy human subjects where a suspension of β-glucan was inhaled and comparison made with saline control. Subjects were examined before exposure, then 24 and 72 h later by using spirometry (Thorn et al., 2001). Results from this type of study yield valuable information regarding essentially uncharacterized responses elicited by glucans and permit comparison with endotoxin exposure.

Experimental cardrooms have also been used as a research tool for the assessment of human risk from dust exposure (Castellan et al., 1984; Haglind and Rylander, 1984). In this context, the exposure levels occurring during commercial fiber processing are simulated to establish the effects on workers or previously unexposed subjects in a controlled environment employing standard spirometry. With this type of methodology, dose–response relationships have been shown between dust levels and FEV_1 decrement over the working shift. Cardroom experiments have also disclosed that the threshold level for producing a 5% decrease in FEV_1 is lower for smokers (Haglind and Rylander, 1984).

In contrast to the simulated environment, data are often collected by studying lung function decrement in the industrial setting, where employees are regularly exposed to dust and endotoxin. This has been done by means of respiratory questionnaire and spirometric testing before and after working shifts. A follow-up study may sometimes be instigated several years later. This type of investigation has been commonly pursued in cotton mills, where occupational endotoxin was first detected (Christiani et al., 1994, 1999; Cinkotai et al., 1988). Risk factors specific to individual workers such as age, type of work, and smoking history have been usefully examined by this style of assessment.

Human studies have generated essential data regarding the actions of dusts and toxicants in the real-life situation. This information is of vital importance to the prevention of occupational lung disease, because knowledge about threshold levels and risk factors relevant to individual workers will assist in the implementation of safety limits for specific dust contaminants such as endotoxin and glucans.

31.6 ANIMAL STUDIES

The majority of information available concerning inhalation toxicology of organic dusts and their contaminants has been derived from animal studies. Acute and subchronic animal toxicology models of inflammation are well established and these models were originally used to elucidate the exact components of organic dusts responsible for the respiratory effects so long observed in humans. They

have also been used to determine exposure–response relationships to investigate disease mechanisms and provide quantitative data to enable risk assessments to be carried out (Thorne, 2000). *In vivo* studies are widely employed and mainly involve guinea pigs because they have sensitive airways that readily demonstrate bronchoconstriction, which can be measured using plethysmography. Dose-dependent effects of glucan exposure have been shown using nebulized baker's yeast glucan in concentrations from 1 to 300 μg/ml, guinea pigs were exposed daily for 1 h over 5 d and responses measured using whole-body plethysmography; airway hyperreactivity was shown on subsequent histamine challenge (Nicholls et al., 2002). This technique has been used successfully in a number of studies, including the investigation of the timescale of induced hyperreactivity by endotoxin (Davey and Nicholls, 1994). However, some differences in results arise, probably because of the different aspects of the experimental protocols, including specific type and concentration of toxicant as well as exposure duration and subject history. These differences are highlighted in Table 31.6, which details *in vivo* studies carried out by a number of research groups (Gregory, 2002).

Perfused lung and tracheal spiral protocols have been successfully utilized (mainly with guinea pig tissue). These isolated tissues have been used to analyze the direct actions of suspect agents, allowing the simultaneous collection and investigation of experimentally released mediators. Alternatively, these systems are often used to examine the phenomenon of bronchial hyperresponsiveness, where the degree of bronchoconstriction caused by known mediators such as histamine or methacholine increases on incubation with a suspect agent. The response of *in vitro* tracheal spirals to endotoxin has been shown to be comparable to the response seen *in vivo* (Young et al., 1994). These experiments involve simple equipment; hence, sophisticated techniques are not required. However, there are drawbacks: the entire respiratory system cannot be examined as isolated tracheal spirals and lung perfusions do not include the nasal passages; this may be significant when using obligate nose breathers such as guinea pigs (Young and Nicholls, 1994).

Macrophages control the initial inflammatory response to inhaled aerosols; hence, these cells are routinely isolated and exposed to dusts, toxicants, and other contaminants *in vitro*. Macrophages are commonly obtained by bronchoalveolar lavage, followed by density gradient centrifugation. These cells may be harvested from unexposed animals, which are subsequently challenged *in vitro* with, for example, LPS, or they may be examined for increased production of inflammatory indicators, such as cytokines and TNF-α. The technique utilized in Figures 31.3a and b was to isolate macrophages from healthy guinea pigs. These cells were then incubated with 100 μg/ml baker's yeast glucan for 1 h. In a purely visual assessment of the effects of glucan, healthy macrophages are shown by electron micrograph to adhere to a microscope slide coverslip, whereas those damaged by glucan incubation are unable to do this, resulting in reduced cell density compared with the control micrograph, where only fragments of these cells remain. Alternatively, cells taken from pretreated animals can be characterized or enumerated and compared with those collected from controls. In a variation of this, chemotactic assays have been carried out using guinea pig alveolar macrophages, as cellular recruitment is characteristic of an immune response (Milanowski et al., 1995).

Using a variety of *in vivo* and *in vitro* paradigms as described above, it has also been found (Nicholls, 1999) that LPS exerts a marked toxic effect on the airway epithelium, leading to its shedding from the basement membrane. Such an action may be responsible, in part, for the increased reactivity of the airway to inhaled materials following exposure to LPS. Thus, animal studies have served as the foundation of much investigational discovery centered around occupational and environmental toxicants. Increased standardization of experimental protocols will extend the value of extrapolation from animal to human even further. Notwithstanding this notion, animal studies will continue to complement human-based research in this area.

31.7 CONCLUSIONS

The undeniable volume of literature published on endotoxin and glucan inhalation toxicology is a consequence of the intricate nature of the biochemistry and immunology of these agents at the cellular

TABLE 31.6 The Different Exposure Protocols and Effects Obtained *In Vivo* on Exposure to LPS and (1,3)-β-D-Glucan, Alone or in Combination in Guinea Pigs and Humans[a]

Species	Exposure protocol	Reported effect on lungs	Reference
Guinea pig nsens healthy	LPS alone.	Increase in neutrophils.	Rylander (1994b)
	LPS and curdlan (chronic 40 h, 5 d/week for 5 weeks).	Increase in neutrophils reduced compared with LPS alone.	
Guinea pig nsens healthy	Grifolan insoluble 100 μg/ml.	Increase in eosinophils.	Fogelmark et al. (1998)
	LPS 25 μg/ml.	Increase in TCC and neutrophils.	
	Grifolan and LPS at above concentrations (4 h/d, 5 d/week, 5 weeks).	Increase in lymphocytes higher than LPS or grifolan alone, greater increase in eosinophils than grifolan alone.	
Guinea pig nsens healthy	Endotoxin from *E. agglomerans*, 10 μg/ml (3 h).	Increase in breathing rate, increase in TCC, N, L, Er, and M; also increase in superoxide anion.	Milanowski (1997)
	Baker's yeast glucan 200 μg/ml (3 h).	Small increase in breathing rate, increase in TCC, N, L, Er, Et and superoxide anion.	
Human healthy	LPS, four inhalations 6 μg/ml or 0.9 μg/ml (1 h).	Neutrophilia, BAL release TNFα and IL-8, FEV_1, decline.	Jagielo et al. (1996)
Human healthy	LPS, 40 μg/ml (20 breaths, 4 μl per dose, from 500 μg/ml LPS solution.	Decline in FEV_1, and increase in N. MPO, ECP, N, and L increased in sputum.	Thorn and Rylander (1998)
Human asthmatic	LPS, 20 μg (5 breaths, 4 μl per dose, repeated 15–360 min to 20 μg LPS).	BHR, increase in TNFα in blood at 1 h also increase in TCC and N.	Michel (1992)
Human healthy	Endotoxin doses of 20, 30, and 200 μg/ml (15 min nebulized inhalations).	BHR at high concentrations, dose-dependent response change in FEV_1, only at 30 and 200 μg/ml.	Rylander et al. (1989)
Human healthy/atopic	Grifolan solubilized 6 μg/ml (3 h), two groups from high and low β-glucan homes.	No change in airway responsiveness, increase in blood N, MPO, and TNFα.	Beijer (1998)

[a] Abbreviations: sens, sensitized; nsens, nonsensitized; TCC, total cell count; N, neutrophils, L, lymphocytes; Er, erythrocytes; M, macrophages; BAL, broncoalveolar lavage; MPO, myeloperoxidase; ECP, eosinophil cationic protein; BHR, bronchial hyperresponsiveness.

level. Despite these research efforts, many of the underlying mechanisms involved in the toxicology of these biological agents have yet to be adequately resolved, and this issue is compounded by discordant findings from research groups.

In the occupational environment, safety limits for dusts exist; however, concentration alone does not define the overall dust-exposure risk to employees. Other factors require consideration, with respect to the exposure parameters and composition of dust contaminants along with individual

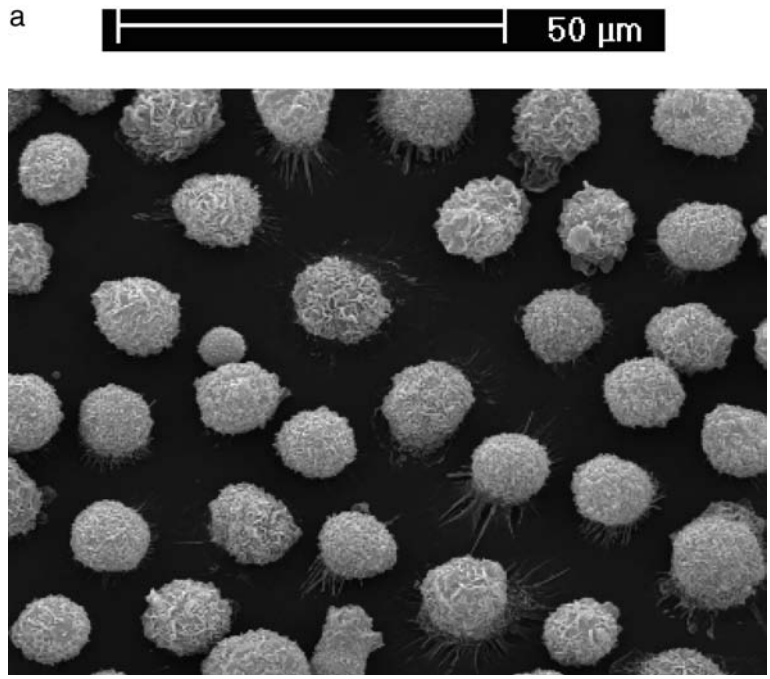


FIGURE 31.3a Electron micrograph showing healthy guinea pig macrophages adhered to a microscope slide coverslip. Figure courtesy of Matthew Lewis-Lakelin.

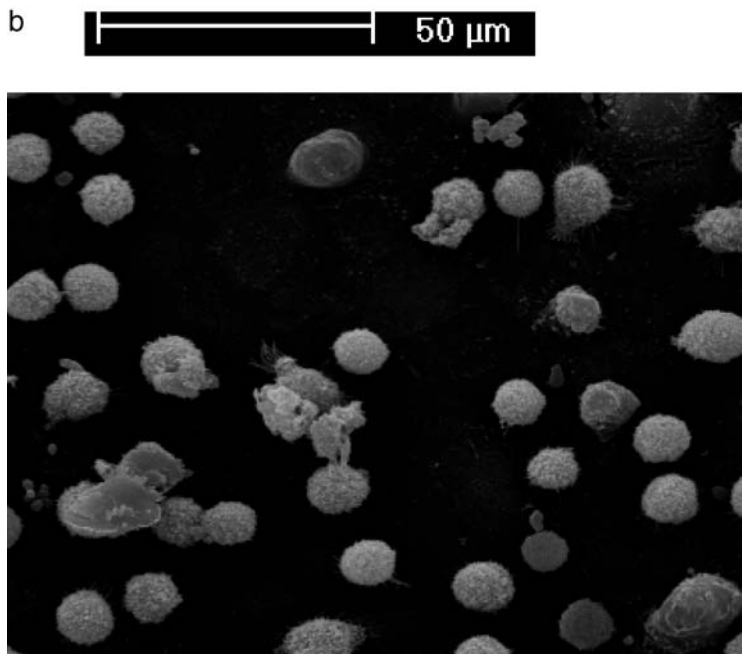


FIGURE 31.3b Electron micrograph showing guinea pig macrophages incubated with 100 μg/ml baker's yeast glucan for 1 h. Figure courtesy of Matthew Lewis-Lakelin.

worker-related characteristics. Endotoxin risk has traditionally focused on the industrial perspective, though this must be tempered by the fact that it is also present in house dust. The implication of endotoxin in the domestic situation has significant repercussions, especially when the duration of time spent in this environment is considered. By the same token, glucan is more frequently associated with the built environment and evidence of its role in "sick building syndrome" will inevitably continue to mount.

Standardization of toxicant measurement protocols and monitoring methods will be a vital step toward prevention of toxicant-induced lung disorders, because this will aid development and implementation of routine toxin screening both in the domestic and occupational setting. Such advances, however, must be coupled with deeper understanding of toxicant biological response mechanisms and their impact on respiratory function.

ACKNOWLEDGMENTS

This work was sponsored by the British Cotton Growing Association and the Liverpool Cotton Research Corporation, Liverpool, UK. The authors thank Clive Gregory for his assistance during the preparation of this manuscript.

REFERENCES

Abel, G., and Czop, J.K., Stimulation of human monocytess beta-glucan receptors by glucan particles induces production of TNF-α and IL-1 beta, *Int. J. Immunopharmacol.*, 14, 1363–1373, 1992.

Akira, S., Mammmalian Toll-like receptors, *Curr. Opin. Immunol.*, 15, 1–7, 2003.

Beijer, L., Thorn, J., and Rylander, R., Inhalation of the (1–3)-β-D-glucan in humans, in *Proceedings of the 22nd Cotton and Other Organic Dust Research Conference*, Wakelyn, P.J., Jacobs, R.R., and Rylander, R., Eds., Beltwide Cotton Conferences, NCC, Memphis, TN, 1998, pp. 251–253.

Bohn, J.A. and BeMiller, J.N., (1→3)-β-D-Glucans as biological response modifiers; a review of structure-functional activity relationships, *Carbohydr. Polym.*, 28, 3–14, 1995.

Brown, G.D., Taylor, P.R., Read, D.M., Wilment, J.A., Williams, D.L., Martinez-Pomares, L., Wong, S.Y.C., and Gordon, S., Dectin-1 is a major beta-glucan receptor on macrophages, *J. Exp. Med.*, 196, 407–412, 2002.

Buick, J.B. and Magee, T.R.A., Microbial contamination of flax dust, *Resour. Conserv. Recycl.*, 27, 99–104, 1999.

Caroff, M., Kariban, D., Cavaillon, J.M., and Haeffner-Cavaillon, N., Structural and functional analyses of bacterial lipopolysaccharides, *Microbes Infect.*, 4, 915–926, 2002.

Castellan, R.M., Olenchock, S.A., Hankinson, J.L., Millner, P.D., Cocke, J.B., Bragg, C.K., Perkins, H.H., and Jacobs, R.R., Acute bronchoconstriction induced by cotton dust: dose-related responses to endotoxin and other dust factors, *Ann. Intern. Med.*, 101, 157–163, 1984.

Christiani, D.C., Ye, T.-T., Wegman, D.H., Eisen, A.E., Dai, H-L., and Lu, P-L., Pulmonary function among cotton textile workers, *Chest*, 105, 1713–1721, 1994.

Christiani, D.C., Ye, T.-T., Zhang, S., Wegman, D.H., Eisen, E.A., Ryan, L.A., Olenchock, S.A., Pothier, L., and Dai, H.L., Cotton dust and endotoxin exposure and long-term decline in lung function: results of a longitudinal study, *Am. J. Ind. Med.*, 35, 321–331, 1999.

Cinkotai, F.F., Seaborn, D., Pickering, C.A.C., and Faragher, E., Airborne dust in the personal breathing zone and prevalence of byssinotic symptoms in the Lancashire textile industry, *Ann. Occup. Hyg.*, 32, 103–113, 1988.

Cloutier, M.M. and Rohrbach, M.S., Effects of endotoxin and tannin isolated from cotton bracts on the airway epithelium, *Am. Rev. Respir. Dis.*, 134, 1158–1162, 1986.

Cook, J.A., Dougherty, W.J., and Holt, T.M., Enhanced sensitivity to endotoxin induced by the RE stimulant, glucan, *Circ. Shock*, 7, 225–238, 1980.

Czop, J.K. and Austen, K.F., Generation of leukotrienes by human monocytes upon stimulation of their β-glucan receptor during phagocytosis, *Proc. Natl. Acad. Sci. USA*, 82, 2751–2755, 1985.

Davey, A.K. and Nicholls, P.J., Airway reactivity and inflammation in the guinea pig following a single endotoxin exposure, in *Proceedings of the 18th Cotton Dust Research Conference*, Wakelyn, P.J., Jacobs, R.R., and Domelsmith, L.N., Eds., Beltwide Cotton Conferences, NCC, Memphis TN, 1994, pp. 298–303.

Dobrovolskaia, M.A. and Vogel, S.N., Toll receptors, CD14, and macrophage activation and deactivation by LPS, *Microbes Infect*., 4, 903–914, 2002.

Douwes, J., Zuidhof, A., Doekes, G., van der Zee, S., Wouters, I., and Boezen, H.M., (1–3)-β-D-glucan and endotoxin in house dust and peak flow variability in children, *Am. J. Respir. Crit. Care Med*., 162, 1348–1354, 2000.

Erridge, C., Bennett-Guerrero, E., and Poxton, I.R., Structure and function of lipopolysaccharides, *Microbes Infect*., 4, 837–851, 2002.

Fernández-Caldas, E., Dust mite allergens: mitigation and control, *Curr. Allergy Asthma Rep*., 2, 424–431, 2002.

Fogelmark, B., Sjorstrand, B.M., and Rylander, R., Pulmonary inflammation induced by repeat inhalations of β(1–3)-D-glucan and endotoxin, *Int. J. Exp. Pathol*., 75, 85–90, 1994.

Fogelmark, B., Thorn, J., and Rylander, R., Inhalation of (1–3)-β-D-glucan (grifolan) causes airway eosinophilia, in *Proceedings of the 22nd Cotton and Other Organic Dusts Research Conference*, Wakelyn, P.J., Jacobs, R.R., and Rylander, R., Eds., Beltwide Cotton Conferences, NCC, Memphis TN, 1998, pp. 251–253.

Fogelmark, B., Thorn, J., and Rylander, R., Inhalation of (1–3)-β-D-glucan causes airway eosinophilia, *Mediat. Inflamm*., 10, 13–19, 2001.

Gehring, U., Douwes, J., Doekes, G., Koch, A., Bischof, W., Fahlbusch, B., Richter, K., Wichmann, H-E., and Heinrich J., β(1–3)-glucan in house dust of German homes: housing characteristics, occupant behavior and relations with endotoxins, allergens and molds, *Environ. Health Perspect*., 109, 139–144, 2001.

Gehring, U., Bischof, W., Fahlbusch, B., Wichmann, H-E., and Heinrich, J., House dust endotoxin and allergic sensitization in children, *Am. J. Respir. Crit. Care Med*., 166, 939–944, 2002.

Gill, C.I.C., Byssinosis in the cotton trade, *Br. J. Ind. Med.*, 4, 48–55, 1947.

Gregory, C.J., Investigation of the Effect of β(1,3)-D-Glucan and Lipopolysaccharide on the Mast Cell, Tissue and Airways of the Lung, Ph.D. thesis, University of Wales, Cardiff, UK, 2002.

Haglind, P. and Rylander, R., Exposure to cotton dust in an experimental cardroom, *Br. J. Ind. Med*., 41, 340–345, 1984.

Hasday, J.D., Bascom, R., Costa, J.J., Fitzgerald, T., and Dubin, W., Bacterial endotoxin is an active component of cigarette smoke, *Chest*, 115, 829–835, 1999.

Heederik, D. and Douwes, J., Towards and occupational exposure limit for endotoxins? *Ann. Agric. Environ. Med.*, 4, 17–19, 1997.

Heinrich, J., Gehring, U., Douwes, J., Koch, A., Fahlbusch, B., Bischof, W., and Wichman, H.E., Pets and vermin are associated with high endotoxin levels in house dust, *Clin. Exp. Allergy*, 31, 1839–1845, 2001.

Henson, P.M., Doherty, D.E., Riches, D.W.H., Parsons, P.E., and Worthen, G.S., LPS and cytokines in lung injury in *Endotoxin and the Lungs*, Brigham, K.L., Ed., Marcell Dekker Inc., New York, 1994, pp. 229–253.

Herbert, A., Carvalheiro, M., Rubenowitz, E., Bake, B., and Rylander, R., Reduction of alveolar-capillary diffusion after inhalation of endotoxin in normal subjects, *Chest*, 102, 1095–1098, 1992.

Heumann, D. and Thierry, R., Initial responses to endotoxins and Gram-negative bacteria, *Int. J. Clin. Chem*., 323, 59–72, 2002.

Hoffman, O.A., Olson, E.J., and Limper, A.H., Fungal β-glucans modulate macrophage release of tumor necrosis factor-α in response to bacterial lipopolysaccharide, *Immunol. Lett*., 37, 19–25, 1993.

Holst, O., Ulmer, A.J., Brade, H., Flad, H-D., and Rietschel, E.T., Biochemistry and cell biology of bacterial endotoxins, *FEMS Immunol. Med. Microbiol*., 16, 83–104, 1996.

Hsieh, V., Amoruso-Marchat, B., Rylander, R., and Polla, B.S., Oxygen metabolites from lavage and interstitial lung cells after inhalation of endotoxin in guinea-pigs, *Int. Arch. Allergy Immunol*., 104, 42–47, 1994.

Jacobs, R., Endotoxins in the environment, *Int. J. Occup. Environ. Health*, 3, S3–S5, 1997.

Jagielo, P.J., Thorne, P.S., Watt, J.L., Frees, K.L., Quinn, T.J., and Schwartz, D.A., Grain dust and endotoxin challenges produce similar inflammatory responses in normal subjects, *Chest*, 110, 263–270, 1996.

Kirikae, T., Lei, M-G., Chen, T-Y., and Morrison, D.C., Interactions of endotoxin with membranes and membrane receptors, in *Endotoxin and the Lungs*, Brigham, K.L., Ed., Marcell Dekker Inc., New York, 1994, pp. 21–39.

Lombardo, L.J. and Balmes, J.R., Occupational asthma: A review, *Environ. Health Perspect*., 108, 697–704, 2000.

Makhlouf, M., Durando, M., Cook, J.A., and Halushka, P.V., Role of eicosanoids in endotoxin-induced lung dysfunction, in *Endotoxin and the Lungs*, Brigham, K.L., Ed., Marcell Dekker Inc., New York, 1994, pp. 153–163.

Medzhitov, R., Preston-Hurlburt, P., and Janeway, J., A human homologue of the Drosophila Toll protein signals activation of adaptive immunity, *Nature*, 388, 394–396, 1997.

Michel, O., Systemic and local airways inflammatory response to endotoxin, *Toxicology*, 152, 25–30, 2000.

Michel, O., Ginanni, R., Le Bon, B., Content, J., Sergysels, R., and Duchateau, J., Inflammatory response to acute inhalation of endotoxin in asthmatic patients, *Am Rev. Respir. Dis*., 146, 352–357, 1992.

Milanowski, J., Experimental studies on the effects of organic dust-derived agents on respiratory system: comparison between endotoxins and glucans, *Inhalation Toxicol*., 9, 369–388, 1997.

Milanowski, J., Sorenson, W.G., Dutkiewicz, J., and Lewis, D.M., Chemotaxis of alveolar macrophages and neutrophils in response to microbial products derived from organic dust, Environ. Res., 69, 59–66, 1995.

Miyake, K., Innate recognition of lipopolysaccharide by CD14 and toll-like receptor-4-MD-2: unique roles for MD-2, *Int. Immunopharmacol*., 3, 119–128, 2003.

Morrison, D.C. and Ryan, J.L., Endotoxins and disease mechanisms, *Ann. Rev. Med*., 38, 417–432, 1987.

Mueller, A., Raptis, J., Rice, P.J., Kalbfleisch, J.H., Stout, R.D., Ensley, H.E., Browder, W., and Williams, D.L., The influence of glucan polymer structure and solution conformation on binding to (1–3)-beta-D-glucan receptors in a human monocytes-like cell line, *Glycobiology*, 4, 339–346, 2000.

Nicholls, P.J., Vegetable dusts and lung disease, in *Indoor Air Pollution; Problems and Priorities*, Leslie, G.B. and Lunau, F.W., Eds., Cambridge University Press, London, 1992, pp. 161–182.

Nicholls, P.J., The wrecking of the wrapping: the airway epithelium, a prime target for inhaled organic dusts, in *Proceedings of the 23rd Cotton and Other Organic Dusts Research Conference*, Wakelyn, Jacobs, and Rylander, Eds., Beltwide Cotton Conferences, NCC, Memphis TN, 1999, pp. 200–202.

Nicholls, P.J., Gregory, C.J., and Lewis-Lakelin, M.B., Acute and chronic exposure to inhaled (1–3)-β- D-glucan causes airway inflammation in the conscious guinea pig, *J. Pharm. Pharmacol*., 53, S100, 2002.

Niven, R.McL., Fletcher, A.M., Pickering, C.A.C., Simpson, J.C.G., Oldham, L.A., and Bakirci, N., Repeatability of sampling for endotoxin and dust within 3 cotton textile mills, in *Proceedings of the 22nd Cotton and Other Organic Dust Research Conference*, Wakelyn, P.J., Jacobs, R.R., and Rylander, R., Eds., Beltwide Cotton Conferences, NCC, Memphis TN, 1998, pp. 199–202.

Ohno, N., Miura, N.N., Adachi, Y., and Yadomae, T., Inflammatory and immunotoxicology activities of (1–3)-β-D-glucans, in *Proceedings of the 22nd Cotton and other Organic Dusts Research Conference*, Wakelyn, P.J., Jacobs, R.R., and Rylander, R., Eds., Beltwide Cotton Conferences, NCC, Memphis, TN, 1998, pp. 240–242.

Ohno, N., Uchiyama, M., Tsuzuki, A., Tokunaka, K., Miura, N.N., Adachi, Y., Aizawa, W., Tamura, H., Tanaka, S., and Yadomae, T., Solubilization of yeast cell wall (1→3)-β-D-glucan by sodium hypochlorite oxidation and dimethyl sulfoxide extraction, *Carbohydr. Res*., 316, 161–172, 1999.

Park, J.H., Spiegelman, D.L., Burge, H.A., Gold, D.R., Chew, G.L., and Milton, D., Longitudinal study of dust and airborne endotoxin in the home, *Environ. Health Perspect*., 108, 1023–1028, 2000.

Parkes, W.R., *Occupational Lung Disorders*,. Butterworths, London, 1983.

Raetz, C.R.H., Biochemistry of endotoxins, *Annu. Rev. Biochem*., 59, 129–170, 1990.

Ramazzini, B., *Diseases of Workers*, transl. from the Latin text *De Morbis Artificum* of 1713, Hafner, New York, 1964.

Rose, C., Hypersensitivity pneumonitis, in *Occupational and Environmental Respiratory Disease*, Harber, P., Schenker, M.B., and Balmes, J., Eds., Mosby, St. Louis, 1996, pp. 201–215.

Rylander, R., Diseases associated with exposure to plant dusts: focus on cotton dust, *Tuber. Lung Dis*., 73, 21–26, 1992.

Rylander, R., Pulmonary immunotoxicology of inhaled (1→3)-β-D-glucan, in *Proceedings of the 18th Cotton and Other Organic Dusts Research Conference*, Wakelyn, P.J., Jacobs, R.R., and Domelsmith, L.N., Eds., Beltwide Cotton Conferences, NCC, Memphis TN, 1994a, 347–348.

Rylander, R., Section 2, in *Organic Dusts: Exposure Effects and Prevention*, Rylander, R., and Jacobs, R.R., Eds., CRC Press, Boca Raton, FL, 1994b, pp. 73–79.

Rylander, R., Airway responsiveness and chest symptoms after inhalation of endotoxin or (1–3)-β-D-glucan, *Indoor Built Environ*., 5, 106–111, 1996.

Rylander, R., Airborne (1→3)-β-D-glucan and airway effects in a day care center before and after renovation, *Arch. Environ. Health*, 52, 281–285, 1997.

Rylander, R., and Holt, P.G., (1–3)-β-D-glucan and endotoxin modulate immune response to inhaled allergen, *Mediat. Inflamm.*, 7, 105–110, 1998.

Rylander, R. and Lin, R-H., (1→3)-β-D-glucanslationship to indoor air-related symptoms, allergy and asthma, *Toxicology*, 47–52, 2000.

Rylander, R., Haglind, P., and Lundholm, M., Endotoxin in cotton dust and respiratory function decrement among cotton workers in an experimental cardroom, *Am. Rev. Respir. Dis.*, 131, 209–213, 1985.

Rylander, R., Bake, B., Fischer, J.J., and Helander, I.M., Pulmonary function and symptoms after inhalation of endotoxin, *Am. Rev. Respir. Dis.*, 140, 981–986, 1989.

Rylander, R., Norrhall, M., Engdahl, U., Tunsalter, A., and Holt, P.G., Airway inflammation, atopy and (1–3)-β-D-glucan exposures in two schools, *Am. J. Respir. Crit. Care Med.*, 158, 1685–1687, 1998.

Sandula, J., Kogan, G., and Ka uráková, E.M., Microbial (1–3)-β-D-glucans, their preparation, physico-chemical characterization and immunomodulatory activity, *Carbohydr. Polym.*, 38, 247–253, 1999.

Schilling, R.S.F., Hughes, J.P.W., Dingwall-Fordyce, I., and Gilson, J.C., An epidemiological study of byssinosis among Lancashire cotton workers, *Br. J. Ind. Med.*, 12, 217–227, 1955.

Schilling, R. and Rylander, R., Cotton dust, in *Organic Dusts—Exposure, Effects and Prevention*, Rylander, R., and Jacobs, R.R., Eds., CRC Press, Boca Raton, FL, 1994, pp. 177–191.

Schwartz, D.A., Inhaled endotoxin a risk for airway disease in some people, *Respir. Physiol.*, 128, 47–55, 2001.

Simpson, J.C.G., Niven, R.McL., Pickering, C.A.C., Oldham, L.A., Fletcher, A.M., and Francis, H.C., Comparative exposures to endotoxin in workers exposed to organic dusts, in *Proceedings of the 20th Cotton and Other Organic Dust Research Conference*, Wakelyn, P.J., Jacobs, R.R., and Rylander, R., Eds., Beltwide Cotton Conferences, NCC, Memphis TN, 1996, pp. 212–215.

Su, H-J., Wu, P-C., Chen, H-L, Lee, F-C., and Lin, L-L., Exposure assessment of indoor allergens, endotoxins, and airborne fungi for homes in Southern Taiwan, *Environ. Res. Sect. A*, 85, 135–144, 2001.

Thorn, J., The inflammatory response in humans after inhalation of bacterial endotoxin: a review, *Inflamm. Res.*, 50, 254–261, 2001.

Thorn, J. and Rylander, R., Inflammatory response after inhalation of bacterial endotoxin assessed by the induced sputum technique, *Thorax*, 53, 1047–1052, 1998.

Thorn, J., Beijer, L., and Rylander, R., Effects after inhalation of (1–3)-β-D-glucan in healthy humans, *Mediat. Inflamm.*, 10, 173–178, 2001.

Thorne, P.S., Inhalation toxicology models of endotoxin and bioaerosol-induced inflammation, *Toxicology*, 152, 13–23, 2000.

Wakelyn, P.J., Greenblatt, G.A., Brown, D.F., and Tripp, V.W., Chemical properties of cotton dust, *Am. Ind. Hyg. Assoc. J.*, January, 22–31, 1976.

Williams, D.L., Overview of (1–3)-β-glucan chemistry, immunology and toxicology, in *Proceedings of the 20th Cotton and Other Organic Dusts Research Conference*, Wakelyn, P.J., Jacobs, R.R., and Rylander, R., Eds., Beltwide Cotton Conferences, NCC, Memphis TN, 1996, pp. 285–288.

Williams, D.L., Mueller, A., Raptis, J., Rice, P., and Kalbfleisch J., Receptor binding of fungal cell wall (1–3)-β-D-glucan to human macrophage and neutrophil cell lines, in *Proceedings of the 23rd Cotton and Other Organic Dusts Research Conference*, Wakelyn, P.J., Jacobs R.R., and Rylander, R., Eds., Beltwide Cotton Conferences, NCC, Memphis TN, 1999, 176–179.

Williams, D.L., Mueller, A., Raptis, J., Rice, P., and Quillen, J.H., Binding of fungal and plant glucans to the human macrophage receptor, in *Proceedings of the 21st Cotton and Other Organic Dusts Research Conference*, Wakelyn, P.J., Jacobs R.R., and Rylander, R., Eds., Beltwide Cotton Conferences, NCC, Memphis TN, 1997, pp. 169–171.

Yi, E.S., Hypersensitivity pneumonitis, *Crit. Rev. Clin. Lab. Sci.*, 39, 581–629, 2002.

Young, R.S. and Nicholls, P.J., Hypereactivity of the airways: a minireview, in *Proceedings of the 18th Cotton Dust Research Conference*, Wakelyn, P.J., Jacobs, R.R., and Domelsmith, L.N., Eds., Beltwide Cotton Conferences NCC, Memphis TN, 1994, pp. 286–289.

Young, R.S., Davey, A.K., and Nicholls, P.J., Airway responses to methacholine in the guinea pig following a single dose of endotoxin: comparison of in vitro and in vivo data, in *Proceedings of the 18th Cotton Dust Research Conference*, Wakelyn, Jacobs, and Domelsmith, Eds., Beltwide Cotton Conferences NCC, Memphis TN, 1994, pp. 295–297.

Young, R.S., Jones, A.M., Nicholls, P.J., Something in the air: Endotoxins and glucans as environmental troublemakers, *J. Pharm. Pharmacol*., 50, 11–17, 1998.

Young, S., Robinson, V.A., Barger, M., Ziedler, P., Porter, D.W., Frazer, D.G., and Castranova, V., Modified endotoxin responses in rats pretreated with 1–3-β-glucan (zymosan A), *Toxicol. Appl. Pharmacol*., 178, 172–179, 2002.

Young, S-H., Robinson, V.A., Barger, M., Whitmer, M., Porter, D.W., Frazer, D.G., and Castranova, V., Exposure to particulate 1→3-β-glucans induces greater pulmonary toxicity than soluble 1→3-β-glucans in rats, *J. Toxicol. Environ. Health Part A*, 66, 25–38, 2003.

32 Fluorocarbon Alternatives—Methodologies for Special Studies and Results

Darol E. Dodd and William J. Brock

CONTENTS

32.1 INTRODUCTION

32.1.1 History and Development of Fluorocarbons

The year 2004 marked the 30th anniversary of the landmark paper by Molina and Rowland (1974), which describes their hypothesis on the depletion of stratospheric ozone by chlorine liberated from long-lived chlorofluorocarbons (CFCs). Nearly ten years later, the Vienna Convention was adopted, an international treaty for the protection of the ozone layer. The Vienna Convention set in motion the negotiations that culminated in the Montreal Protocol agreements of 1987. The Montreal Protocol provided for the immediate freeze and future phasedown of CFC global production. Since that time additional agreements established accelerated schedules of the phasedown and phaseout of CFC production. The Montreal Protocol was the beginning of an end for CFCs that started nearly 70 years before.

The use of chemicals in refrigeration systems began in the mid- to late-1800s when it was found that ammonia in combination with water was capable of producing temperature sufficient to produce ice (Downing, 1988). Sulfur dioxide was found to have properties similar to ammonia and could also be used in this same technology. In the 1880s and 1890s, other chemicals were found to have some refrigeration use and included methyl chloride and carbon dioxide. Because of the increase in refrigeration technology that used ammonia and sulfur dioxide, there were rapidly growing complaints, injuries, and deaths as home refrigerators and air-conditioning equipment sales grew rapidly in the United States into the early 1900s. The compounds that were being used were either toxic or flammable, which limited their use to large-scale commercial refrigeration systems. Searching for alternatives was somewhat limited, primarily because of other technology limitations at the time, but in the 1920s with greater access to electricity and other technological advancements, the search for better refrigeration chemicals began to expand.

Fluorine chemistry was in its infancy in the 1920s when General Motors's chief chemist, Thomas Midgely, was assigned the task of finding a safe substitute for ammonia and sulfur dioxide refrigerants. As a chemist, he knew that he had to find a stable molecule with very high bond energies and, therefore, he focused on chlorine and fluorine chemistry. Within a few short months, CFC-12 (difluorodichloromethane) was selected as the refrigerant of choice. This compound met the criteria set by Midgely, i.e., a compound that was not flammable and had very low toxicity. In 1930, GM and DuPont formed a joint venture, Kinetic Chemical, to produce CFC-12 on a commercial scale. General Motors's refrigerator line Frigidaire® began in 1931. General Motors chose to give the technology to society rather than patent it, so CFC-12 was quickly adopted as the global standard refrigerant. Shortly after the introduction of CFC-12, other CFCs were introduced over the next 5 years, primarily as refrigerants, and included CFC-11, CFC-114, and CFC-113. With World War II, further research into other CFCs slowed, although HCFC-22 was developed as another refrigerant with large-scale production occurring in the late 1940s.

32.1.1.1 Development of Fluorocarbons as Propellants, Solvents, and Fire Suppression Agents

The physical and chemical properties of this class of compounds resulted in substantial commercial growth over the next 30 years and included new applications, including propellants for consumer products, fire-extinguishing applications, solvents for cleaning applications, and chemical intermediates. During WWII, there was a need for insecticide repellants for combat soldiers in the Pacific. The Department of Agriculture at that time developed and used CFC-12 in a new application—aerosol propellant. With this application, others began to use CFCs for similar products, including consumer products (deodorants), handheld fire extinguishers, and portable glass chillers. Other applications emerged. Compounds such as CFC-113 were found to have chemical properties ideal for a solvent and specialized electronic cleaning agent because it evaporated quickly at room temperature, leaving essentially no residue. At one point in commercial development, there was consideration for use of

some fluorocarbons as personal cleaning agents, e.g., shampoos. However, these applications were quickly discounted because of costs and potential personal injury.

32.1.1.2 Environmental Fate of CFCs, Ozone Depletion, and the Montreal Protocol

The rapid market growth for the CFCs and the recognized chemical stability of these compounds prompted Dr. Ray McCarthy, then DuPont's Freon Products Laboratory Director, in 1970 to pose questions regarding the environmental fate of the CFCs once released to the atmosphere. Dr. McCarthy's questions led Dr. James Lovelock to develop an instrument, using an electron capture detector, to measure parts per trillion concentration of CFC in the atmosphere (Lovelock, 1971). He placed the instrument on a ship, making a round-trip voyage from England to South Africa, enabling him to obtain a latitudinal concentration profile. With this information, Dr. McCarthy determined, as a first approximation, that atmospheric accumulation of CFCs equaled total global production. That is, there were no significant sinks, and there was no significant atmospheric degradation of CFCs. The research of Dr. Lovelock led to other research into the atmospheric chemistry of the CFCs that included the work of Molina and Rowland. Molina and Rowland (1974) suggested that the chlorine (or bromine) atoms of the CFCs reacted with ozone in the upper atmosphere, resulting in the formation of chlorine (or bromine) oxides, thereby reducing the levels of ozone. For their work, Molina and Rowland shared the Nobel Prize in Chemistry in 1995.

The 1970s were an active time for research into the effects of CFCs on the stratospheric ozone levels, as well as international policy changes. A number of researchers became integral in the search for a mechanism into ozone depletion, whereas different levels of federal governments began to shape laws and regulations that effectively reduced intentional release of the CFCs. In 1977, the U.S. Food and Drug Administration (FDA) banned the use of aerosol propellants for certain consumer products, e.g., deodorants, and the U.S. Environmental Protection Agency (EPA) developed regulations in conjunction with the Clean Air Act to reduce the nonessential uses of CFCs. Regulatory changes such as these eventually lead to international discussions and cooperation between governments, all culminating in 1987 with the adoption of the Montreal Protocol along with the Copenhagen amendments that outlined a process and timeline for the phaseout of the use of the CFCs.

Evidence from atmospheric research demonstrated that an increasing amount of fluorocarbons was reaching the stratosphere and that there was a relationship between production and the time when the fluorocarbon reached the stratosphere. McFarland and Kaye (1992) estimated a 3–4 year delay. Hence, policy changes implemented in one year would not have an immediate effect on reducing ozone depletion for several years. This science became recognized in the proceedings of the Montreal Protocol and all subsequent amendments. Developed countries were to eliminate the production of CFCs by 2000. Developing countries, then known as Article 5 countries, were exempt for a period of 10 years from this phaseout schedule. The Copenhagen amendment of 1992 to the Montreal Protocol accelerated the phaseout among developed countries to 1995. In addition, the CFC alternatives, hydrochlorofluorocarbons (HCFCs) were added to the list of compounds to eliminate. The phaseout of HCFCs was delayed to 2030.

32.1.2 Toxicology Programs

32.1.2.1 Program for Alternative Fluorocarbon Testing—An Industry Consortium

From the foregoing, it is clear that alternatives to the CFCs were needed to fill the potential void for refrigerants, propellants, fire-extinguishing agents, etc. Many of the fluorocarbon-producing companies had very active research and development programs in place to find new CFC alternatives, but these companies recognized that the expense for toxicological testing could limit commercial production. In 1987, in concert with the signing of the Montreal Protocol, DuPont,

Allied-Signal (now Honeywell), Atochem (now Arkema), and ICI agreed to form a consortium made up of toxicologists, but with oversight from a management committee of representatives from each company. This consortium became known as PAFT, Program for Alternative Fluorocarbon Toxicity Testing. Over time, participation in PAFT by other companies expanded from the original four companies to nearly 15 companies, representing the major industrialized regions of the world—United States, Europe, and Japan. Within PAFT, the toxicology database for eight separate fluorocarbon substitutes was developed ranging from acute toxicity studies through two-year inhalation bioassays, metabolism and mechanistic studies (Table 32.1). PAFT completed its work in 1998.

PAFT Group M (Mechanisms) was created in response to potentially adverse responses observed in the two-year carcinogenicity studies conducted with HCFC-123 and HFC-134a. An increased incidence of benign tumors of the liver, testes, or pancreas were observed in rats. The data from other testing with primarily HCFC-123 suggested a mechanism of peroxisome induction (see Results section). In addition, findings from the two-generation reproduction study with HCFC-123 created health hazard concerns. Hence, this PAFT group conceived research programs in explaining the results of these studies.

32.1.2.2 International Pharmaceutical Aerosol Consortium

In addition to the basic toxicology programs developed by PAFT, another consortium was created because of its interest in finding alternatives to CFC-12 for use in metered dose inhalers (MDIs) for administration of pharmaceuticals in the treatment of, for example, asthma. This consortium was the International Pharmaceutical Aerosol Consortium (IPAC). The toxicology programs established by this group were designed for a specific purpose. The first two compounds this group investigated were HFC-134a and HFC-227ea. Although IPAC and PAFT operated under different missions, there was a great deal of cooperation between the two groups, including the sharing of data, assistance in procuring compounds for testing, and the development of protocols.

32.1.3 Regulatory Environment

32.1.3.1 Creation of Significant New Alternatives Policy

Under Title VI of the U.S. Clean Air Act (CAA) of 1990 (Public Law 101–549), the U.S. EPA is mandated to evaluate alternatives to Class I ozone-depleting substances, including halon fire and explosion protection agents, to reduce the overall risk to human health and the environment. Section 612 of the CAA requires EPA to develop a program to evaluate the risks to human health and the environment posed by the substitutes. EPA's program to carry out the mandate is called Significant New Alternatives Policy (SNAP). The SNAP program became effective April 18, 1994 (59 FR 13044) and allows EPA to make decisions on a proposed substitute in a particular end use (e.g.,

Table 32.1 Program for Fluorocarbon Toxicity Testing Groups

PAFT Group	Compound examined	Intended replacement
I	HCFC-123, HFC-134a	CFC-11, CFC-12, Halon 1211
II	HCFC-141b	CFC-11
III	HFC-125, HCFC-124	CFC-114, CFC-115, Halon 1301
IV	HCFC-225, HCFC-225cb	CFC-113
V	HFC-32	CFC-115, HCFC-22 and refrigerant blends
M	Mechanistic Studies	See text

household refrigerators) within a sector (e.g., refrigeration and air conditioning). The SNAP program not only evaluates chemical substitutes but also alternative manufacturing processes such as water mist systems for fire and explosion protection technologies. Evaluation criteria used by EPA for making decisions on substitutes include environmental impact, health and safety, efficacy, and market potential. Acceptable substitutes are categorized further on the basis of limitations imposed by EPA. The categories include acceptable, acceptable subject to narrowed use limits, and acceptable subject to use conditions. Examples of categorization under SNAP in the fire and explosion protection sector for Halon 1301 total flooding agent are given in Table 32.2.

32.1.3.2 Relationship of SNAP with TSCA and Other Regulations

EPA is empowered by the Toxic Substances Control Act (TSCA; Public Law 94–469) to promulgate standards for different types of toxicological studies. In concert with SNAP, EPA believes that the statutory language included in section 612 of the CAA is written broadly to allow for a reasonably comprehensive evaluation of substitutes that will be introduced as replacements for ozone-depleting chemicals. Section 612(e) requires producers of chemicals, both new and existing, to notify EPA before introducing such chemicals into interstate commerce for significant new uses as class I alternatives. As stated previously, section 612(c) requires EPA to produce lists of acceptable and unacceptable substitutes. These interrelated provisions of section 612 serve as the basis for EPA's belief that all substitutes, whether "new or existing" chemicals, should be subject to SNAP review. This regulatory purview extends to those chemicals already listed on the TSCA inventory of existing chemicals. EPA believes SNAP review is critical for such chemicals given the differing statutory objectives of TSCA and the CAA, and the new and expanded applications of many existing chemicals as class I and II ozone-depleting substances (ODS) replacements, which could alter existing release and exposure profiles.

EPA works in concert with several agencies on assessing environmental and health risks associated with ODS substitutes. As seen from Table 32.2, to control exposure, EPA patterned use restrictions after

TABLE 32.2 Halon 1301 Substitute, EPA SNAP Category, and Limitations or Conditions

Halon 1301 substitute	EPA SNAP category	Limitations or conditions
Powdered Aerosol A (Trade Name SFE)	Acceptable	For use in unoccupied areas only
Perfluoropropane (Trade Name PFC-218 and CEA-308)	Acceptable substitute subject to narrowed use limits	For nonresidential uses where other alternatives are not technically feasible due to performance or safety requirements (a) because of their physical or chemical properties, or (b) where human exposure to the extinguishing agents may result in failure to meet applicable use conditions
HCFC-22	Acceptable substitute subject to use conditions	Where egress from an area cannot be accomplished within 1 min, the employer shall not use this agent in concentrations exceeding its cardiotoxic NOAEL of 2.5%; where egress takes longer than 30 sec but less than 1 min, the employer shall not use the agent in a concentration greater than its cardiotoxic LOAEL of 5.0%; HCFC-22 concentrations >5.0% are only permitted in areas not normally occupied by employees provided that any employee in the area can escape within 30 sec, and the employer shall assure that no unprotected employees enter the area during agent discharge

the Occupational Safety and Health Administration (OSHA) standards for the use of Halon 1301 in fixed systems (29 CFR 1910.162). EPA interpreted OSHA's egress times as mandatory in workplaces, although some users have taken issue with EPA on this position. In the fire and explosion protection sector, EPA works with the National Fire Protection Association (NFPA). In general, NFPA qualifies how and when substitutes should be used, including safety standards as outlined in NFPA 2001 Standard for Clean Agent Fire Extinguishing Systems. Recently, EPA rescinded use conditions imposed under the SNAP program that limit human exposure to halocarbon and inert gas agents used in the fire suppression and explosion protection industry to avoid redundancy with NFPA 2001. On occasion, EPA partners with associations in an effort to minimize impact of ODS substitutes on environment or health. For example, the Fire Equipment Manufacturers' Association (FEMA), Fire Suppression Systems Association (FSSA), Halon Alternatives Research Corporation (HARC), and the National Association of Fire Equipment Distributors (NAFED) worked with EPA to develop a voluntary code of practice to minimize unnecessary emissions of HFCs. As part of this partnership, industry and EPA initiated the HFC Emissions Estimating Program (HEEP) to monitor emissions of HFCs from the fire protection sector.

32.1.3.3 International Regulations

The Montreal Protocol was signed by 24 nations and the European Economic Community. International harmonization of toxicological requirements for ODS substitutes is best represented by the PAFT (described previously). The PAFT represents a unique effort by most CFC producers from the United States, Asia, and Europe to evaluate the toxicology of limited production or research chemicals to support the introduction and use of these chemicals as substitutes for widely used compounds. In addition to toxicology evaluation, management and product quality are part of the PAFT effort.

32.2 TOXICOLOGY METHODS FOR FLUOROCARBON ALTERNATIVES

The phaseout of the CFCs brought about significant changes to the fluorocarbon market for companies. The compounds that had for decades been made available because of their stability were the principal reason why they were being eliminated. Research and development into the alternatives took on a frantic pace during the 1980s and 1990s as companies attempted to replace the phased-out CFCs. Much of the data that were generated during this time had been published in peer-reviewed journals and presented at professional meetings and symposia. Indeed, a basic tenet of PAFT at the time was to publish the data, making it available to everyone that had an interest in this class of compounds. There is, however, no single source of data for all of these compounds. The purpose of this section on fluorocarbon alternatives is to review the toxicology data for many of these alternative compounds, with special attention to those data generated by inhalation exposures. In addition, the variety of regulations that are in effect for these compounds will be discussed as well as those protocols developed to examine certain end points of toxicological interest.

32.2.1 Toxicity-Testing Guidelines

Numerous testing guidelines have been published over the past 20 or so years, and these guidelines for testing have become essentially "required" rather than just "guidelines" for the development of protocols for testing. Guidelines for inhalation toxicology studies are specifically referenced in the OECD, TSCA, and FIFRA Guidelines (Table 32.3). In all of these guidelines, however, the generation of inhalation atmospheres is not specifically recommended other than to indicate that the chamber atmospheres should be homogeneous and stable. For compounds that require the generation of particles, some guidance is provided to ensure that respirable particle sizes are created during

inhalation exposures. Overall, the specifics of atmosphere generation are left to the study director to ensure a properly designed study.

These toxicity-testing guidelines make reference to conducting studies by the most appropriate route of potential human exposure, although the inhalation route is not mandated *per se.* The FIFRA guideline is the only guideline that specifies an inhalation study for fibrous materials. Detailed descriptions of the inhalation toxicity and methods are provided in other chapters.

32.2.1.1 Methodology Considerations

The commonality across all of these guidelines involves the need for at least three exposure concentrations; measuring of clinical observations before, during, and after exposure; and measuring other in-life parameters, e.g., food consumption, body weights. As the study duration increases, other parameters are to be included such as hematological and clinical chemistry measures. Over the past several years, greater emphasis has been placed on determining the effects of chemicals on neurological development, e.g., developmental neurotoxicity studies, as well as assessing neurological effects of the adult animal. A functional observational battery (FOB) and Irwin testing have become more common in repeated-exposure studies such that many of the regulatory authorities specify FOB within 90-d studies. For the fluorocarbons, however, FOB determinations during exposure are difficult. Indeed, many FOBs are conducted postexposure, usually within one hour of termination of exposure. There are little, if any effects ever observed on FOB determinations because of the rapid elimination of these compounds. In assessing the neurotoxicity of one compound HFC-4310mee, Malley et al., (1995b) exposed rats in an air-tight modified glove box. In this procedure, the investigators were able to manipulate the animals to examine for righting reflex, open-field exploratory behavior, grip strength, and tail pinch.

Inhalation exposures and generation of exposure atmospheres with the fluorocarbons are not as challenging as might occur with powdered substances or fibrous materials. Most of these compounds are gases at room temperature or are volatile liquids. The physical and chemical properties of some selected fluorocarbons are shown in Table 32.4.

For most of the fluorocarbons listed in Table 32.4, the compounds have low boiling points and vapor pressures that allow easy generation of inhalation atmospheres. For compounds such as HFE-7100 and HFC-4310mee, heating of the holding vessels would be needed to generate the vapor atmosphere in the inhalation chamber. Stevens and Green (1987) thoroughly describe various methods of atmosphere generation for different physical forms of compounds, e.g., gases and liquids.

TABLE 32.3 Inhalation Toxicology Guidelines

Testing Guideline	Description and Reference
Organization of Economic Cooperation and Development (OECD)	No. 403: Acute Inhalation Toxicity
	No. 412: Repeated Inhalation Toxicity; 28-day or 14-day Study
	No. 413: Subchronic Inhalation Toxicity; 90-Day Study
TSCA Health Effects Testing Guideline	40CFR 798.2450: Subchronic Inhalation Toxicity
	40CFR 798.4350: Inhalation Developmental Toxicity
FIFRA Health Effects Test Guidelines	40CFR 870.1300: Acute Inhalation Toxicity
	40CFR 870:3465 90-Day Inhalation Toxicity
	40CFR 870:8355 Combined Chronic Toxicity/Carcinogenicity Testing of Respirable Fibrous Particles

Knowledge of the above-mentioned physical and chemical properties is essential for conducting an inhalation toxicity study.

32.2.2 Specialized Studies

32.2.2.1 Cardiac Sensitization

In the early 1900s, Levy and Lewis (1911) reported that cats lightly anesthetized with chloroform were unexpectedly sensitive to injected epinephrine. In these studies, the investigators administered chloroform at 0.5% or 2% in air followed by a bolus intravenous injection of epinephrine (up to 65 μg total dose). The authors described the electrocardiographic (ECG) pattern as "heterogenetic," short pauses in heart rate followed by tachycardia. Continued administration of chloroform ultimately resulted in ventricular fibrillation. Levy followed up on this initial work and reported variations in cardiac sensitivity that were dependent on the duration and degree of anesthesia (Levy, 1913). He found that cats under light anesthesia were more susceptible to the cardiotoxic effects of chloroform than deeper surgical anesthesia. In a review of the literature, Levy found a number of cases where humans had been overcome by chloroform. In these cases, he reported that medical treatment consisted of injecting epinephrine (to stimulate the cardiovascular system) but, in many cases, the patient subsequently died after exhibiting tachycardia followed by ventricular fibrillation. This increased sensitivity of the heart to epinephrine brought about by exposure to a specific organic chemical was referred to as cardiac sensitization.

Little experimental work on cardiac sensitization was reported in the literature after the initial work of Levy until Meek et al. (1937) refined the experimental protocol of Levy and used dogs as the experimental animal. Like the work of Levy, Meek and his collaborators demonstrated an increased sensitivity of the heart to hydrocarbons (cyclopropane) followed by intravenous injections of epinephrine.

Based on these studies, the potential hazard associated with administering hydrocarbon anesthetic agents followed by epinephrine became clearly recognized. It was not until the 1960s with the increased commercial use of CFCs as aerosol propellants and their deliberate misuse that resulted in

TABLE 32.4 Physical and Chemical Properties of Selected Fluorocarbons

Fluorocarbon	Physical and Chemical Properties			
	Form at 22° C	Molecular Weight	Boiling Point (°C)	Vapor Pressure (mmHg) at 25°C
CF_3I	Gas	196	–8.5	3,293
HCFC-123	Liquid	153	27.9	718
HCFC-124	Gas	136	–12	2,888
HCFC-141b	Liquid	117	32	600
HCFC-22	Gas	86	–41	7,250
HCFC-225ca	Liquid	202	25	240
HFC-125	Gas	120	–48.5	10,500
HFC-134a	Gas	102	–26	4,730
HFC-227ea	Gas	170	–17	3,375
HFC-236fa	Gas	152	–1.4	2,043
HFC-32	Gas	52	–51.6	12,525
HFC-4310mee	Liquid	196	55	226
HFE-7100	Liquid	Mixture[a]	61	202

[a] HFE 7100 is a mixture of methyl nonafluoroisobutyl ether and methyl nonafluorobutyl ether.

numerous sudden deaths did cardiac sensitization become once again a phenomenon needing further investigation.

In the 1960s the CFC propellants were being illegally abused to achieve a light anesthesia. In this application, the user attempted to get a "high" (stage I anesthesia) similar to that described for illicit drug use. However, there were numerous fatalities from "sniffing"[1] of the propellants (Reinhardt et al., 1971). The deliberate, abusive "sniffing" of aerosol products and propellants to achieve a state of euphoria began in the early- to mid-1960s and peaked in the late 1960s and early 1970s (Bass, 1970). Deaths from aerosol "sniffing" were always sudden, occurred during or shortly after inhalation of high concentrations of the aerosols, and were generally accompanied by physical exertion or some type of stress (for example, extreme excitation). The mechanism for most of these deaths was thought to be due to cardiac sensitization occurring from the inhalation of high concentrations of aerosol propellants, coupled with corresponding high blood levels of endogenous adrenaline, resulting in the sudden onset of ventricular fibrillation. Autopsy of those that died of this abuse generally do not present any unusual findings. No anatomical changes are observed in the heart, brain, or other organs. The diagnosis of cardiac sensitization is usually based on circumstantial evidence at the scene, i.e., position of the body, empty aerosol cans, and a lack of autopsy findings that might otherwise be responsible for the death. Because of the increased illicit use of the CFC propellants, additional research was undertaken to describe this phenomenon in more detail.

Because of the reported increase in deaths from aerosol "sniffing," many different groups undertook a search for an animal model and the development of a methodology to predict cardiac sensitization potential. The most active groups during the period of the late 1960s and into the 1970s were Reinhardt and colleagues at DuPont's Haskell Laboratory and Clark and Tinston at ICI's Central Toxicology Laboratory. The research conducted by both groups centered primarily on selection of an appropriate animal model and simulating increases in blood adrenaline levels by intravenous injection of appropriate doses of epinephrine.

The method developed by Reinhardt is shown in Figure 32.1. In brief, a conscious beagle dog, fitted with a flow-through mask, is exposed to various gaseous concentrations of the test compound in air and given an intravenous epinephrine injection before and during exposure to a potential sensitizing agent. The ECG response is continuously monitored. For the first 7 min of the experiment, the dog is allowed to breathe air alone. A control intravenous injection of epinephrine (8 μg/kg) is administered at 2 min over a 9-sec interval with exposure to air continued to 7 min. From 7 to 17 min, the dog is then allowed to breathe a specific concentration of a compound-air mixture. After 5 min of exposure to the compound-air mixture (12 min into the study), a challenge injection of epinephrine (8 μg/kg) is given. If the compound at the specified concentration is a cardiac sensitizer, a life-threatening arrhythmia would be seen on the ECG. After 5 more minutes of exposure to the compound, the study is stopped (17-min point of the test protocol).

Because the normal pharmacological action of high doses of epinephrine alone causes ventricular fibrillation, it was necessary to use a smaller dose in this assay. The dose of epinephrine used in the protocol of Reinhardt et al. (1971) was 8 μg/kg, which is similar to that used by other investigators (Meek et al., 1937; Chenoweth, 1946; Raventos, 1956; Clark and Tinston, 1973). This dose of epinephrine, given intravenously in 1 ml of saline over a 9-sec period, provides a dose rate of about 50 μg/kg/min. This dose rate of epinephrine is about ten times the dose calculated to occur in humans (5 μg/kg/min) during times of stress (Cannon, 1919; Mullin et al., 1972).

Length of exposure to the sensitizing agent was also investigated because it is an important variable relative to the induction of cardiac sensitization. In a study on CFC-12 (Reinhardt et al., 1971), exposure to 13.5% for 30 sec induced cardiac sensitization in 2 of 7 dogs. In the standard 17-min protocol, where exposure lasts for 5 min before an epinephrine challenge, the threshold for cardiac sensitization was 5.0%. However, for more than 5 min of exposure, the threshold for sensitization appears to be independent of exposure duration. For example, with CFC-12, exposures of 30 or

[1] Aerosol "sniffing" is a misnomer. "Sniffing" is the deliberate, deep inhalation of highly concentrated vapors.

even 60 min prior to epinephrine challenge did not lower the cardiac sensitization threshold of 5.0% seen in the standard 17-min protocol (Reinhardt et al., 1971). Because of this finding and the rapid equilibration in blood observed with fluorocarbons, a 5-min exposure to the test agent (prior to epinephrine challenge) was chosen for the Reinhardt protocol.

As mentioned previously, in the 1980s and into the 1990s, a significant amount of toxicological investigation was undertaken to find alternatives to the CFCs. Much of this work was done under the coordination of an international industry consortium (PAFT). Cardiac sensitization studies were part of PAFT's research programs. The protocol used for these studies was similar to that described by Reinhardt et al. (1971). However, the dose of epinephrine used was titrated for each experimental animal and this modification was expected to control for individual dog variation in response to cardiac sensitizers.

Prior to exposing the experimental animal (dog) to the fluorocarbon, epinephrine was administered (i.v.) at doses ranging from 2 to 12 μg/kg while the dog was exposed to air. The purpose of this procedure was to determine the dose of epinephrine that would be minimally arrhythmogenic. If an arrhythmia were observed, the dose of epinephrine would be decreased, or, if no arrhythmia was observed, the dose of epinephrine was increased up to a maximum dose of 12 μg/kg. Once the dose of epinephrine was established, that dose would be used when the dog was exposed to the fluorocarbon following the same 17-min protocol (Figure 32.1). Although this approach addresses biological variation, it can result in selecting an epinephrine dose that is too high, resulting in a fatal arrhythmia, or too low, resulting in an insufficient challenge. Thus, if the same chemical were to be tested using the fixed-dose method and the titration method, it is possible that the test results would differ relative to a no observable adverse effect level (NOAEL) or lowest observable adverse effect levels (LOAEL). Indeed, in studies with HCFC-141b, the cardiac sensitization LOAEL using the epinephrine-titration method was 9000 ppm (epinephrine dose, 10 μg/kg). However, when tested in the Reinhardt protocol (fixed epinephrine dose, 8 μg/kg), the LOAEL was 5000 ppm (Brock et al., 1995). In a third

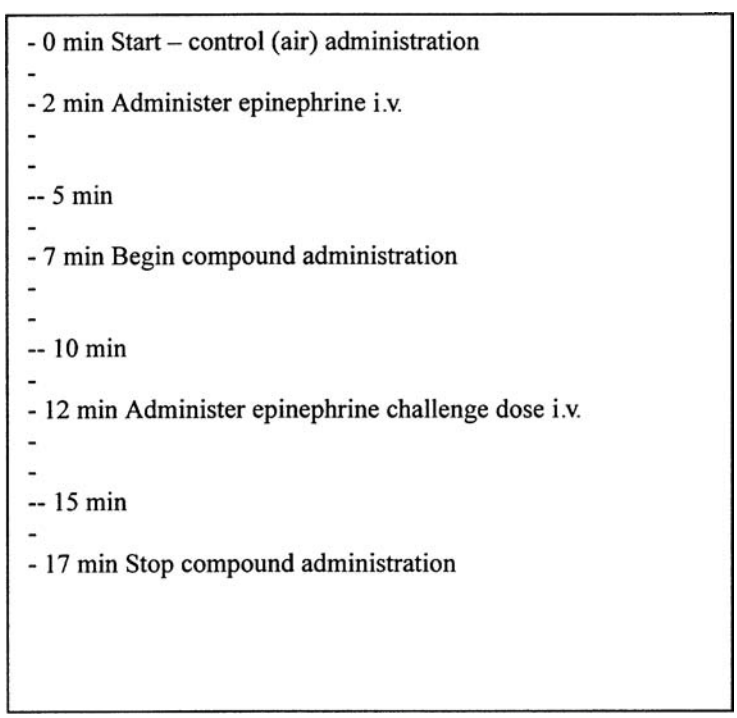


FIGURE 32.1 General protocol for cardiac sensitization.

unpublished study, (G.M. Rusch, personal communication, 2002) the cardiac sensitization threshold was 20,000 ppm (epinephrine dose, 10 μg/kg). These three studies were conducted over a 30-year period. Clearly, the threshold for a cardiac sensitization response from these three studies varies, and this variation cannot be explained solely on the basis of epinephrine dose.

An issue raised with the use of the titration method was the possible severity of the response to a potential sensitizer. In the presence of a sensitizer, the challenge dose of epinephrine might result in a fatal response, an event that investigators attempted to avoid. To address this concern, an escalating epinephrine dose procedure was developed. With this procedure, the dog is challenged with increasing epinephrine doses of 1.2, 2.4, 4.8, and 6 μg/kg during a 30-min exposure to air or test agent. Approximately 90 sec is required for "wash out" of the pharmacological effect of epinephrine. Once a positive sensitization response is observed, then an additional challenge with epinephrine could be repeated by using a lower dose, thereby conclusively determining whether a compound was a sensitizer. To our knowledge, this procedure has been used with only one fluorocarbon, HFC-227ea (Chengelis, 1993).

Although this methodology may help to distinguish spurious findings, administration of multiple epinephrine doses, coupled with an exposure duration of 30 min, could result in an overly sensitive test. Therefore, the NOAEL or LOAEL for cardiac sensitization for the same chemical could prove to be lower by using this method compared with the fixed epinephrine and titration methods.

32.2.2.2 Neurotoxicity

In the toxicity-testing guidelines referenced above, guidance is provided indicating the need to physically examine animals prior to and following exposure. Indeed, the guidance refers to the use of FOB and Irwin methodologies for detecting neurological effects. Although such examinations and testing would be useful for those compounds with long clearance times, i.e., the half-life of elimination may be hours, this testing becomes more difficult with fluorocarbons, particularly those with half-lives measured in minutes. In studies with HCFC-123 and HFC-134a, no changes in FOB measurements were observed in either subchronic or chronic studies (Malley et al., 1995a; Collins et al., 1995). For each compound, these materials are eliminated within about 30 minutes following exposure.

In studies with HFC-4310mee, Malley et al. (1995b) showed that this compound was potentially neurotoxic when administered by inhalation. During exposure, rats began to exhibit tremors and convulsions, and these generally lasted about 20–30 min. After this time, the animals became lethargic and sedated. On termination of the exposure, the animals recovered fully, not showing any long-lasting toxicity. When the inhalation exposures were repeated the next day, this pattern of convulsions followed by sedation was again evident. Changes in FOB parameters were not, however, observed in these studies primarily because of the compound being rapidly eliminated following termination of exposure. Clearly, such a conclusion of no effects on FOB parameters appears contrary to the effects observed during the inhalation exposures.

Additional studies with HFC-4310mee were undertaken to examine further the neurotoxicity of this compound. In these studies, an air-tight Plexiglas chamber, a modified glove box, was constructed that allowed manipulation of the animals during exposure. Although all FOB parameters could not be assessed, the investigators were able to observe the rats in an open-field test, and for tail-pinch and righting reflex. These parameters were affected by exposure.

32.2.2.3 Genotoxicity

The purpose of genotoxicity testing is to screen chemicals for potential mutagenic and carcinogenic activity. Genotoxicity assays are short-term tests performed primarily in cell culture systems. The prediction of a chemical to produce cancer in humans is more reliable when information is available from several genotoxicity test systems, i.e., a "battery of tests." For this reason, a battery of three or more assays is commonly performed for assessing genotoxicity. There are a wide variety of tests to

consider when selecting an assay. The *Salmonella typhimurium* reverse mutation assay, commonly referred as the Ames assay, is probably the most widely used genotoxicity test for assessing mutagenicity. Results provide information on whether the test substance causes point and frameshift mutations in the genome of this bacterial organism. Details of the assay are available in OPPTS Series 870.5265. Currently, four test strains (TA 1535, TA 1537, TA 98, and TA 100) are designated for testing, but a fifth strain (TA 102) is recommended (OPPTS Series 870.5100). In addition, testing in an *Escherichia coli* strain is required when testing is to be submitted to regulatory authorities. Each strain is tested in the presence and absence of a metabolic activation system with appropriate positive control reference substances to ensure the efficacy of the activation system. A preliminary "range-finding" experiment is required to determine the upper limits of a candidate's concentration that will produce cytotoxicity and/or, for relatively insoluble chemicals, the limits of solubility. A maximum of 5 mg of test substance per plate is acceptable by EPA.

The Ames assay is fairly straightforward for liquid or solid test materials at ambient conditions. The assay is more complicated for gas or vapor replacement candidates, because of the time and skill required for exposing bacteria cultures to the test substance. Exposure chambers may have to be designed or modified. Vapor generation systems and analytical methods to measure the exposure concentration of the test substance have to be developed and implemented. For example, Tedlar® bags may be employed for preparation of vapor test atmospheres and exposure of bacteria. A determination of test substance solubility is desirable, particularly when no cytotoxicity is observed in the "range-finder" experiment.

Conduction of a mammalian cell assay will provide greater confidence in predicting a potential genotoxicity health hazard. Again, there are several assays to consider, but the two most common are the *in vitro* gene mutation assays in Chinese hamster ovary (CHO) cells and mouse lymphoma (L5178Y) cells. EPA guidelines for these tests are available (OPPTS 870.5300). Two references for testing volatile substances in CHO cells are Krahn et al. (1982) and Zamora et al. (1983). A reference for testing vapor of halon replacement candidates in L5178Y cells was reported by Dodd et al. (1997a). Only one assay needs to be selected for chemical testing, because both assays give a similar type of genotoxicity information. The choice of an assay depends primarily on the historical background and familiarity of the laboratory performing the test. Similar to the Ames assay, a preliminary "range-finding" experiment is performed to determine the upper limits of a candidate's concentration that will produce cytotoxicity. The use of an exogenous source of metabolic activation to mimic mammalian *in vivo* conditions is part of the study design.

Conducting an *in vivo* mammalian cell genotoxicity test will complete the "battery of tests." An *in vivo* mammalian cell test is especially relevant to assessing mutagenic hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics, and DNA-repair processes, although these may vary among species, among tissues, and among genetic endpoints. The *in vivo* mammalian cell system used frequently in a genotoxicity testing battery is the mouse micronucleus assay. The mouse micronucleus test detects the damage of chromosome or mitotic apparatus caused by a chemical in immature (polychromatic erythrocyte, PCE) cells of treated animals. Micronuclei are believed to be formed from chromosomes or chromosome fragments left behind during cell division. After cell division, these fragments may not be included in the nuclei of the daughter cells and form single or multiple micronuclei in the cytoplasm. The micronucleus test can serve as a rapid screen for clastogenic agents and test materials that interfere with normal mitotic cell division, effecting spindle fiber function or formation. The assay is based on the increase in the frequency of micronucleated PCEs in bone marrow of the treated animals.

General guidelines to be followed in the mouse micronucleus assay are described in EPA's Health Effects Test Guidelines OPPTS 870.5395 or in the OECD Guidelines. More specifically, the assay is conducted using animals of both sexes and 3 to 4 concentrations of the test agent or negative control (e.g., air-only exposed or saline dosed). Treatment of animals is either by inhalation (volatile test chemicals) or by gavage for three consecutive days. A positive control agent, cyclophosphamide, dissolved in physiological saline, is given by intraperitoneal injection as a single dose. Twenty-four

hours following the last exposure/dose, mice are sacrificed, and bone marrow cells are collected and processed. The frequency of micronucleated cells is observed by counting 2000 PCEs per animal. Animal mortality and the ratio of PCE to mature erythrocytes (normochromatic erythrocyte, NCE) are used as indicators of test agent toxicity. The difference in the micronucleated PCE frequency and the ratio of PCE/NCE between treated and control animals are statistically evaluated by Chi-square analysis, and the dose-dependent response is examined by linear regression. A test agent is considered to have elicited a positive response in the mouse micronucleus test if there is a dose-related increase in micronuclei and if one or more of the doses induces a statistically significant ($p < 0.05$) increase in micronuclei induction.

Results from the genotoxicity test battery provide moderate to strong confidence in predicting carcinogenic potential of a chemical. If the *in vivo* mouse micronucleus test is carried out at either a limit dose or the maximum tolerated dose, or if it can be demonstrated that the test substance is reaching the target tissue, the results of this study normally would override the results of *in vitro* assays for the purpose of health hazard assessment. If results from the genotoxicity test battery remain equivocal and significant exposure of unprotected personnel is possible, then lifetime studies in two species of animals may be required to determine the carcinogenic potential of exposure to a chemical. Also, important biological end points such as developmental toxicity and reproductive toxicity remain to be addressed more thoroughly.

32.2.2.4 Mechanistic Studies

In general, toxicology studies that investigate a chemical's mechanism of toxic action, including its metabolism and toxicokinetics, are termed mechanistic studies. Mechanistic studies are not mandated by regulatory agencies, such as EPA, but they are encouraged by regulatory groups because the type of information gained is valuable for a better understanding of a chemical's toxicity profile and thus prediction of its health risk. Three mechanistic studies that are noteworthy for fluorocarbon alternatives are those that investigate metabolism/toxicokinetics, peroxisome proliferation, and idiosyncratic hepatitis. Of these three, test guidelines prepared by either EPA's Office of Prevention, Pesticides, and Toxic Substances (OPPTS; Series 870.7485) or by OECD (Section 417 of Health Effects Guidelines) are available for metabolism and pharmacokinetics.

The primary objectives of metabolism/toxicokinetic studies are to (1) identify toxicologically important metabolites, (2) examine distribution of metabolites and the parent compound, (3) quantitate the amount of metabolites formed, (4) determine the time course of metabolite formation and elimination, and (5) identify the factors influencing metabolite formation and elimination. Analyzing fluoride ion concentration in tissues and fluids of laboratory animals treated with HFCs, HCFCs, or perfluorocarbons is common. Measurement of concentration of trifluoroacetic acid (TFA) in biosamples is also common. Urinary fluoride detection assists in predicting metabolic pathways, identifying toxic metabolites, and estimating blood/tissue half-lives. A brief description of an experimental approach on metabolism and pharmacokinetics of select halon replacement candidates is by Dodd et al. (1993).

A large number of fluorocarbons and fluorocarbon alternatives are capable of inducing liver enlargement, proliferation of peroxisomes, and induction of microsomal fatty acid oxidation in laboratory animals. Chemicals that produce these characteristics are called peroxisome proliferators. Long-term administration of peroxisome proliferators to rats and mice can cause liver tumors. The proposed mechanisms associated with the production of these tumors include sustained oxidative stress, enhanced cell replication, inhibition of apoptosis, and promotion of spontaneous preneoplastic lesions (Dzhekova-Stojkova et al., 2001). To date, humans treated with drugs that are categorized as peroxisome proliferators do not appear to be susceptible to the hepatocarcinogenic effects. In mice, peroxisome proliferation is mediated through the peroxisome proliferator-activated receptor alpha (PPARalpha), a member of the nuclear receptor superfamily. Low levels of PPARalpha in humans may explain species differences in the carcinogenic response to peroxisome proliferators.

To determine peroxisome proliferation potential, liver samples from animals treated with the test agent are collected at necropsy and measured for β-oxidation activity. Increased activity of β-oxidizing enzymes is considered to be an indication of increase in size (activity) and numbers of cytoplasmic peroxisomes (Moody et al., 1991). In addition, livers are weighed, and liver histopathology is performed. Because lipid metabolism is associated with peroxisomes, measures of blood cholesterol, lipids, trigyceride, and glucose are included in clinical chemistry determinations.

Since the 1960s, inhalation anesthetics, such as halothane, enflurane, and isoflurane have been associated with two types of toxic hepatitis, mild (nonfatal) and fulminant (often fatal) hepatic necrosis. The incidence of the mild form varies from 20 to 25% of anesthetized patients (Thompson and Friday, 1978), and the fulminant form (also referred to as idiosyncratic hepatitis) is 1 of 10,000–30,000 patients (Bunker et al., 1969). There are a number of fluorocarbon alternatives with structures similar to halothane (e.g., HCFC-123, HCFC-124, HFC-125) and they are, therefore, considered suspect for producing toxic hepatitis.

Halothane-induced idiosyncratic hepatitis is thought to result from immunologically mediated allergic or hypersensitivity reactions. Metabolism of halothane leads to the formation of a reactive intermediate, trifluoroacetyl (TFA) chloride that binds with liver proteins to produce a neoantigen product that can illicit an immune response. Patients diagnosed with halothane hepatitis produce antibodies that recognize TFA-protein adducts (Hubbard et al., 1988). Fluorocarbon alternatives containing a geminal dihalomethyl group (-CHX_2) may be metabolized to an acyl halide reactive intermediate. Harris et al. (1992) measured the rates of metabolism for select HCFCs and HFCs in rats and observed that urinary excretion of trifluoroacetic acid correlated with levels of liver TFA-proteins. Further, no hepatic TFA-proteins were detected in rats treated with HFC-134a, a fluoroethane that does not metabolize to a TFA halide.

Potential biomarkers for toxic hepatitis include levels of urinary trifluoroacetic acid, presence of TFA-protein adducts in hepatic fractions, and serum enzyme concentrations of alanine aminotransferase (ALT) and isocitrate dehydrogenase (ICDH). The basis for the measurement of serum ALT and ICDH is because of an association between these liver-derived plasma enzymes and liver injury (determined histologically) in laboratory animals exposed to halothane (Lind and Gandolfi, 1991). The guinea pig is considered a representative animal model for idiosyncratic hepatitis; however, a quantitative association between these biomarkers and individual susceptibility to toxic hepatitis remains at large from a risk assessment perspective.

32.2.2.5 QSAR Analysis

Quantitative structure–activity relationships (QSARs) may be used as an integral part of health hazard assessment. This approach relies on an experienced toxicologist or chemist to fit the new chemical alternative into a category of existing chemicals because of similarities in molecular structure or chemical functionality. For this approach to be of value, the existing chemicals category or close structural analog must have its own robust toxicology database. QSAR analysis programs have been formalized and computerized for some health end points (e.g., cancer, mutagenicity, teratogenicity) and may be useful with appropriate recognition of the limitations of these programs. A review of the various computerized QSAR programs available commercially is beyond the scope of this chapter, but this subject has been reviewed recently (Dearden et al., 1997). The EPA has grouped chemical substances with similar physical-chemical, structural, and toxicological properties into working categories. Additional candidate categories for EPA's new chemical review process are proposed by TSCA New Chemicals Program (NCP) staff based on available data and experience of reviewing premanufacturer's notifications on related substances. These groupings enable the user of the NCP Chemical Categories guidance document to benefit from the accumulated data and previous decisions within EPA's new chemicals review process since 1987 to identify areas of health hazard concern. Currently, there are 45 chemical categories listed in the table of contents of the EPA document, the detailed summaries of which may be found at http://www.epa.

gov/oppt/newchems/. The Organization for Economic Co-operation and Development (OECD) prepared a monograph on the evaluation of the predictive power of the QSAR techniques used by EPA in context of the new chemicals notification scheme established by TSCA (http://www.epa.gov/oppt/newchems/ene4147.pdf).

Recently, Nyden and Skaggs (1999) prepared a general listing of chemical classes, functional groups, possible toxic end points, and references of possible QSARs as part of a document on screening methods for fire suppression alternative agents.

For close structural analogs that have been used for several years, relevant information may be gained from historical experience with human exposure from normal handling. For many existing chemicals with many years of widespread industrial use, no adverse health effects have been observed. On the other hand, in some cases of overexposure or where unexpected toxicity was discovered, adverse effects in occupational populations have occurred. When they are available, retrospective (or case-control) epidemiological studies for close structural analogs may provide insight as to the potential for certain health effects by the new chemical.

32.3 RESULTS OF SPECIFIC STUDIES

In this section, the results of the toxicology programs for both HCFC-123 and HFC-134a will be briefly described in comparison with the known toxicity of the CFC replacements, CFC-11 and CFC-12, respectively. The toxicity of other fluorocarbons, such as the perfluoroalkanes, HFE-7100, CF_3I, Novec™ 1230, and perfluorooctane sulfonate will also be summarized.

Although structurally related, HCFC-123 and HFC-134a have different toxicity profiles apparently related to the incorporation of the chlorine in the ethane molecule. During the early 1990s a great deal of commercial debate ensued between various fluorocarbon producers and the users and producers of air-conditioning systems. In these "chiller wars," debate was centered on the differential toxicity of HCFC-123 compared with HFC-134a. It is clear, however, that given the different molecular makeup of the molecule, differences in toxicity would be expected, thereby resulting in the commercial debate that one compound was superior to another. In retrospect and because of the need to limit fugitive emissions from air-conditioning systems, exposures to either of the compounds in this application would have been limited. In contrast, exposures during the manufacturing of these compounds would have been greater, but still somewhat limited because of the engineering controls instituted for production.

32.3.1 HCFC-123

HCFC-123 has very low oral toxicity with an approximate lethal dose (ALD) in the rat of 9000 mg/kg. On the skin of guinea pigs, a 50% solution in propylene glycol produced no irritation or sensitization and no irritation was observed when applied on the skin of rabbits. No deaths occurred in rats and rabbits topically administered 2000 mg/kg of HCFC-123 for 24 h under an impervious cover. No irritation was noted in the rats but slight to moderate erythema was noted in 6 of 10 rabbits. In the rabbit eye, a 50% solution of HCFC-123 in propylene glycol produced mild, temporary irritation and corneal opacity. HCFC-123 also has very low inhalation toxicity in rats with a 4-h LC_{50} of 32,000 ppm. In experimental screening studies for cardiac sensitization with dogs, HCFC-123 produced a cardiac sensitization response following an intravenous challenge with epinephrine at 20,000 ppm and above (ECETOC, 1996).

In a 4-week, subchronic inhalation study, groups of 10 male and 10 female rats were exposed 6 h a day, 5 days a week to 1000, 5000, 10,000, or 20,000 ppm of HCFC-123. Exposures to 5000 ppm and above resulted in anesthetic-like effects during the exposure period. Body weights were significantly lower in most of the exposed groups. The primary systemic effect observed in this study was an effect on the liver. Relative liver weights were higher in females at all exposure concentrations and in males exposed to 20,000 ppm. Male rats of the 20,000 ppm group had slightly higher liver

enzyme activities. However, no gross or microscopic pathological liver changes were noted in any of the exposed rats (ECETOC, 1996).

Three separate 90-d inhalation studies were conducted with HCFC-123 (Rusch et al., 1994). The concentrations used for these studies ranged up to 10,000 ppm with exposures occurring for 6 h a day, 5 days a week. The rat was used in each of these studies, and the dog was used in one study. Overall, the primary finding with exposure to HCFC-123 was an effect on the liver with increases in relative and absolute liver weights occurring at concentrations of 5000 ppm and above. Increases in liver enzymes were also a common finding in these 90-d studies. Other findings in these 90-d studies included kidney and heart weight changes and anesthetic effects at 5000 ppm and above. No microscopic changes were noted in rats in these studies, although liver necrosis and hypertrophy were observed in dogs exposed at 10,000 ppm.

In the third 90-d inhalation study, serum triglyceride, cholesterol, and glucose concentrations were significantly reduced compared with controls at all exposure concentrations. Serum cholesterol was also significantly lower in females exposed to 1000 or 5000 ppm. These effects, along with the increases in liver weights, were suggestive of an effect on lipid biosynthesis and possibly an effect on peroxisome production. Higher peroxisome β-oxidation activity was noted in all exposed rats in this study. Comparisons of liver sections from the control and high-level exposure groups by electron microscopy revealed that peroxisome number in high-dose animals were about 2-fold greater. This higher enzyme activity and peroxisome number indicate an induction of hepatic peroxisome proliferation. This finding was the first in a series of findings for HCFC-123 that led to development of a hypothesis that this compound may induce tumors in a two-year bioassay (see below). Furthermore, the findings at that time continued to fuel debate over the human relevance of peroxisome induction and the occurrence of a "triad" of tumors generally observed with this class of compounds.

In a two-year inhalation study, male and female rats were exposed 6 h a day, 5 days a week, to 300, 1000, or 5000 ppm of HCFC-123 (Malley et al., 1995a). After 12 months of exposure to HCFC-123, male and female rats exposed to 5000 ppm and females exposed to 1000 ppm had lower body weight and body weight gain. Rats exposed to 5000 ppm appeared sedated during exposure but quickly recovered after the end of the daily exposure. Serum triglyceride and glucose concentrations were significantly decreased as they were in the 90-d study. Serum cholesterol was significantly lower in all the exposed females and in the males exposed to 5000 ppm. Rats exposed to 5000 ppm had significantly higher mean relative liver weights without any gross or microscopic pathological changes.

After 24 months of exposure, decreased body weights were observed in males exposed to 5000 ppm of HCFC-123 and in females exposed to 1000 or 5000 ppm. A slight, reversible lethargy was noted in the 5000 ppm males during exposure, a finding consistent with the subchronic studies. Serum triglyceride, cholesterol, and glucose continued to be decreased in a dose-related manner. Liver weights were increased at 5000 ppm. A positive, dose-related effect on survival was noted in the HCFC-123 exposed groups. Histopathological examination revealed an increased incidence of benign tumors of the testes, pancreas (exocrine), and liver (Table 32.5).

When hepatocellular adenomas were statistically analyzed, only a significant increase was observed in the animals of the 5000 ppm group, and the dose–response curve was flat, affording no statistical significance. Indeed, the shape of the dose–response curve suggested a threshold phenomenon, a response that has been observed in long-term bioassays with other peroxisome-proliferating compounds, e.g., clofibrate, WY-14,643.

Interstitial cell adenomas in the testes, unilateral and bilateral combined, exhibited only a marginal statistically significant increase in the 5000 ppm group. As with hepatocellular adenomas, the shape of the dose–response curve suggested a threshold phenomenon. Furthermore, the response that was observed is a typical age-related response with the difference between control and high-dose groups in this study related to lower survival in the control group. Age-adjusted statistics supports this contention.

The pancreas was a target tissue in both males and females. However, the incidence of benign tumors was statistically significantly increased in males only. There were increases in the incidence

TABLE 32.5 Incidence of Benign Tumors in Rats Exposed to HCFC-123 for Two Years

0 ppm (control)	300 ppm	1000 ppm	5000 ppm
	Leydig Cell Adenomas		
4/67 (6%)[a]	12/66 (18%)	9/66 (14%)	14/66 (21%)
	Pancreatic Acinar Cell Adenomas		
Males			
1/67 (1%)	4/66 (6%)	12/66 (19%)	14/66 (21%)
Females			
0/65 (0%)	2/66 (3%)	0/67 (0%)	2/69 (3%)
	Hepatocellular Adenomas		
Males			
3/67 (4%)	2/66 (3%)	2/66 (3%)	8/66 (12%)
Females			
0/65 (0%)	5/67 (7%)	2/67 (3%)	7/69 (10%)

[a] Incidence of tumors/number of rats examined (percent incidence).

of nonneoplastic hyperplasia in both sexes. In males, the incidence was increased significantly in a dose-related manner. In females, while increases were noted, when adjusted for mortality (incidental tumor test and life table analysis) this increase was not statistically significant. Acinar cell hyperplasia and acinar cell adenomas represent a continuum of toxicity. When the incidence of adenomas and hyperplasia were combined, the incidence in the high-dose group females was significantly different from controls by pairwise comparison. However, no significant dose–response trend was noted with the age-adjusted tests.

In addition to the tumor incidence above, the combined incidence of cholangiofibromas was increased in female rats only at the high concentration (0 of 67 for control; 6 of 70 for the 5000 ppm group). However, the shape of the dose–response curve, like those of the other benign tumors observed in this study, exhibited a "hockey-stick" shape. When accounting for the changes in survival, the occurrence of this tumor was not statistically significant by life table tests ($p = 0.063$). The diagnosis of cholangiofibroma as a benign tumor is supported in the literature.

HCFC-123 was not embryotoxic or teratogenic in groups of pregnant rats exposed 6 h/d on days 6 through 15 of gestation to 5000 or 10,000 ppm (Malinverno et al., 1996). In rats exposed to 10,000 ppm, maternal toxicity was evident at the end of each daily exposure and indicated as slight anesthesia. HCFC-123 was also not embryotoxic or teratogenic in groups of pregnant rabbits exposed on days 6 through 18 of gestation to 500, 1500, or 5000 ppm. Dose-related maternal toxicity was characterized by reduced weight gain and reduced food consumption (Malinverno et al., 1996).

In a two-generation reproduction study, groups of male and female rats were exposed 6 h/d, 7 d/week to 30, 100, 300, or 1000 ppm (Malinverno et al., 1996). The rats were exposed for 12 weeks before being mated, throughout a 2-week mating period, during the 20-d gestation period, and until the offspring were weaned. Mating performance and duration of pregnancy were not affected throughout the study. No adverse clinical signs of toxicity were observed. Body weights were slightly lower between weeks 7 and 15 in males exposed to 1000 ppm and in females exposed to 300 or 1000 ppm. Liver weights were slightly increased in F_0 rats exposed to 300 or 1000 ppm, but liver weights of the pups were not affected. Litter size of F_1 pups was slightly decreased at 1000 ppm. F_1 pup body weights were reduced at $\geqslant$100 ppm during lactation days 4–21 only. In the F_2 generation, a decrease in pup weights was also observed during the period of lactation days 4–21 at $\geqslant$30 ppm. The reduced total body weight is thought to be related to the decrease in maternal fat.

To further examine this hypothesis, a cross-fostering study of control and HCFC-123 exposed rats was undertaken.

In the cross-fostering study (Buschmann et al., 2001), pregnant and lactating rats were exposed to air (control) or 1000 ppm for 6 h/d on gestation days 6–15 and from days 5 to 21 postpartum. Pups were cross-fostered to new dams within the first 2 days after birth. In the HCFC-123-exposed maternal animals, decreases in body weights, cholesterol, and triglycerides were observed, and these findings were consistent with previous studies with HCFC-123. Pups cross-fostered to HCFC-123 maternal animals also had reduced body weights and triglyceride levels compared with pups cross-fostered to control maternal animals. Milk production in HCFC-123 maternal animals was similar to controls, and total fat, glucose, and protein content were unaffected by HCFC-123 exposure. An increase in urinary trifluoroacetate, the major metabolite of HCFC-123, was observed in cross-fostered pups. Overall, the effects observed in this study were considered to be due to maternal effects during lactation, not a direct effect on the pups, and were related to possible elimination of HCFC-123 or TFA through milk.

32.3.2 HFC-134a

HFC-134a has low acute toxicity by the inhalation route in several species. In the rat, the 15-min LC_{50} of >800,000 ppm and a 4-h LC_{50} >500,000 ppm were reported (ECETOC, 1995). During exposure rats showed incoordination, pumping respiration, unresponsiveness, cyanosis, convulsion, and death. Surviving rats regained their coordination within 5 min after exposure and appeared normal. In the dog, HFC-134a did not produce mortality following a 3- to 5-h exposure of 700,000 or 800,000 ppm (ECETOC, 1995). A 10-min -EC_{50} for anesthetic effects, measured by the loss of the righting reflex, was 280,000 ppm in the rat and 270,000 ppm in the mouse (ECETOC, 1995).

In repeated-exposure studies, male rats were exposed to 0 or 100,000, ppm HFC-134a (6 h/d, 5 d/wk), over a 14-d period, or 1000, 10,000, or 50,000 ppm over a 28-d period. In the 14-d study, no treatment-related abnormalities were observed on body weight, clinical signs, clinical pathology, or histopathology. In the 28-d study, slight changes in liver, kidney, and gonad weights were noted in rats exposed to 50,000 ppm, and an increase in liver weight was observed at 10,000 ppm. No pathological changes were observed in these organs and, therefore, the changes in organ weights were not considered to constitute toxic responses to treatment (ECETOC, 1995).

Male and female rats were exposed to concentrations of 0, 2000, 10,000, or 50,000 ppm HFC-134a (6 h/d, 5 d/wk) for 13 weeks. Animals were sacrificed either at the end of the exposure period or 4 weeks after the last exposure to evaluate for reversibility of any potential compound-related effects. No significant findings were observed at any of the concentrations (ECETOC, 1995). In a one-year dog study, no effects were observed at inhalation concentrations of up to 120,000 ppm (Alexander, 1995).

A combined chronic toxicity/carcinogenicity study (104 weeks duration) was carried out in rats with exposure levels of 2500, 10,000, or 50,000 ppm HFC-134a (Collins et al., 1995). Ten rats of each sex from each group were designated for interim kill after 52 weeks. All groups had a similar survival rate, and there were no differences in in-life measures across all groups. No clinical pathology changes were noted except of a slight increase in urinary fluoride levels among rats exposed to 10,000 and 50,000 ppm. The only treatment-related effect of toxicological significance was confined to the testes of male rats exposed to 10,000 and 50,000 ppm. There was a statistically significant increase in the weight of the testes of controls and there was an increased incidence of Leydig cell hyperplasia and benign Leydig cell tumors (Table 32.6). The no-effect level was considered to be 10,000 ppm HFC-134a (ECETOC, 1995).

The benign tumors of the testicular interstitial cells (Leydig cell adenoma) are common in the aging rat and are not life threatening. The spontaneous incidence of this tumor type is variable from one strain to another, ranging from a few percent in Sprague–Dawley rats up to 100% in some Wistar derived and in Fisher 344 rats (Bar, 1992). However, these tumors are not likely to progress

TABLE 32.6 Effect of HFC-134a on the Pathology of the Rat Testis

Number of animals (n = 85) with finding[b]	Exposure concentration (ppm)[a] 0	2,500	10,000	50,000
Leydig cell hyperplasia	27	25[c]	31	40
Leydig cell adenoma	9	7[c]	12	23**

[a] Exposure was for 6 h/d, 5 d/wk for up to 104 weeks

[b] Data includes all animals from interim, intercurrent and terminal killings

[c] Data from 79 animals

** Significantly different from control values $p < 0.01$ (Fisher's exact test)

to malignancy in the rat, because malignant Leydig cell tumors have not been observed in control Fisher rats (Boorman et al, 1990; Iawata et al, 1991; Cook et al., 1999).

In the two-year bioassay, male and female rats were exposed to inhalation concentrations of 0, 2500, 15,000, or 50,000 ppm. For mice, exposure concentrations were 0, 2500, 15,000, or 75,000 ppm. Exposures were 1 h daily for 2 years. As noted previously, IPAC conducted a number of inhalation toxicity studies on HFC-134a because this compound was intended to replace CFC-12 and other compounds as the propellant in metered dose inhalers. Because of this application, the duration of exposure was limited to 1 h versus the 6 h used in the PAFT program. No treatment-related effects on in-life measures, e.g., survival, body weights, clinical observations were observed, and no clinical pathology or histopathological effects were observed (Alexander et al., 1996).

Male CD-1 mice were exposed 6 h/d for 5 d to levels of HFC-134a up to 212,000 mg/m^3 (50,000 ppm) and mated with unexposed female mice. No effects on fertility were observed, as shown by investigating the parameters for reproductive performance (ECETOC, 1995).

The developmental toxicity of HFC-134a was tested in rats at 0, 1000, 10,000, and 50,000 ppm (Collins et al., 1995). Groups of pregnant Alpk/ApfSD, Wistar derived rats were exposed 6 h/d to HFC-134a from day 6 to 15 of pregnancy. The exposure to HFC-134a produced abnormal clinical signs in animals but did not affect maternal body weights. Mean fetal weights were slightly but significantly lower in the 50,000 ppm group. Embryonic and fetal survival were unaffected by the treatment, and there was no evidence for teratogenicity except for a slight retardation of skeletal ossification at 50,000 ppm (ECETOC, 1995). In another study using similar exposure conditions to the above, groups of Sprague–Dawley rats were tested at concentrations of 0, 30,000, 100,000, or 300,000 ppm HFC-134a. No teratogenic effects were observed although some maternal growth and fetal development retardation in the form of delayed ossification occurred. The minimum maternal effect dose was demonstrated to be 100,000 ppm, and the minimum embryofetal effect was demonstrated to be 300,000 ppm (ECETOC, 1996).

Groups of New Zealand White rabbits were exposed by inhalation to target atmospheric concentrations of 0, 2500, 10,000, and 40,000 ppm HFC-134a for 6 h/d from days 7 to 19 of gestation. Exposure levels of 40,000 and 10,000 ppm HFC-134a were associated with slight maternal toxicity manifest as reduced body weight gain and food consumption. There was no evidence for maternal toxicity at the exposure level of 2500 ppm. There was no evidence of embryotoxicity or fetotoxicity at any concentration levels (Collins et al, 1995).

In addition to the preclinical studies, some authors have published short-term clinical studies to evaluate the safety of HFC-134a as a propellant in MDIs. In a 28-d double blind study conducted by Harrison et al. (1996), human subjects were exposed to HFC-134a or chlorofluorocarbon propellant. (CFC-12 and CFC-11, 2.3:1, v/v). No significant effects on cardiovascular or respiratory parameters were observed. In a separate study (Emmens et al., 2000), subjects were exposed, whole-body, to inhalation concentrations of 2000 ppm for 1 h during which the subjects were evaluated for blood pressure, pulse, EKG, and psychological performance tests. No effects were observed in any of the subjects in this study.

32.3.3 Other Fluorocarbons

In addition to HFCs and HCFCs, several other fluorocarbons have either replaced previously used fluorocarbons, especially the CFCs, or are being considered as alternatives to currently used fluorocarbons. In some cases, a considerable amount of toxicology data is available on the fluorocarbon agent, because the chemical manufacturer performed a battery of toxicity tests on the agent (e.g., HFE-7100). Physiologically based pharmacokinetic models have been developed for a few agents (e.g., CF_3I). The environmental impact of PFOS is a most interesting risk assessment scenario that led to the halting of its production by the chemical manufacturer.

32.3.3.1 Perfluoroalkanes

Perfluorination of alkanes makes these chemicals relatively biologically inert, therefore a number of candidate agents (e.g., perfluoroethane [FC-116], perfluoropropane [FC-218], perfluorobutane [FC-3110], perfluorohexane [FC-5114], etc.) have been considered for replacement of CFCs or halons. Toxicity studies in mammals confirm the low toxicity associated with these chemicals. Acute toxicity and cardiac sensitization values are typically in the hundreds of thousands ppm range. For example, the NOAEL for cardiac sensitization of FC-3110 is 400,000 ppm. Similar to inert gases, the concern for minimum oxygen levels (approximately 20%) becomes a factor in experimental design of toxicity studies with perfluoroalkanes. Ozone depletion potential is negligible. The major drawback that limits this family of chemicals for further consideration as CFC or halon replacement candidates is their atmospheric lifetime and global warming potential (GWP). FC-3110 has an atmospheric lifetime approximately 25 times greater than CO_2, and a GWP that is approximately 5000 times greater than CO_2. Because of the environmental impact of PFCs, EPA restricts their use to when other alternatives are not technically feasible.

32.3.3.2 Hydrofluoroether 7100 (HFE-7100)

HFE-7100 is a fluorocarbon replacement agent intended for a variety of applications, such as cleaning and rinsing agents, lubricant carrier, drying agent, or heat transfer medium. It is a mixture of 1-methoxy-1,1,2,2,3,3,4,4,4-nonafluorobutane (40%) and 1-methoxy-2-trifluoromethyl-1,1,2,3,3,3-hexafluoropropane (60%) with a vapor pressure of 170 mmHg at 20°C. Currently, EPA accepts HFE-7100 for use in several applications, such as refrigeration, heat transfer, and nonaerosol cleaning agents.

The acute inhalation LC_{50} (4-h) value in rats is greater than 100,000 ppm (highest concentration tested), and the acute oral LD_{50} value in rats is >5 g/kg (highest dose tested). Undiluted HFE-7100 is not appreciably absorbed through the skin (rabbits) and is minimally irritating to the eyes or skin and is not a skin sensitizer (guinea pigs). The major metabolite of both isomers is heptafluorobutyric acid. This metabolite was no longer detected in the serum of rabbits 48 h after a single intravenous injection of HFE-7100 at doses up to 10 mg/kg.

Cardiac sensitization potential was tested in beagle dogs at concentrations up to 100,000 ppm. The protocol was similar to that of Reinhardt et al. (1971), including intravenous injections of epinephrine before and after inhalation of the test agent. HFE-7100 is not a cardiac sensitizer at $\leqslant$100,000 ppm (NOAEL). Results of an *in vitro* bacterial assay, an *in vitro* mammalian cell assay, and an *in vivo* mouse micronucleus test with the liquid state of HFE-7100 were negative for mutagenicity. Rats were used to determine and evaluate the potential for HFE-7100 to produce developmental toxicity. Exposure concentrations ranged from 0 to 30,000 ppm (6 h/d, gestation days 6 through 19). A mild increase in the number of fetuses with supernumerary ribs was observed at $\geqslant$15,000 ppm, but the effect was attributed to maternal stress. The NOAEL for developmental toxicity would be 4500 ppm. Twenty-eight-day and 90-day inhalation studies were performed in rats. In general, the effects observed in these studies were attributed to weak induction of peroxisomal proliferation (e.g., increased liver weight, palmitoyl-CoA oxidase activity, and incidence of

hypertrophy of centrilobular hepatocytes). The NOAEL was 7500 ppm. Urinary fluoride concentrations were monitored during both studies. Extrapolation of the urinary fluoride data indicated that a urinary fluoride value of 5 mg/l would be achieved at an exposure concentration of 1800 ppm for an 8-h work period. The 5 mg/l value is well below the 10 mg/l end-of-shift biological exposure index (BEI) for urinary fluoride (ACGIH, 2000). The WEEL Guide (AIHA, 2002) recommends an occupational exposure level of 750 ppm.

32.3.3.3 Iodotrifluoromethane (CF_3I)

Because of its similarity in chemical structure to bromotrifluoromethane (Halon 1301), CF_3I was a likely candidate to be considered for replacement of Halon 1301, a common and widely used fire suppressant. CF_3I had both low ozone depletion potential and short atmospheric lifetime. However, from a cardiotoxic perspective, CF_3I was much more potent than Halon 1301. Currently, EPA accepts the use of CF_3I only in normally unoccupied areas. Except for its cardiac sensitization potential, CF_3I has low acute inhalation toxicity. The 4-h LC_{50} value in rats is approximately 160,000 ppm (Ledbetter, 1994). The LOAEL for cardiac sensitization in epinephrine-challenged dogs is 4000 ppm; the NOAEL is 2000 ppm (Dodd and Vinegar, 1998). In comparison, similar values for cardiac sensitization for Halon 1301 are 75,000 ppm (LOAEL) and 50,000 ppm (NOAEL). Genotoxicity assays with CF_3I indicated a positive mutagenic effect in bacteria, no mutagenic effect in mammalian cells, and a NOAEL of 25,000 ppm for micronuclei formation in mouse peripheral blood cells (Dodd et al., 1997a). Ninety-day inhalation toxicity and reproductive toxicity studies in rats indicate CF_3I is not a reproductive hazard with a NOAEL of 20,000 ppm (Dodd et al., 1997b, 1999).

A physiologically based pharmacokinetics (PBPK) model was developed to simulate blood concentrations of CF_3I during short-term ($\leqslant$5 min) inhalation exposure (Vinegar et al., 2000). Based on the 5-min blood concentration of dogs exposed to CF_3I at the LOAEL of 4000 ppm, acceptable human exposure times of 0.35 to 5.0 min results in CF_3I concentrations of 5000 to 3000 ppm, respectively. The NFPA 2001 Standard allows exposure to halocarbon agents in normally occupied areas at a design concentration up to the LOAEL for a length of time determined using a PBPK model. Additionally, the PBPK model was used to simulate blood levels of CF_3I in personnel exposed during its release from an F-15 jet engine nacelle (Vinegar et al., 1999). Blood levels reached during these exposures were compared with the blood level associated with the LOAEL (for cardiac sensitization) to evaluate the possibility of safe egress. Some egress paths resulted in peak blood concentrations that exceeded the LOAEL target blood concentration, whereas other egress paths resulted in lower than the LOAEL target blood concentration.

32.3.3.4 Novec™ 1230

A relatively new replacement alternative to HFCs and PFCs is Novec™ 1230 (1,1,1,2,2,4,5,5,5-nonafluoro-4-[trifluoromethyl]-3-pentanone). It is a liquid at room temperature and has a fairly low vapor pressure of approximately 246 mmHg at 20°C. In a fire situation, the fluid rapidly gasifies to extinguish a fire. Other characteristics of Novec™ 1230 include zero ozone depletion potential, a 5-d atmospheric lifetime, and a global warming potential of one. It has been reviewed by EPA for fluorocarbon applications. The acute inhalation LC_{50} (4-h) value in rats is greater than 98,600 ppm (highest concentration tested), and the acute oral LD_{50} value in rats is $>$2g/kg (highest dose tested). Undiluted Novec™ 1230 is practically nonirritating to the eyes or skin of rabbits and is not a skin sensitizer (guinea pigs).

Cardiac sensitization potential was tested in epinephrine-challenged dogs at concentrations up to 155,000 ppm. Severe clinical signs other than cardiotoxicity were observed at 155,000 ppm, thus the NOAEL for cardiac sensitization of Novec™ 1230 is $\leqslant$100,000 ppm. Results of an *in vitro* bacterial assay and an *in vitro* mammalian cell assay with the liquid state of Novec™ 1230 were considered negative for mutagenicity. A 28-d inhalation study was performed in rats. Pulmonary effects were observed in male and female rats exposed to 10,000 or 20,000 ppm. Hepatic effects were observed in

male rats at these concentrations and at the lower concentrations of 4000 and 1000 ppm. The hepatic effects were attributed to peroxisomal proliferation, thus the NOAEL for all other exposure related effects was 4000 ppm.

32.3.3.5 Perfluorooctane Sulfonate (PFOS)

PFOS is a perfluorinated organic acid manufactured primarily by the 3M Company and is used as a surfactant in products, such as fire-fighting foams that are used to extinguish Class B fires that involve flammable fuels. However, PFOS is also formed from the degradation of other fluorocarbon products that are common in household items such as clothing, carpets, and food containers. PFOS is very stable and does not appear to be further degraded environmentally or by humans. Technical advances in analytical chemistry allowed for measurements of fluorine in human serum and the environment at part per billion (ppb) levels. The potential health effects of PFOS have been assessed from a number of oral toxicity studies in rats and non-human primates, but the results in laboratory studies have not been associated with identifiable adverse effects on human health, wildlife, or the environment. Therefore, PFOS toxicity and its persistence have created considerable scientific debate regarding its health risk assessment. The 3M Company stopped manufacturing PFOS chemicals in December 2000. Products that once contained PFOS have been replaced with products that do not contain PFOS chemicals.

The majority of evidence suggests that PFOS toxicity may be related to a primary metabolic disorder. In laboratory animals, characteristic effects due to PFOS exposure include hepatomegaly, marked decreases in serum triglyceride and cholesterol, and accumulation of lipid vacuoles in the liver. Because PFOS is a perfluorinated fatty acid, its toxicity may be related to alterations in fatty acid metabolism. A possible mechanism of action is activation of peroxisome proliferation, but not all species respond to PFOS-induced peroxisome proliferation. Results of reproductive toxicity studies in rats indicate sensitivity of neonates to PFOS, due to *in utero* exposure. The NOAEL for second-generation pups is 0.1 mg/kg/d. A two-year bioassay in rats resulted in a higher incidence of hepatocellular adenomas and thyroid follicular cell adenomas.

The dispersion of PFOS is global, including humans and various species of wildlife. Serum concentrations of PFOS in nonoccupational human samples are $\leqslant$0.1 ppm, but range from 2 to 11 ppm in occupational workers. The serum half-life of PFOS in retired workers is on the order of years, but is 100 days in rats and 200 days in monkeys. Serum concentrations of PFOS approximate liver tissue concentrations, which is useful information for assessing risk. Yet, our lack of understanding of the mode of action of PFOS, the nature and extent of its toxicity in humans and wildlife, the potential genetic variations in its metabolism, and its susceptibility to adverse effects, provide scientific challenges for a complete risk characterization of PFOS. Also, it is unclear whether the hazard concerns of PFOS can be extrapolated to other perfluorinated chemicals except where the chemical degrades to PFOS.

32.3.4 Combustion Products of Fluorocarbon Alternatives

Halons are the predominant chemicals used for fire suppression. Halon 1301 is the primary total flooding agent, and Halon 1211 is the primary streaming agent. For the safety of unprotected fire fighters and other personnel coming in contact with a fire-fighting scenario, halon replacement chemicals need to be evaluated for potential toxicity of combustion products. However, combustion toxicology is a complex discipline with many uncertainties as to test procedures and data evaluation. Some biological end points of combustion toxicology testing, e.g., incapacitation, are difficult to assess in laboratory animal models and then extrapolate to humans. Currently, EPA does not mandate a hazard evaluation of combustion products of halon substitutes. The most commonly analyzed and evaluated combustion product for toxicity potential is carbon monoxide (CO). For fluorocarbons, there is also concern for the generation of hydrogen fluoride (HF) and perfluoroisobutylene (PFIB), because of their toxic potencies. Exposure concentrations above 50 ppm or 0.3 ppm of HF or PFIB, respectively, for short periods become a concern regarding either human performance or

life-threatening health effects. Subsequently, the health hazard code assigned by the National Fire Protection Association (NFPA) on a fluorocarbon's fire and explosion characteristics includes possible HF or PFIB production and production rates. Manufacturers of fluorocarbon products, such as 3M Company, carry out studies to determine HF or PFIB generation rates of select products. Results of these studies help rank products on the basis of HF or PFIB generation rates and provide generation rates versus temperature curves for extrapolating data on products.

32.4 DISCUSSION AND CONCLUSIONS

The search for fluorocarbon alternatives was initiated more than 30 years ago following the discovery of the effects of CFCs on the stratospheric ozone levels and the subsequent potential for serious health and environmental complications. The 1987 Montreal Protocol and its amendments were agreed to by most countries and established a schedule to phase out the worldwide production of ozone-depleting substances. Inhalation toxicologists have been very active researching fluorocarbon alternatives because of their inherent physicochemical properties (e.g., high volatility) and their potential to produce toxicity (e.g., cardiac sensitization). Toxicology programs initiated by the chemical and pharmaceutical industries, such as PAFT and IPAC, worked closely with government regulatory agencies, such as U.S. EPA and OSHA, and built an extensive database of toxicology information on intended fluorocarbon replacements. Most of these toxicology studies were performed via the inhalation route and have followed the health effects testing guidelines established by OECD, TSCA, and/or FIFRA.

Specialized toxicity studies were designed to assess a wider range of potential health effects of fluorocarbon alternatives. The purpose of this chapter was to identify these studies, describe their methodologies, their experimental approach and rationale, and provide examples of results for prototype chemicals. For example, the test for cardiac sensitization is described in detail, including information on how the experimental design evolved and comments on interpretation of test results. Methods for neurotoxicity evaluation have expanded to include behavioral assessments, and the *in vitro* genotoxicity of volatile agents is relatively new in approach. Inhalation chamber design and test agent generation play important roles in exposure methodology for both neurotoxicty and genotoxicity assessments of fluorocarbon alternatives. Mechanistic studies are crucial in understanding the toxicity of chemicals including replacements for CFCs. In particular, evaluation of peroxisome proliferation of several HCFC and HFC replacement candidates in rodent studies broadened the scientific understanding on the health implications of this phenomenon. The same could be said about the occurrence of Leydig cell adenomas that were observed in several 2-year fluorocarbon bioassays. In this case, knowledge about their potential to progress to malignant tumors has been gained and put into a risk perspective.

Toxicity summaries were provided for several fluorocarbon replacement candidates, HCFC-123, HFC-134a, perfluoroalkanes, HFE-7100, CF_3I, Novec™ 1230, and PFOS. A wide range of toxicity concerns were identified from this small set of examples, such as cardiac sensitization, peroxisome proliferation, heptaocellular adenomas, Leydig cell tumors, and reproductive/developmental effects. However, most of these concerns were not considered serious health risks, except for cardiac sensitization under acute high-concentration exposure conditions. To date, hundreds of ODS alternatives have been identified and many of them have had inhalation toxicity tests (predominantly acute exposure) performed on them. In the United States, the driver behind much of the toxicity testing is EPA's SNAP program. The EPA has an excellent Web site on their SNAP program. The Web site provides general information, decisions on particular substitutes on their end use (e.g., household refrigeration) within a sector (e.g., refrigeration and air conditioning), limits and conditions applied to acceptable substitutes, and a listing of SNAP submissions. Documents can be downloaded and printed. The Web site is www.epa.gov/Ozone/snap.

The future on toxicity testing and research of fluorocarbon alternatives remains strong, in part, due to the continuous search for the ideal ODS replacement for a specific end use. In addition, the

phaseout for interim ODS replacements (e.g., HCFCs) is in progress (under the Montreal Protocol and its agreements), and in 2010, developing countries must cease production of ODS (under Article 5 of Montreal Protocol). Thus, inhalation toxicology of fluorocarbon alternatives will continue to grow for several more years.

REFERENCES

Alexander, D.J., Safety of propellants, *J. Aerosol Med.*, 8 (Suppl 1), S29–S34, 1995.

Alexander, D.J., Libretto, S.E., Adams, M.J., Hughes, E.W., and Bannerman, M., HFA-134a (1,1,1,2-tetrafluoroethane): effects of inhalation exposure upon reproductive performance, development and maturation of rats, *Hum. Exp. Toxicol.*, 15, 508–517, 1996.

ACGIH (American Conference of Governmental Industrial Hygienists), *2000 TLVs® and BEIs®,* ACGIH, Cincinnati, OH, 2000, p. 98.

AIHA (American Industrial Hygiene Association), *The AIHA 2002 Emergency Response Planning Guidelines and Workplace Environmental Exposure Level Guides Handbook.* AIHA, Fairfax, VA, 2002. p. 38.

Bar, A., Significance of Leydig cell neoplasia in rats fed lacitol or lactose, *J. Am. Coll. Toxicol.*, 11, 189–207, 1992.

Bass, M., Sudden sniffing death, *J. Am. Med. Assoc.*, 212, 2075–2079, 1970.

Boorman, G.A., Chapin, R.E., and Mitsumori, K., Testis and epididymis, in *Pathology of the Fischer Rat*, Boorman, G., Eustis, S., Montgomery, C., Elwell, M., and MacKenzie, W., Eds., Academic Press, New York, 1990.

Brock, W.J., Trochimowicz, H.J., Millischer, R.J., Farr, C., Kawano, T., and Rusch, G.M., Acute and subchronic toxicity of 1,1-dichloro-1-fluoroethane, *Food Chem. Toxicol.*, 33, 483–490, 1995.

Bunker, J.P., Forrest, W.H., Moswteller, F., and Vandam, L.D., Eds., *The National Halothane Study,* National Institutes of Health, National Institute of General Medical Sciences, Bethesda, MD, 1969.

Buschmann, J., Bartsch, W., Dasenbrock, C., Fuhst, R., Pohlmann, G., Preiss, A., and Berger-Preiss, E., Cross-fostering inhalation toxicity study with HCFC-123 in lactating Sprague-Dawley rats, *Inhalation Toxicol.*, 13, 671–687, 2001.

Cannon, W.B., Studies on the conditions of activity in endocrine glands, *Am. J. Physiol.*, 50, 399–342, 1919.

Chengelis, C.P., Acute cardiac sensitization study in dogs with FM-200 by inhalation, WIL Research Laboratories, WIL Study WIL-12265, unpublished data, 1993.

Chenoweth, M.B., Ventricular fibrillation induced by hydrocarbons and epinephrine, *J. Ind. Hyg. Toxicol.*, 28, 151–158, 1946..

Clark, D.G. and Tinston, D.J., Correlation of the cardiac sensitizing potential of halogenated hydrocarbons with their physiochemical properties, *Br. J. Pharmacol.*, 49, 355–357, 1973.

Collins, M.A., Rusch, G.M., Sato, F., Hext, P.M., and Millischer, R.J., 1,1,1,2-Tetrafluoroethane: repeat exposure inhalation toxicity in the rat, developmental toxicity in the rabbit, and genotoxicity in vitro and in vivo, *Fundam. Appl. Toxicol.*, 25, 271–280, 1995.

Cook, J.C., Klinefelter, G.R., Hardisty, J.F., Sharpe, R.M., and Foster, P.M., Rodent Leydig cell tumorigensis: a review of the physiology, pathology, mechanisms and relevance to humans, *Crit. Rev. Toxicol.*, 29, 169–261, 1999.

Dearden, J.C., Barratt, M.D., Benigni, R., Bristol, D.W., Combes, R.D., Cronin, M.T.D., Judson, P.N., Payne, M.P., Richard, A.M., Tichy, M., Worth, A.P., and Yourick, J.J., *The Development and Validation of Expert Systems for Predicting Toxicity*, The Report and Recommendations of ECVAM Workshop 24, Atlanta, 25, 223–252, 1997

Dodd, D.E., Brashear, W.T., and Vinegar, A., Metabolism and pharmacokinetics of selected halon replacement candidates, *Toxicol. Lett.*, 68, 37–47, 1993.

Dodd, D.E., Kinkead, E.R., Wolfe, R.E., Leahy, H.F., English, J.H., and Vinegar, A., Acute and subchronic inhalation studies on trifluoroiodomethane vapor in Fischer 344 rats, *Fundam. Appl. Toxicol.*, 35, 64–77, 1997b.

Dodd, D.E., Leahy, H.F., Feldmann, M.L., English, J.H., and Vinegar, A., Reproductive toxicity screen of trifluoroiodomethane (CF_3I) in Sprague-Dawley rats, *Inhalation Toxicol.*, 11, 1041–1055, 1999.

Dodd, D.E., Ledbetter, A.D., and Mitchell, A.D., Genotoxicity testing of the halon replacement candidates trifluoroiodomethane (CF_3I) and 1,1,1,2,3,3,3-heptafluoropropane (HFC-227ea) using the *Salmonella*

typhimurium and L5178Y mouse lymphoma mutation assays and the mouse micronucleus test, *Inhalation Toxicol.*, 9, 111–131, 1997a.

Dodd, D.E. and Vinegar, A., Cardiac sensitization testing of the halon replacement candidates trifluoroiodomethane (CF_3I) and 1,1,2,2,3,3,3-heptafluoro-1-iodopropane (C_3F_7I), *Drug Chem. Toxicol.*, 21, 137–149, 1998.

Downing, R.C., *Fluorocarbon Refrigerants Handbook*, Prentice Hall, Englewood Cliffs, NJ, 1988.

Dzhekova-Stojkova, S., Bogdanska, J., and Stojkova, Z. Peroxisome proliferators: their biological and toxicological effects, *Clin. Chem. Lab. Med.*, 39, 468–474, 2001.

ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals), Joint Assessment of Commodity Chemicals, No. 33, 1,2-Dichloro-2,2,2-trifluoroethane (HCFC-123), Brussels, 1996.

ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals), Joint Assessment of Commodity Chemicals, No. 31, 1,1,1,2-Tetrafluoroethane (HFC-134a), Brussels, 1995.

Emmen, H.H., Hoogendijk, E.M., Klopping-Ketelaars, W.A., Muijser, H., Duistermaat, E., Ravensberg, J.C., Alexander, D.J., Borkhataria, D., Rusch, G.M., and Schmit, B., Human safety and pharmacokinetics of the CFC alternative propellants HFC 134a (1,1,1,2-tetrafluoroethane) and HFC 227 (1,1,1,2,3,3, 3-heptafluoropropane) following whole-body exposure, *Regul. Toxicol. Pharmacol.*, 32, 22–35, 2000.

Harris, J.W., Jones, J.P., Martin, J.L., LaRosa, A.C., Olson, M.J., Pohl, L.R., and Anders, M.W., Pentahaloethane-based chlorofluorocarbon substitutes and halothane: correlation of in vivo hepatic protein trifluoroacetylation and urinary trifluoroacetic acid production with calculated enthalpies of activation, *Chem. Res. Toxicol.*, 5, 720–725, 1992.

Harrison, L.I., Donnell, D., Simmons, J.L., Ekholm, B.P., Cooper, K.M., and Wyld P.J., Twenty-eight-day double-blind safety study of an HFA-134a inhalation aerosol system in healthy subjects, *J. Pharm. Pharmacol.*, 48, 596–600, 1996.

Hubbard, A.K., Roth, T.P., Gandolfi, A.J., and Brown, B.R., Halothane hepatitis patients generate an antibody response toward a metabolite of halothane, *Anesthesiology*, 68, 791–796, 1988.

Iawata, T.J., Hirouchi, Y., Koike, Y., Yamakawa, S., Kobayashi, K., Yamamoto, T., Inoue, H., and Enomoto, M., Historical control data of non-neoplastic and neoplastic lesions in the F344/DuCrj rats, *J. Toxicol. Pathol.*, 4, 1–24, 1991.

Krahn, D.F., Barsky, F.C., and McCooey, K.T., CHO/HGPRT mutation assay: evaluation of gases and volatile liquids, in *Genotoxic Effects of Airborne Agents*, Tice, R.R., Costa, D.L., and Schaich, K.M., Eds., Plenum, New York, 1982, pp. 91–103.

Ledbekkers, A.D., Mantech Environmental Technology, Inc., Final Report, Project 1530-001, 1994.

Levy, A.G., The exciting causes of ventricular fibrillation in animals under chloroform anesthesia, *Heart*, 4, 319–378, 1913.

Levy, A.G. and Lewis, T., Heart irregularities resulting from the inhalation of low percentages of chloroform vapour and their relationship to ventricular fibrillation, *Heart*, 3, 99–111, 1911.

Lind, R.C. and Gandolfi, A.J., Subanesthetic halothane is hepatoxic in guinea pigs; plasma ICDH vs ALT as an index of injury, *Toxicologist*, 11, 63, 1991.

Lovelock, J.E., Air pollution and climatic change, *Atmosph. Environ.,* 5, 403–411, 1971.

Malinverno, G., Rusch, G.M., Millischer, R.J., Hughes, E.W., Schroeder, R.E., and Coombs, D.W., Inhalation teratology and reproduction studies with 1,1-dichloro-2,2,2-trifluoroethane (HCFC-123), *Fundam. Appl. Toxicol.*, 34, 276–287, 1996.

Malley, L.A., Carakostas, M., Hansen, J.F., Rusch, G.M., Kelly, D.P., and Trochimowicz, H.J., Two-year inhalation toxicity study in rats with hydrochlorofluorocarbon 123, *Fundam. Appl. Toxicol.*, 25, 101–114, 1995a.

Malley, L.A., Murray, S.M., Elliott, G.S., Van Pelt, C., Marrs, G.E., and Brock, W.J., Subchronic toxicity, neurotoxicity, and teratogenicity of 1,1,1,2,2,3,4,5,5,5-decafluoropentane (43–10mee) by inhalation exposure to rats, *Int. J. Toxicol.*, 7, 19, 1995b.

McFarland, M. and Kaye, J., Chlorofluorocarbons and ozone, *Photochem. Photobiol.*, 55, 911–929, 1992.

Meek, W.J., Hathaway, H.R., and Orth, O.S., The effects of ether, chloroform and cyclopropane on cardiac automaticity, *J. Pharmacol. Exp. Ther.*, 61, 240–252, 1937.

Molina, M.J. and Rowland, F.S., Stratospheric sink for chlorofluoromethanes: Chlorine atom-catalysed destruction of ozone, *Nature*, 249, 810–812, 1974.

Moody, D.E., Reddy, J.K., Lake, B.G., Popp, J.A., and Reese, D.H., Peroxisome proliferation and nongenotoxic carcinogenesis: commentary on a symposium,. *Fundam. Appl. Toxicol.*, 16, 233–248, 1991.

Mullin, L.S., Azar, A., Reinhardt, C.F., Smith, P.E., Jr., and Fabryka, E.F., Halogenated hydrocarbon-induced cardiac arrhythmias associated with release of endogenous epinephrine, *Am. Ind. Hyg. Assoc. J.*, 33, 389–396, 1972.

Nyden, M.R. and Skaggs, S.R., *Screening Methods for Agent Compatibility with People, Materials, and the Environment*, National Institute of Standards and Technology Interagency Report 6323, April 1999, 67 pp.

Raventos, J., The action of fluothane—a new volatile anesthetic, *Br. J. Pharmacol.*, 11, 394–410, 1956.

Reinhardt, C.F., Azar, A., Maxfield, M.E., Smith, P.E., and Mullin, L.S., Cardiac arrhythmias and aerosol sniffing, *Arch. Environ. Health*, 22, 265–279, 1971.

Rusch, G.M., Coombs, D., and Hardy, C.J., Acute, genetic, developmental, and inhalation toxicology of 1,1,1, 3,3-pentafluoroethane (HFC-245fa). *Toxicol Sci.,* 52, 289–301, 1999.

Rusch, G.M., Trochimowicz, H.J., Malley, L.J., Kelly, D.P., Peckham, J., Hansen, J., and Charm, J.B., Subchronic inhalation toxicity studies with hydrochlorofluorocarbon 123 (HCFC 123), *Fundam. Appl. Toxicol.*, 23, 169–178, 1994.

Stevens, J.T. and Green, J.D., Test article administration, in *Inhalation Toxicology: Research Methods, Applications and Evaluation*, Chapter 3, Salem, H., Ed., Marcel Dekker, Inc., New York, 1987.

Thompson, D.S. and Friday, C.D., Changes in liver enzyme values after halothane and enflurane for surgical anesthesia, *South. Med. J.*, 71, 779–782, 1978.

Vinegar, A., Jepson, G.W., Hammann, S.J., Harper, G., Dierdorf, D.S., and Overton, J.H., Simulated blood levels of CF_3I in personnel exposed during its release from an F-15 jet engine nacelle and during intentional inhalation, *Am. Ind. Hyg. Assoc. J.*, 60, 403–408, 1999.

Vinegar, A., Jepson, G.W., Cisneros, M., Rubenstein, R., and Brock, W.J., Setting safe acute exposure limits for halon replacement chemicals using physiologically based pharmacokinetic modeling, *Inhalation Toxicol.,* 12, 751–763, 2000.

Zamora, P.O., Benson, J.M., Li, A.P., and Brooks, A.L., Evaluation of an exposure system using cells grown on collagen gels for detecting highly volatile mutagens in the CHO/HGPRT mutation assay, *Environ. Mutagen,* 5, 795–801, 1983.

33 Animal Models for Three Major Cigarette-Smoke-Induced Diseases

Raymond R. Schleef, Patrick M. Vanscheevwijck, Walter K. Schlage, Joseph F. Borzelleca, Christopher R.E. Coggins, and Hans-Juergen Haussmann

CONTENTS

33.1 INTRODUCTION

Cigarette smoking produces considerable morbidity and mortality from many diseases, with three main categories (U.S. Department of Health and Human Services, 1982, 1983, 1984). Since the early epidemiological studies linking smoking with these diseases, scientists have performed animal studies to examine the links in a laboratory setting. In the classic toxicology rationale, well-designed animal studies eliminate those confounding factors inherent in epidemiology but also bring into question the extrapolation of results from laboratory animals (especially rodents) to humans.

This chapter is not concerned with the use of animal studies to establish causation of the examined diseases. Our aim was to review and (hopefully) establish the underlying mechanisms for these diseases. Given the mechanisms, scientists could then work on ways to test diagnostic tools, chemopreventative treatments, and designs for potentially reduced-exposure/risk products (PREP) (U.S. Institute of Medicine, 2001). In a recent report (U.S. Institute of Medicine, 2001), the U.S. Institute of Medicine determined that the concept of PREP cigarettes is feasible. It was also determined that manufacturers of PREP cigarettes should be required to conduct appropriate toxicological testing in *in vitro* and *in vivo* models, as well as appropriate clinical testing, to support any potential claims. Given the complexity of diseases such as lung cancer (LC), cardiovascular disease (CVD), and chronic obstructive pulmonary disease (COPD), animal models are an essential part of a comprehensive pharmacological and toxicological assessment for the above-mentioned objectives, as part of a matrix together with analytical chemistry and *in vitro* assessments.

The initial phase of animal tests with cigarette mainstream smoke (MS) was in retrospect quite basic, preferentially using the mouse skin painting assay (see below) for carcinogenicity. Since then, sophisticated nose-only inhalation techniques have been introduced with MS. Even with these techniques, one disease state (CVD) has largely been omitted in the face of attempts to produce experimental LC, with COPD falling somewhere between the two. For CVD, many of the published studies have avoided exposures to MS, studying instead the effects of inhalation exposures to surrogates for environmental tobacco smoke (ETSS). Although qualitatively similar, the concentration of ETSS is much more dilute than that of MS. Many studies used whole-body exposures, introducing complicating factors due to oral and dermal absorption of smoke components. Exposures using routes of exposure other than inhalation suffer another inherent limitation in extrapolating to human smokers and are beyond the scope of this chapter.

33.2 CARDIOVASCULAR DISEASE: PAST APPROACHES AND NEW IDEAS

33.2.1 Epidemiology

There are numerous epidemiological studies that link smoking with varying types of CVD (coronary heart disease, stroke, peripheral vascular disease, and congestive heart failure), with an increased risk for both thrombosis of narrowed vessels and increased atherosclerosis in those vessels (Burns, 2003). The relationships between smoking and other cardiovascular risk factors have recently been reviewed in the Third National Health and Nutrition Examination Survey (NHANES III), a major conclusion being that inflammation may be a common underlying mechanism (Bazzano et al., 2003).

The contribution of oxidant stress to smoking-induced vascular injury has also been considered (Burke and FitzGerald, 2003). Endothelial abnormalities may also be involved at an early stage in the atherogenic and thrombotic change (Puranik and Celermajer, 2003), especially in the coronary endothelium (Czernin and Waldherr, 2003). There are clearly genetic factors involved in MS-induced CVD (Wang et al., 2003a).

It may be possible to develop models of MS-induced disease, based on the techniques used in the examination of cardiovascular responses in animals exposed to ETSS. The issue here is a paradox: epidemiological studies of nonsmokers whose spouses smoke have shown a small excess (30%, or an odds ratio of 1.3) in the risk of ischemic heart disease (Denson, 1999), whereas the

overall excess risk from smoking 20 cigarettes per day is reported to be 80% (or an odds ratio of 1.8, for a presumably much higher exposure than that of the nonsmoking spouse of a smoker) (Law and Wald, 2003). Others (Burns, 2003) have attempted to explain this paradox.

33.2.2 Basic Pathways Mediating MS-Induced CVD

To set the stage for a discussion on animal models for cigarette smoke-related CVD, this section will highlight the effects of cigarette smoke-derived constituents on diverse tissues. Access of gaseous and dissolved constituents of cigarette smoke to the circulatory system and subsequent distribution throughout the body is permitted by the proximity of the blood-containing capillary endothelial cells with the pulmonary epithelial lining of the lung. A textbook example of a compensatory response to a constituent in MS is the binding of carbon monoxide (CO) in MS to hemoglobin, reducing its oxygen-binding capacity, which can result in a compensatory increase in the production of red blood cells and blood viscosity.

Blood vessels are lined by a single layer of endothelial cells, which are recognized as a metabolically active tissue. Exposure of these cells to constituents of MS both *in vitro* and *in vivo* alters the production of several proteins and small molecular weight molecules, which also play key roles in inflammatory processes. Current information suggests that endothelial cell activation during inflammation is mediated by a diverse set of cells, ligands, receptors, and small molecular weight molecules (Szmitko et al., 2003a, 2003b). A classic example is the release of leukocyte-derived cytokines in response to an invading organism and the accompanied cytokine-induced expression of several endothelial adhesive glycoproteins (e.g., intercellular adhesive glycoprotein-1, ICAM-1), which facilitates the binding and trans-endothelial migration of leukocytes into the surrounding tissue.

The inflammatory response of the body to cigarette smoke is represented by a series of inhalation experiments clarifying the role of tumor necrosis factor-alpha (TNFα) in this process. More specifically, nose-only MS inhalation experiments (a single exposure) with mice deficient in the human receptor for TNFα revealed that these mice do not develop lung inflammation, indicating that TNFα is important in the acute response to MS (Churg et al., 2002a). Experiments with macrophage metalloelastase-deficient (MMP-12$^{-/-}$) mice indicated that both neutrophils and MMP-12 are required for MS-induced inflammation and matrix breakdown (Churg et al., 2002b). Subsequent experiments with MS-exposed mice (a single exposure) revealed that levels of E-Selectin, an endothelial activation marker, were increased in only wild-type (MMP-12$^{+/+}$) mice (Churg et al., 2003a). This same group has proposed a mechanistic model in which MMP-12 mediates MS-induced inflammation by releasing TNFα from macrophages, which activates endothelial cells by binding to the TNFα-receptor on the cell surface of endothelial cells (Churg et al., 2003a). This results in the expression of cell surface adhesion glycoproteins that are required for leukocyte binding and efflux into the surrounding tissues.

Acute exposure to MS can directly affect a number of tissues resulting in altered production of inflammatory cytokines and other mediators that can subsequently indirectly affect the vasculature. For example, it has been reported (Zhang et al., 2001) that a 4-month whole-body exposure of 13-month-old C57Bl/6 mice to sidestream smoke (30 min/day, 2 cigarettes every 10 min, 5 days/week) resulted in increases in both splenocyte interleukin-6 production and hepatic lipid peroxide production. Multiple antioxidants given orally effectively prevented these changes.

A cascade initiated by the activation of endothelial cells, the binding and trans-endothelial migration of monocytes into the subendothelial space and their subsequent transformation into macrophages induced by constant inhalation of MS leads to a chronic inflammatory process that sets the stage for the destructive modification of blood vessels. The ability of MS-derived constituents to activate platelets further support conditions that are favorable for enhanced deposition of leukocytes and platelets on the vessel wall and occlusive thrombosis formation. The negative effects of MS-derived constituents are not restrictive to the vessel wall but act in a similar manner on endothelial cells that line the myocardium. MS-derived constituents have been observed to directly affect the oxygen-requiring myocytes and alter cardiac function and structure in a manner that predisposes

the myocardial tissue to ischemia and enhanced myocardial infarction. Thus, MS-induced CVD is not a single phenomenon but occurs as a combination of alterations or aberrant responses in the circulatory system that negatively impact mortality and morbidity and can be categorized as endothelial dysfunction, atherogenesis, thrombosis, and impaired cardiac function.

33.2.3 Difficulty in Developing Animal Models for Smoke-Induced CVD

Although multiple epidemiological, clinical, and pathological studies have strongly related cigarette smoking to the development of CVD, most species of laboratory animals appear to be resistant to the effects of MS and only minor changes in cardiovascular function are observed following chronic exposure to MS, even after exposure of several years. This situation has resulted in the use of compromised animal models (Kodavanti and Costa, 2001) in an attempt to further investigate the mechanisms by which MS affects the cardiovascular system. The following sections summarize observations obtained following the exposure of either ETSS or MS in both normal and compromised animal models.

33.2.4 Animal Models to Investigate the Impact of Smoke on Endothelial Dysfunction (Including Vasodilation)

Cigarette smoke exerts both positive and negative effects on the synthesis/activity of proteins and other molecules. Endothelial-dependent vasodilation is decreased by MS in humans, and one mechanism is decreased production (or increased degradation) of the endothelium-derived vasodilator nitric oxide. A decreased vasodilatory capability of the vessels increases blood pressure, which can negatively impact the circulatory system. Because endothelial-dependent vasodilation is currently recognized as a basic marker for endothelial dysfunction, the effect of MS on vasodilation has been investigated in a series of different animal models.

An organ bath system is one useful assay for measuring the vasoconstrictor and vasodilator properties of a blood vessel after exposure to MS: typically, the thoracic aorta is used in this *in vitro* assay. Exposure for 10 weeks, 6 h per day, to ETSS has been shown to reduce endothelial-dependent relaxation in normocholesterolemic rabbits (Hutchison et al., 1997a, 1997b) and rabbits with diet-induced hypercholesterolemia (Hutchison et al., 1997b, 1999).

In addition to the thoracic aorta, several other vessels (e.g., carotid artery, infrarenal artery, superficial femoral artery) have also been analyzed in an organ bath system after exposure of animals to MS. For example, it was reported in rabbits (Hutchison et al., 2001) that 10 weeks of ETSS exposure also induced endothelial dysfunction in the pulmonary artery, possibly by decreasing arginine in the endothelium because dietary supplementation with arginine prevented the dysfunction.

An organ bath system has shown that 8 weeks of MS exposure impaired the endothelium-dependent relaxation to acetylcholine in rabbit superficial femoral arteries and saphenous veins (Traul et al., 1995). These observations were extended by investigating the effect of an antioxidant (ascorbic acid) on the MS-mediated vascular injury of the superficial femoral artery (8-week exposure, no smoke concentrations provided) (Mays et al., 1999). Acetylcholine-dependent relaxation was significantly reduced in the rings from the MS animals and the combined ascorbic acid and MS group. However, there were differences with regard to the sensitivity of a particular vessel to MS. For example, rabbits exposed to MS or supplied a diet supplemented with a high content of cholesterol did not exhibit a defective response to acetylcholine when the infrarenal aorta was analyzed (Mudaliar et al., 1997). However, endothelial damage, as measured by acetylcholine-mediated vasorelaxation, was observed in the infrarenal aorta harvested from rabbits that were exposed to both MS and elevated dietary cholesterol (Mudaliar et al., 1997). The authors suggest that differences in the structure of vessels (e.g., content of elastin) might explain the ability of MS alone or the combination of MS and hypercholesterolemia to achieve an affect on vasorelaxation

in a particular animal model (Mudaliar et al., 1997). Other studies from the same group (Johnson et al., 1999) expanded upon this concept and demonstrated that cholesterol, but not an 8-week exposure to MS, decreased carotid artery endothelial-dependent relaxation in rabbits.

Conflicting reports using vessels harvested from MS-exposed rats have also been published. One group (Ribeiro-Jorge et al., 1995) observed impaired endothelial-dependent vasodilation in thoracic aorta rings harvested from Wistar rats that were exposed to ETSS for 30 days in a whole-body chamber, whereas others (Zhu et al., 1996) reported that 6-week whole-body exposure to ETSS in Sprague–Dawley rats increased the endothelial sensitivity of aortic ring to acetylcholine and the maximal relaxation of the rings to acetylcholine was significantly greater. These authors (Zhu et al., 1996) suggested that abnormal aortic ring vasodilation may have been mediated by chronic exposure to nitric oxide and CO. Nose-only exposure of Sprague–Dawley rats to MS for 10 weeks was shown to augment endothelium-derived vasorelaxation of the thoracic aorta precontracted with norepinephrine (Nene et al., 1997).

33.2.5 Endothelial Regeneration and Angiogenesis

The concept of endothelial dysfunction as a central point for smoke-induced vascular disease is further represented by the inhibition of new endothelial cell growth, required during multiple physiological processes, including those for development (Magers et al., 1995) and wound healing (Ma et al., 2000). For example, a MS-induced reduction in the angiogenic response in acetic acid-induced gastric ulcers was correlated with a reduction in nitric oxide synthesis and epidermal growth factor (EGF) biosynthesis (Ma et al., 2000). The adverse effects of MS on blood vessels can also be demonstrated by exposing the chick chorioallantoic membrane to smoke solutions for 4–5 days (Melkonian et al., 2000). Solutions of whole- or gas-phase MS and SS smoke caused a disruption in angiogenesis and extracellular matrix deposition in the chick chorioallantoic membrane. It may be possible to modify this assay to permit direct exposure of fertilized chicken eggs and develop a rapid assay to further dissect the effects of smoke on endothelial function.

Cigarette smoke has been found to stimulate endothelial cell growth in several model systems. First, Sarkar et al. (1999) subjected rats to balloon injury of the thoracic aorta and exposed the rats to MS at 6 cigarettes per day for 2 weeks. Exposure to smoke significantly increased aortic endothelial regeneration and was associated with an increase in serum nitric oxide levels. These authors suggested that the increased nitric oxide levels, possibly derived from the MS, may stimulate the vascular endothelium. Second, Sekhon et al. (1994) exposed rats to smoke from 7 cigarette per day for 1, 2, and 7 days and noted that the exposures produced proliferation of the endothelium of the pulmonary vasculature. Third, a pathological model for angiogenesis using Lewis lung cancer cells injected into mice (Zhu et al., 2003) showed that ETSS could increase tumor size and capillary density (see below).

33.2.6 Animal Models to Investigate the Impact of Smoke on Atherogenesis

Rabbits are a useful model for atherosclerotic research, with a high-fat diet stimulating this process. ETSS in whole-body exposures has been observed to further promote the atherosclerotic process in normal and hypercholesterolemic New Zealand white rabbits (Zhu et al., 1993; Hutchison et al., 1997a). Apparently, the nicotine in the smoke does not contribute to the arterial lipid lesions in ETSS-exposed rabbits (Sun et al., 2001). Cockerels are another animal species that responds with enhanced atherosclerosis in response to ETSS (Penn and Snyder, 1993; Penn et al., 1994, 1996), with increased atherosclerosis reported after a 16-week exposure to one cigarette per day, but (as a nonmammal) with questionable relevance to humans.

Rodents appear to be resistant to the effects of a high-fat diet and do not develop atherosclerosis on a high-fat diet for 1 or 2 years. This situation has led to the development of transgenic

mice deficient in genes important in lipid transport and metabolism, including the gene coding for apopolipoprotein E ("ApoE") (Breslow, 1996). This molecule is important in the transport of cholesterol from tissues into the liver; the absence of ApoE results in the formation of lipid-rich plaques in murine vasculature, which is further enhanced by a high-fat diet. The mice develop vascular lesions that are reported to be very similar to those noted in humans (Breslow, 1996).

Gairola et al. (2001) exposed $ApoE^{-/-}$ mice to ETSS in whole-body chambers for 6 h/d, 5 days per week for 14 weeks, using a particulate concentration of 25 mg/m^3. Exposures resulted in significant elevations in blood COHb concentrations (values were approximately 10%). Morphometric assessments showed increased plaque development in the intimal area of the aorta in the smoke-exposed groups, confirmed by measurement of tissue cholesterol concentration.

The aortic root plaques of $ApoE^{-/-}$ mice exposed for 8 weeks to MS from unfiltered cigarettes (no details given on smoke concentrations) have been shown to have higher levels of immunoreactivity for tissue factor, vascular cell adhesion molecule-1, and macrophages, as compared with nonexposed mice (Matetzky et al., 2000). Subgroups of smoke-exposed mice treated with aspirin showed reductions in tissue factor expression.

Exposure of $ApoE^{-/-}$ mice to MS for 42 days, 6 h/d was shown to not only increase lesion formation but also increased mitochondrial DNA damage and protein nitration, while decreasing the specific activities of several mitochondrial enzymes (Knight-Lozano et al., 2002). Thus, the $ApoE^{-/-}$ mouse may be useful for understanding the role of MS in fostering atherosclerosis.

There are only sparse reports in the literature on modeling of human cardiovascular disease in larger (nonrodent) animals exposed to MS. For example, Marshall (1986) briefly summarized observations on the use of minipigs as models for atherosclerosis, work that suggested an effect of MS on platelet adhesion and atherosclerosis. A major exception is the National Cancer Institute study "Effects of nicotine and carbon monoxide on atherogenesis" (Hazleton, 1981). The purpose of the study was to "assess the relative and combined contributions of nicotine and carbon monoxide in cigarette smoke as co-factors in the genesis of diet-induced atherosclerosis in the male beagle dog." The study involved hundreds of dogs, each with a permanent tracheotomy. Several of the dogs died during the experiment, primarily as a result of the diet (i.e., malnutrition and/or advanced atherosclerosis).

The study used cigarettes containing tobacco with different concentrations of nicotine (no data given), and some groups of animals were exposed to smoke enriched with added CO, or to CO alone, for 2 years. The results of this study "lent no support to the suggestion that cigarette smoking increases the rate of development of atherosclerosis." The authors stated, in fact, that "these results appear to be more indicative of a possible protective effect from cigarette smoking and/or CO inhalation than of an atherogenic effect" (Hazleton, 1981). However, the results of this study have not appeared in the peer-reviewed literature.

Another study used baboons exposed to smoke for 3.3 years (Rogers et al., 1988), with again some groups being fed an atherogenic diet. Animals were taught to inhale smoke through the mouth, using operant conditioning with water rewards. Cigarette smoking did not increase the extent of diet-induced atherosclerosis, shown also in another non-human primate (Raymond et al., 1982).

33.2.7 Models to Detect the Impact of Smoke on Intimal Hyperplasia

Cigarette smoking has been identified as a risk factor for restenosis and thrombosis in patients who undergo vascular surgical procedures and angioplasty. Petrik et al. (1995) and Law et al. (1996) examined the effect of MS on balloon catheter injury of the left common carotid artery. Intimal hyperplasia was measured by planimetry and smoke exposure was shown to accelerate carotid artery hyperplasia (Petrik et al., 1995; Law et al., 1996). Petrik et al. (1995) exposed rats to a target dose of smoke from 1 to 8 cigarettes per day, 5 days per week for 4 weeks, in an attempt to understand

the dose–response relationship of smoke and intimal hyperplasia. The data appear to demonstrate a threshold effect with no effect below 4 cigarettes per day, whereas a similar increase in intimal hyperplasia after injury was obtained at 6 and 8 cigarettes per day (Petrik et al., 1995). Tani et al. (2004) detected increased intimal thickening in $ApoE^{-/-}$ mice exposed to MS (1 cigarette/day, for 5 weeks, 21 days following placement of carotid arterial cuff). Research into the underlying mechanisms leading to intimal thickening and plaque development have suggested a role of increased inducible nitric oxide synthase (iNOS) expression, using both the specific iNOS inhibitor mercaptoethylguanidine-treated wild-type mice and iNOS-deficient mice exposed to MS from 1 cigarette/day (Anazawa et al., 2004).

33.2.8 Models to Detect the Impact of Smoke on Thrombosis and Platelet Activation

Although MS increases the risk of thromboembolic complications, it has been difficult to reproducibly demonstrate a transient increase in *ex vivo* platelet activity either in humans or animals following exposure to MS (Folts et al., 1990). In an attempt to circumvent the problems associated with the processing of blood for *ex vivo* platelet aggregation studies, Folts and colleagues have developed an *in vivo* animal model of arterial stenosis (Keller and Folts, 1988; Folts et al., 1990). In brief, anesthetized dogs were mechanically ventilated and the periodic formation of acute platelet thrombi measured in a mechanically stenosed (70% diameter reduction) coronary artery using an electromagnetic flow probe.

This latter group have utilized this canine model to investigate the platelet activation ability of MS in comparison with smoke generated with a new type of cigarette where the tobacco was only heated (by a burning charcoal heat source) (Gering and Folts, 1990). The rate of flow declined similarly in dogs exposed to both kinds of smoke, suggesting that the MS produced from both cigarette types had similar effects on acute platelet thrombus formation and myocardial oxygen supply.

A second model for detecting platelet activation following MS exposure from one cigarette using an observation chamber implanted onto the dorsal skin of hamsters and intravital microscopic examination of the microcirculation (Lehr et al., 1999). Exposure of hamsters to MS resulted in aggregation and adhesion of platelets and/or leukocytes to the microvascular endothelium in venules and arterioles (Lehr, 2000). Leukocyte adhesion to the vascular wall appears to be inhibited by platelet-activating factor receptor antagonist (Lehr et al., 1997) and by pretreatment with superoxide dismutase (Lehr et al., 1993).

Platelet function was measured in a 10-week study with cholesterol-fed rabbits, exposed whole-body to two different concentrations of ETSS, 6 h/d (Zhu et al., 1993). At the end of the experiment, bleeding time measured at the end of the exposure was about 20% shorter in both ETSS groups than in controls. There was no effect on bleeding time preexposure; changes in platelet aggregation and platelet count were equivocal (Zhu et al., 1993).

33.2.9 Animal Models to Investigate the Impact of Smoke on the Myocardium and on Myocardial Function

Because exposures to both MS and ETSS have been reported to damage the heart, a number of laboratories have optimized assays that not only reveal a detrimental effect of MS on heart function but may also demonstrate mechanisms by which MS impacts the heart. Studies in the 1970s investigated the ability of MS to cause myocardial injury (cardiomegaly) in guinea pigs (Lough, 1978). White albino male guinea pigs were restrained in an eight-channel smoking machine and exposed (12–15 weeks, 5 d/week) to two types of MS: (1) unfiltered MS or (2) MS that was passed through a glass fiber filter ("Cambridge filter") to remove the particulate phase of the smoke. Heart weights of the smoking animals were significantly increased in both the unfiltered and Cambridge-filtered MS groups in comparison to non-MS-exposed animals. Although

no differences were apparent upon light microscopic examination of the hearts by using three histological stains (i.e., hematoxylin-eosin, periodic acid-Schiff, and trichrome), electron microscopic examination revealed morphological changes associated with both the nonfiltered and the Cambridge-filtered MS groups. For example, the mitochondria of the MS-exposed animals were shrunken and irregular with frequent separations of the outer mitochondrial membrane, as well as the presence of increased lipid and increased lysosomal residual bodies that were suggestive of increased autophagolysosomal activity.

Because the ultrastructural changes in the myocardium were similar in the nonfiltered and Cambridge-filtered MS groups and resembled those changes that occurred in animals exposed to pure CO, the authors suggested that CO might be the toxic agent causing the cardiomyopathy in the MS-exposed guinea pigs, a suggestion confirmed in later work using rats (Ayres et al., 1989).

Van Jaarsveld and coworkers (1992b, 1992a) investigated if MS-induced abnormalities in cardiac mitochondrial ultrastructure would lead to mitochondrial dysfunction, which could be measured during the response of the cardiac tissue to stress, such as during low oxygen supply (ischemia). These investigators utilized an *in vitro* "working heart model" to examine if MS exposure aggravated the ischemia/reperfusion injury (van Jaarsveld et al., 1992a, 1992b). These latter studies utilized whole-body exposure of Sprague–Dawley rats to ETSS for 2 months. The hearts were isolated and perfused with an oxygenated Krebs buffer. The hearts were subjected to normothermic ischemic cardiac arrest for 10 min and either 5 or 10 min of reperfusion.

Mitochondria were isolated and functional analysis revealed that ischemia followed by reperfusion depressed mitochondrial oxidative function, which was more severe in the smoke-exposed rats. In rats exposed to ETSS, cytosolic low molecular weight iron was observed to be higher and myocardial α-tocopherol (an antioxidant) levels were more severely diminished during ischemia/reperfusion than non-ETSS-exposed animals. Subsequently, dietary supplementation with α-tocopherol and β-carotene was shown to be able to partially protect smoke-exposed rats from myocardial ischemic/reperfusion injury (van Jaarsveld et al., 1992a). More specifically, vitamin supplementation protected the smoke-exposed animals from losses in mitochondrial functional oxidative damage but not from increases in cellular low-molecular-weight iron content.

This group of researchers utilized this ischemic/reperfusion model to investigate the impact of MS exposure on the levels of several other antioxidants (van Jaarsveld et al., 1994). In the nonperfused hearts of MS-exposed rats, cysteine levels were diminished while myocardial glutathione (GSH) concentrations were elevated. Following ischemia/reperfusion, further negative changes in cysteine and GSH levels were detected in the smoke-exposed animals, whereas no changes in these two parameters were detected following ischemia/reperfusion of hearts from control animals.

The negative effect of ETSS on antioxidant levels and heart function has also been examined in mouse models. Zhang et al. (2002) exposed C57Bl/6 mice nose-only to two doses of ETSS (i.e., 1 or 2 h) over a period of 4 months and observed a dose-dependent decrease in α-tocopherol levels in cardiac tissue. Although most cardiac parameters were not affected by exposure to ETSS and agreed with the aforementioned difficulty in detecting functional changes in normal animals, there was an increase in vascular impedance (effective arterial elastance) and a significant reduction in the stroke volume at the higher ETSS dose (Zhang et al., 2002). Moreover, the decreases in cardiac α-tocopherol levels were strongly correlated with the reductions in stroke volume in the ETSS-exposed animals. Both splenic interleukin (IL) β and TNFα secretion exhibited a strong negative correlation with stroke volume (Zhang et al., 2002).

As mentioned above, studies on cardiac function using the $ApoE^{-/-}$ mouse model revealed that MS exposure increases mitochondrial damage (Knight-Lozano et al., 2002).

Zhu et al. (1994) examined the effects of exposure to ETSS on myocardial infarct size in an *in vivo* rat model of coronary artery occlusion and reperfusion. Sprague–Dawley rats were exposed whole-body to ETSS for 3 days, 3 weeks, or 6 weeks. The animals were anesthetized and mechanically ventilated on a Harvard rodent respirator. A reversible coronary artery snare occluder was

placed around the proximal left anterior descending coronary artery via a midline sternotomy, and the animals were subjected to 35 min of left coronary artery occlusion followed by 120 min of reperfusion. Measurement of infarct size was performed by injection of phthalocyanine blue dye into the left ventricular cavity, allowing normally perfused myocardial tissue to stain blue. Utilizing this protocol, the ETSS exposure showed an increase in infarct size compared with the control groups, with longer exposure associated with larger infarct size (Zhu et al., 1994). For example, infarct size nearly doubled with 6 weeks of exposure.

In a follow-up study, this group investigated if decreased myocardial reperfusion in the ETSS-exposed animals could have been mediated by endothelial cell damage and decreased vascular production of endothelium-derived relaxing factor, nitric oxide (Zhu et al., 1996). Because nitric oxide is metabolically derived from arginine and dietary supplementation with arginine has been shown to improve endothelial-dependent vasorelaxation and reduce myocardial and cerebral infarct size, an experiment was performed to examine the effect of arginine on infarct size following ETSS exposure. Sprague–Dawley rats were exposed for 6 weeks to ETSS, either in the presence or absence of 2.25% arginine in their drinking water; excised hearts were subsequently exposed to ischemia and reperfusion injury. Infarct size was similarly increased by ETSS exposure but arginine supplementation significantly reduced the infarct size in ETSS-exposed animals (Zhu et al., 1996).

This same group then utilized this model system to assess the effects of ETSS on infarct size in young rats exposed *in utero* or in the neonatal to adolescent period (Zhu et al., 1997). Exposure to ETSS in the neonatal to adolescent period was also observed to significantly increase the myocardial infarct size; exposure to ETSS *in utero* for 3 weeks resulted only in a tendency for increased infarct size (Zhu et al., 1997).

Because MS is known to correlate with an increased propensity for hypertensive disease, this same group then attempted to define the role of the renin-angiotensin system in the ETSS-induced sensitivity to ischemia/reperfusion injury (Zhu et al., 2002). Changes in the renin-angiotensin system were shown to lead to endothelial dysfunction and increased infarct size in this rat ischemia-reperfusion model; however, the renin-angiotensin system did not mediate the increased infarct size (Zhu et al., 2002).

33.2.10 Conclusion and Outlook

The literature indicates a number of biological processes associated with CVD are affected by the exposure of animals to cigarette smoke. Unfortunately, a lack of standardization exists concerning the generation of smoke (e.g., MS/ETSS, smoking procedure) and the exposure conditions (whole-body/nose-only, concentrations used, length of treatment) which hinder comparisons between studies. Moreover, differences exist not only between species but also in the response of different blood vessels in a particular assay following smoke exposure. For example, smoke has been found to stimulate, inhibit, or have no effect on the vasodilatory properties of blood vessels using the aortic ring assay. However, a preponderance of studies indicates that endothelial function is altered in some manner following smoke exposure (e.g., expression of cell surface markers or alteration of growth). These observations are in accord with the current hypothesis concerning the importance of these cells in maintaining normal homeostasis and with the hypothesis that alterations in endothelial function are involved in the initiation of CVD.

Reports of increased atherosclerosis and thrombosis in inhalation models with smoke provide a further link to the progression of CVD and a basis for future experiments directed at dissecting the mechanisms by which the cigarette smoke initiates and promotes CVD. The detection of alterations in myocardial mitochondrial protein, enzyme, and DNA provide an initial mechanistic understanding concerning the elevation in nonatherosclerotic myocardial infarction in smokers. Because epidemiological data indicate that myocardial infarction and stroke are the two major CVDs elevated in smokers, additional animal model systems that progress to these

end points need to be developed and experimentally employed to fully define the effects of cigarette smoke on CVD.

33.3 CHRONIC OBSTRUCTIVE PULMONARY DISEASE: PAST APPROACHES AND NEW IDEAS

33.3.1 Epidemiology

Chronic obstructive pulmonary disease (COPD) is considered a major health concern (Halbert et al., 2003), with an overall prevalence in adults estimated at between 4 and 10% in countries where it has been rigorously measured (Halbert et al., 2003). A recent estimate for the incidence of COPD in the USA was given as 16 million people (Mahadeva and Shapiro, 2002). The major risk factors for COPD are considered to be cigarette smoking, use of biomass fuels, and air pollution (Halbert et al., 2003). Epidemiological studies however have shown that it is mainly susceptible smokers that develop COPD (Siafakas and Tzortzaki, 2002).

33.3.2 Basic Pathways Mediating MS-Induced COPD

COPD is a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and is associated with an abnormal inflammatory response of the lungs to noxious particles and gases (Pauwels et al., 2001). The "abnormal" or chronic inflammation leads to a narrowing of the small airways (bronchiolitis) and to alveolar wall destruction (Snider, 2003; Hogg and Senior, 2002). The chronic inflammation is characterized by increased numbers of alveolar macrophages, neutrophils, and cytotoxic T lymphocytes, and the release of multiple inflammatory mediators (lipids, chemokines, cytokines, growth factors) (Barnes, 2004, 2003; Rennard, 1998; Barnes and Cosio, 2004). The abnormal inflammatory response may be the key to susceptibility (Agusti et al., 2003). Although many types of inflammatory cells and mediators have been identified in COPD patients, their role in the progression of the disease remains largely unknown (Barnes, 2004).

The chronic obstructive bronchitis with mucus hypersecretion may contribute to but is not necessarily associated with airflow limitation (Barnes, 2003; Cosio-Piqueras and Cosio, 2001). Emphysema is defined as a condition of the lung characterized by abnormal permanent enlargement of airspaces distal to the terminal bronchiole, accompanied by destruction of the lung parenchyma with or without obvious fibrosis and loss of lung elasticity (Snider, 1992a, 1992b; Cosio-Piqueras and Cosio, 2001; Snider, 2003). Subjects with COPD do not often show emphysema without bronchitis and small-airway disease (March et al., 2000).

Cigarette smoke exposure has been shown to cause severe oxidative stress in the lung (MacNee and Rahman, 2001; Aoshiba et al., 2003a). The oxidants present in cigarette smoke, together with abundant infiltration and activation status of inflammatory cells in the smoker's lung, releasing even more oxygen-based free radicals, may be involved in a proteolytic/antiproteolytic imbalance, leading to tissue destruction (Seagrave, 2000; Churg et al., 2003b). The incidence of such an imbalance in human populations was the subject of a recent review (deSerres, 2003).

Oxidative stress has been shown to directly inactivate antiproteinases such as α1-antitrypsin (α1-AT) and secretory leukoprotease inhibitor (SLPI) (Betsuyaku et al., 2002; Hill et al., 2000; Cavarra et al., 2001b), as well as activating MMPs (Selman et al., 2003; Belvisi and Bottomley, 2003). Moreover, oxidative stress induces the transcription of many proinflammatory genes controlled by the transcription factor NFκB (Di Stefano et al., 2002). The histone acetylation status of the redox-sensitive transcription factors (NFκB and AP-1) has been shown to be (at least in part) regulating proinflammatory gene expression (Adcock et al., 2005). Oxidative stress is also thought to be involved in the accumulation of macrophages in the alveolar interstitial spaces, independent of other proinflammatory stimuli (Kirkham et al., 2003). This latter group have hypothesized that the oxidative stress promotes the macrophage accumulation through the production of reactive

carbonyls (particularly acrolein) (Kirkham et al., 2003). Susceptibility of the lung to oxidative injury has been shown to be largely dependent on the upregulation of antioxidant mechanisms in the lung (Rangasamy et al., 2004).

33.3.3 Animal Models

A number of animal models have been reported that exhibit at least one of the features of the complicated pathology of COPD, such as chronic bronchitis (Nikula and Green, 2000) and emphysema (March et al., 2000; Wright and Churg, 2002; Mahadeva and Shapiro, 2002). In these models airspace enlargement has been demonstrated after chronic exposure to MS, and also in shorter exposures to high concentrations of smoke.

Ideally, such models need to represent the various patterns of alveolar wall destruction that have been reported in humans, as well as host factors that parallel the etiology of the pathological condition. Animal models with genetic predisposition (e.g., an inherent α1-AT deficiency or increased sensitivity to oxidative stress) to develop emphysema are probably the most relevant in mimicking the susceptible human population (Kodavanti et al., 1998; Kodavanti and Costa, 2001; deSerres, 2003). The application of genetic engineering strategies in mice offers a great potential to dissect the pathogenetic pathways of emphysema (Kodavanti and Costa, 2001; Mahadeva and Shapiro, 2002). A few examples of susceptible and genetically engineered models are described below.

Promising susceptible animal models have been described that develop emphysema following whole-body exposure to MS (Cavarra et al., 2001a; Takubo et al., 2002; Valenca et al., 2004). C57Bl/6J mice, which have a mild deficiency in their antielastase screen, and DBA/2 mice, which are sensitive to oxidants, developed emphysema following 60 days to 6 months of exposure to cigarette smoke, whereas the mouse strain with normal antielastase screen and nonsensitivity to oxidants (ICR-mouse) did not (Cavarra et al., 2001a).

The pallid mouse (C57Bl/6J, $pa^{+/+}$), with a severe α1-AT deficiency (Martorana et al., 1993; DeSanti et al., 1995), developed panlobular emphysema after only 4 months of whole-body exposure to cigarette smoke (Cavarra et al., 2001a; Takubo et al., 2002). The pallid mice exhibited features similar to the human situation, including a T-lymphocytic inflammatory response and increased lung compliance (after 6 months of exposure).

The AKR mouse has been shown to develop emphysema after 6 months of exposure to MS (Guerassimov et al., 2004). This mouse model has many interesting features resembling human emphysema, including increased numbers of macrophages, neutrophils, interstitial T cells, and up-regulation of Th1 cytokine, and increased elastance (Htis).

The development of spontaneous emphysema has been studied in various transgenic mouse models (Mahadeva and Shapiro, 2002). Most of these models have contributed to the knowledge of certain aspects of the development of emphysema, but unfortunately most of them have not been challenged by exogenous noxious agents.

A transgenic mouse model was established that expresses low levels of human α1-AT (Churg et al., 2003b), as part of an effort to produce a treatment for cigarette smoke-induced emphysema. The transgenic mice were tolerant to exogenously applied human α1-AT. Mice were exposed to MS for up to 6 months; some of them received human α1-AT repeatedly. The latter treatment abolished smoke-induced elevations of neutrophil counts in lavage, as well as the elastin and collagen breakdown products desmosine and hydroxyproline, respectively. Treatment also provided some protection against airspace size. It was concluded that α1-AT therapy reduced the inflammation and partially protects the animals against emphysema. Recently, a promising transgenic mouse model has been developed in which the major murine isoform of α1-AT (the PI2-isoform, similar to the human α1-AT) has been disrupted (Kushi et al., 2004).

A murine model deficient for macrophage elastase ($MME^{-/-}$) has been shown to be protected against development of MS-induced emphysema (Hautamaki et al., 1997). The authors conclude that macrophage elastase is probably sufficient for the development of emphysema that results from chronic inhalation of MS. The role of the macrophage elastase in the smoke-induced inflammation

and tissue destruction has been corroborated by elegant studies carried out by Ofulue and coworkers (1998), and the involvement of MMP-12 in the pathogenesis of MS-induced emphysema has been demonstrated in a rat model (Xu et al., 2004). It has become clear, however, that the idea that a single type of protease or inflammatory cell is responsible for the development of emphysema in humans is unlikely to be true; a recent review (Churg and Wright, 2005) concludes that different cell types and proteases seem to be involved.

Rangasamy et al. (2004) demonstrated that genetic ablation of a nuclear factor, erythroid-derived-2 (Nrf2), involved in the regulation of many detoxification and antioxidant genes, renders mice more susceptible to pulmonary oxidative injury. These mice developed emphysema more rapidly than was found in their nontransgenic littermates (Rangasamy et al., 2004).

33.3.4 Inflammation and Apoptosis

Recent work has suggested that a further consideration should be taken when examining the role of inflammation and excessive proteolysis in the pulmonary tissue destruction (Aoshiba et al., 2003b). This work provided evidence that alveolar epithelial apoptosis causes emphysema in C57Bl/6J mice. The authors used a novel protein transfection agent ("Chariot") to introduce active caspase-3 into bronchial epithelial cells *in vivo.* These findings indicate that inflammation, proteolysis, oxidative stress, apoptosis, or cell hemostasis in general, are interrelated mechanisms contributing to cigarette smoke-induced emphysema (Tuder et al., 2003a, 2003b).

In many of the studies described relatively minor attention was made to the "aberrant" inflammatory process mentioned earlier. Future models should provide a tool to understand the exact role of inflammation on the etiology and progression of the disease.

33.3.5 Dosimetry

In the majority of the animal models described in the literature, few details are given on the actual concentrations of smoke that were used. Typically, animals are exposed for very short periods (minutes) often to undiluted smoke from nonfiltered cigarettes (to obtain high particulate matter concentrations). Usually, no measurement of the composition of the smoke presented, and in some cases it is not clear whether MS, ETSS, or a mixture was in fact used. In this case, investigations are needed to elucidate whether the mechanisms that lead to the development of emphysema under these conditions are representative for the development of emphysema as it occurs in the human situation (long-term exposure to low concentrations of smoke).

33.4 LUNG CANCER: PAST APPROACHES AND NEW IDEAS

33.4.1 Epidemiology

Numerous epidemiological studies have indicated that smokers have a substantially increased risk of LC (Wynder and Hoffmann, 1994). Recent etiological studies have focused on a classification into four major histologies: adenocarcinoma, squamous cell carcinomas, small cell carcinomas, and large cell carcinomas (Kuper et al., 2002). The adenocarcinoma appears to be showing a major disproportional increase in most parts of the world (Khuder, 2001; Devesa et al., 1999), and for some (unknown) reason this may now be the most predominant histology, replacing squamous cell carcinomas. Adenocarcinoma is much more common in women, young subjects, nonsmokers, and subjects from the Far East (Sobue et al., 2002; Toyooka et al., 2003a).

There are several hypotheses regarding this observation (Charloux et al., 1997). One is that lung cancer in former smokers is becoming an increasing percentage of the total number of lung cancer cases (Burns, 2000), and there are suggestions that adenocarcinoma is more likely to occur in former smokers than in current smokers. A second hypothesis suggests that the shift from nonfilter to filter cigarettes may play a role; it has been suggested that increasing amounts of tobacco-specific

nitrosamines could be responsible (Charloux et al., 1997). Diagnostic consistency may also be relevant (Campobasso et al., 1993).

33.4.2 Basic Pathways Mediating MS-Induced LC

The term "lung cancer" subsumes several heterogeneous, complex proliferative diseases of the lung. The common features of most types of lung cancer include malignant tumors originating from different epithelial entities of the airways and the peripheral lung. Epidemiological data support the view that lung tumors, like tumors in other organs and tissues, are the result of a multistep process leading through several stages from a normal cell to a population of transformed cells that have the capacity to proliferate indefinitely and to escape control mechanisms such as apoptosis (Figure 33.1) (Doll and Peto, 1976; Moolgavkar and Knudson, 1981).

In animal models, especially rodents, the multistage carcinogenesis concept has indeed been demonstrated (Maronpot, 1991). Discrete operational steps have been defined, e.g., in the mouse epidermal carcinogenesis (skin painting: see below) and in the rat liver carcinogenesis model. "Initiation" can be achieved by treatment with a genotoxic chemical or agent and is an irreversible, probably mutational event even though most initiated cells are likely to undergo apoptosis before they can develop into a tumor. According to their activity in initiation/promotion bioassays, carcinogens can be classified as initiators (alkylating agents, many polyaromatic hydrocarbons, ionizing radiation) or promoters (phorbol esters, peroxides, okadaic acid). Most initiators can also act as "complete carcinogens" following a single, high dose without the need for any promotion.

Tobacco smoke elicits both initiating and promoting activity in the respective models. The prominent role of promotion in smoking-related lung cancer is supported by the increasing decline in the lung cancer mortality ratio of individuals who stopped smoking with the time after quitting, reaching almost complete reversibility after 20 years of cessation (Halpern et al., 1993; Enstrom and Heath, 1999; Burns, 2000).

A multitude of *in vivo* and *in vitro* investigations have shown that an initiated cell will behave as a "silent" cell like its normal counterparts, unless it is subjected to promoter effects. "Promotion" can be achieved by repeated treatment with an often nongenotoxic agent and is reversible over an extended period. Initiated cells can be clonally expanded by selective growth advantages under the influence of the tumor promoter due to their higher response to its proliferative signals (or their higher resistance to toxic effects of the promoter) as compared with the normal cells in the surrounding tissue. During a prolonged promotion phase with enhanced cell proliferation and turnover, additional irreversible step(s) and functional adjustments may accumulate, e.g., functional losses of

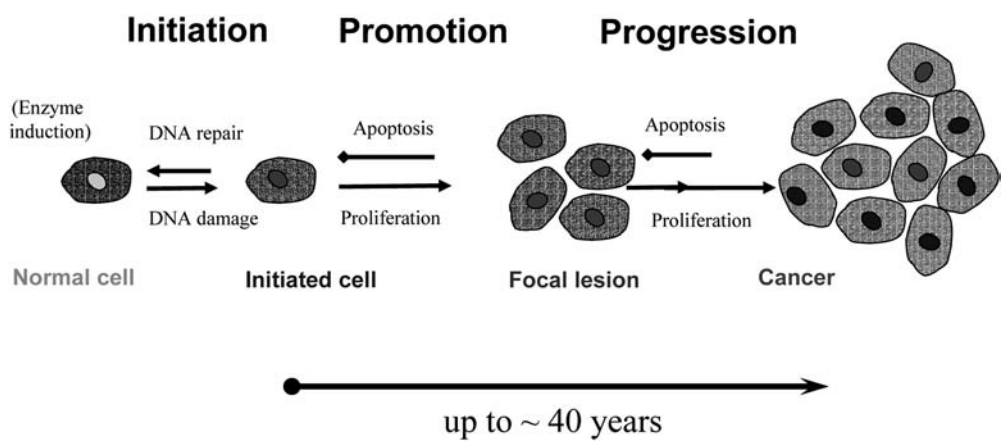


FIGURE 33.1 Carcinogenesis: multistage model.

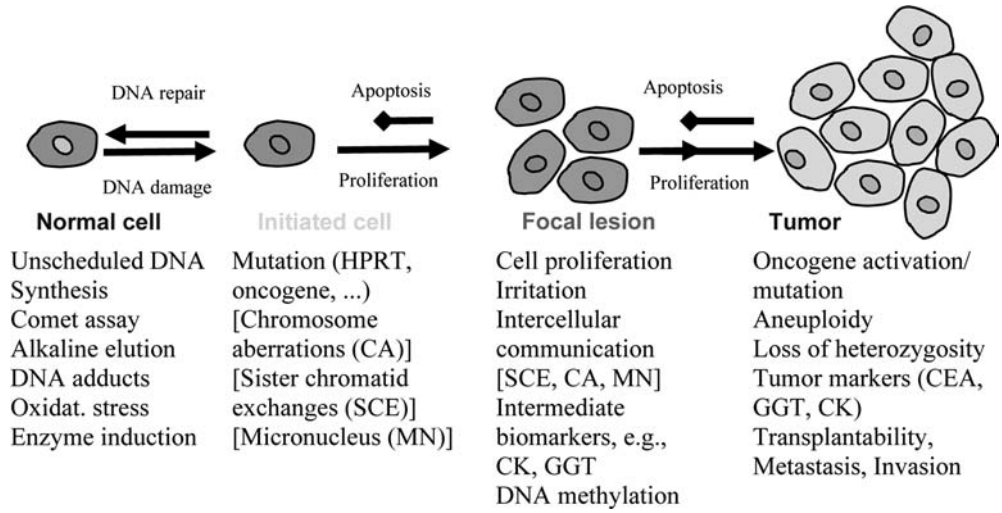


FIGURE 33.2 Biomarkers commonly used as assay end points in carcinogen testing.

the tumor suppressor gene p53 that increase genetic instability (Robles et al., 2002), and thus result in a transformed cell. Once having reached this "progression" stage, the neoplastic cell population continues to proliferate in the absence of promotional stimuli, becomes stepwise autonomous, and induces its own growth signals and vascularization to overcome nutritional restraints. In the final, malignant stage, tumor cells actively invade the surrounding tissue and metastasize into distant organs via the circulation.

On the histological level, a progressive sequence from hyperplasia → metaplasia → dysplasia → carcinoma *in situ* → malignant squamous-cell carcinoma has been proposed for human bronchial carcinoma (Trump et al., 1978; Auerbach et al., 1979). Whereas hyperplasia and metaplasia are in general reversible, the transition to an irreversible, neoplastic lesion may occur in the dysplastic stage. It has been shown that several cytogenetic and molecular markers increase in frequency within this sequence of lesions. These markers can frequently be linked to epigenetic mechanisms of action. Aneuploidy, mutations of p53, and aberrant methylation are associated with the progression from dysplasia to frank carcinoma (Hirsch et al., 2001). For small-cell carcinoma (SCLC), accounting for roughly 15–20% of lung cancer in smokers, the histological etiology from neuroendocrine cells appears to be different compared with bronchial carcinoma. Other molecular markers indicate that different pathways may also be involved, however, p53 mutations are found in over 90% of SCLC (Wistuba et al., 2001).

The histological changes and biomarkers that are specific for the transition to neoplastic transformation occur late in the multistage process. Estimated intervals from the first, initiating damage until clinical diagnosis may last up to several decades (Masui et al., 1986). The apparent lack of "early" biomarkers clearly related to LC makes risk evaluation even more difficult than in the case of CVD, where specific biomarkers of effect often occur early in the pathogenic process (see above). Therefore, animal disease models that mimic the typical and necessary conditions and processes of human lung cancer development in a compressed time frame are needed (Balmain and Harris, 2000) (Figure 33.2).

33.4.3 Animal Models

Although rodent lung cancer inhalation models have successfully been applied for investigations on, e.g., solid particles, nitrosamines, polyaromatic hydrocarbons, and many other respirable materials

Origin	Type	Human	Rat	Mouse
Bronchial	Adenocarcinoma	++	(+)	-
	Large-cell carcinoma	++	(+)	-
	Squamous-cell carcinoma	++	?	-
	Small-cell carcinoma	++	-	-
	Adenoma	?	(+)	-
Bronchiolar-alveolar	Adenocarcinoma/ bronchoalveolar carcinoma	+	+	+
	Adenoma	-	++	++
	Benign keratinizing cyst	-	++	-
	Squamous-cell carcinoma	?	+	-
	Small-cell carcinoma	+	-	-

FIGURE 33.3 Lack of homologous lung tumors in experimental models.

labeled "carcinogens," their tumor spectra differ from the human situation: most rodent lung tumors originate in the peripheral lung (bronchiolo-alveolar adenoma and carcinoma), and it has been difficult to induce bronchogenic carcinomas (except following intratracheal instillation or implantation) in rats and mice (Figure 33.3).

There have been numerous attempts to produce lung tumors by exposing various species of animals to cigarette smoke. Reviews of these attempts are available (Wehner, 1983; Coggins, 1998, 2001, 2002). One review concluded "significant increases in the numbers of malignant tumors of the respiratory tract were not seen in rats, mice, hamsters, dogs, or nonhuman primates exposed for long periods of time to very high concentrations of mainstream cigarette smoke. The results are clearly at variance with the epidemiological evidence in smokers, and it is difficult to reconcile this major difference between observational studies in humans and controlled laboratory studies" (Coggins, 2002).

Another unpublished study (Lovelace Respiratory Research Institute, 1995) used F-344 rats exposed nose-only to MS for 30 months, following an initial treatment with $^{239}PuO_2$. In this study there were severe body-weight effects, and only a marginally significant effect on lung tumorigenesis by MS. There was a so-called "synergistic" or "supra-additive" effect, largely due to MS-induced impairment of ^{239}Pu clearance. Recently, part of the results have been published and demonstrate an incidence of 14% lung tumors in the smoke-exposed females, a statistically significant increase above sham controls (0%) (Mauderly et al., 2004). However, no statistically significant lung tumor induction was observed in the smoke-exposed male rats. The authors discuss that the use of step-serial sections instead of one section per lung could possibly increase the sensitivity of the bioassay (Mauderly et al., 2004).

33.4.4 The A/J Mouse

Recent work (Witschi et al., 1997b) with the A/J strain of mice exposed to ETSS have demonstrated intriguing results: no difference in lung tumor incidences between sham exposed and ETSS-exposed

mice was observed at the end of a five-month exposure period, but a substantially higher incidence was seen in the ETSS-exposed mice at the end of an additional four months without any further treatment. The findings have now been confirmed several times (Witschi et al., 2002, 2004; Obermueller-Jevic et al., 2002) and have been replicated (D'Agostini et al., 2001; Stinn et al., 2005). In some of these studies adenocarcinomas were noted, in addition to the adenomas discussed in Figure 33.3. In one study, mice were exposed only to the vapor phase of the ETSS (using a filter to trap the particulates), and results were very similar to those with unfiltered ETSS (Witschi et al., 1997a). Using mainstream smoke concentrations that were considered "maximally tolerable," in a six-month study with only a five week posttreatment interval, the A/J mouse strain "did not produce or promote lung cancer" (Finch et al., 1996). These two experimental designs were so different that no meaningful comparisons can be made.

The cumulative work with A/J mice was recently reviewed (Witschi et al., 2002), and comparisons were made with two other mouse strains (Balb/c and SWR). In these two strains the response to ETSS exposure was lower than that seen in the A/J strain, as assessed by both tumors per lung and lung tumor incidence. The SWR strain has also been used by other workers, who in a major review confirmed that they are less sensitive than the A/J mice for tumorigenicity studies (De Flora et al., 2003b). In the A/J study (Witschi et al., 2002) no differences were noted in the mutations in the k-*ras* proto-oncogene, despite earlier reports of such a change but with much smaller concentrations of ETSS (Witschi et al., 1995). Mutation in the k-*ras* gene shows a strong association with human pulmonary adenocarcinoma (Ahrendt et al., 2001).

Recent work from our laboratory (Knoerr et al., 2003; Stinn et al., 2002, 2005) has also confirmed the basic model, although no adenocarcinoma was produced in the ETSS-exposed A/J mice (adenomas only). Mutations in the k-*ras* proto-oncogene were identical in sham- and ETSS-exposed mice, despite earlier reports of such a change but with much smaller concentrations of ETSS, as well as with the tobacco-specific nitrosamine NNK (Kawano et al., 1996).

33.4.5 Transgenic Strains

It is well known that lung cancer cells have many genetic and epigenetic changes when compared with healthy cells, and reviews of some of the more frequent molecular genetic changes are available (Mitsuuchi and Testa, 2002; Sekido et al., 2003). One of the most studied genetic changes is the inactivation of the p53 tumor suppression gene (Robles et al., 2002), and transgenic mice with deleted "knockout" and activated "knockin" p53 are available (Lubet et al., 2000; Storer et al., 2003; Wang et al., 2003b). A recent review of aspects of this work is available (Toyooka et al., 2003b). Recent work has combined the "susceptible" A/J strain with p53 transgenics, in whole-body exposures to ETSS for up to 9 months (De Flora et al., 2003a). A weak but significant increase in lung tumor incidence and multiplicity was reported in the p53 mutant mice, with no tumorigenic effect in the wild-type controls (De Flora et al., 2003a).

Genetically engineered mice are being used increasingly in carcinogenicity studies for substances other than MS, in that they can be used in smaller numbers and in shorter studies (Zhao et al., 2000; Storer et al., 2003). The transgenic *ras*H2 mouse carrying the c-Ha-*ras* gene "was created to improve conventional rodent carcinogenicity testing," and results with a variety of materials have been recently reviewed (Tomisawa et al., 2003).

The *ras*H2 mouse is responsive to both nongenotoxic and genotoxic chemicals (Morton et al., 2002), and is considered to be superior to conventional mouse bioassays for cancer hazard identification (Takaoka et al., 2003). Other alterations are being evaluated, including those genetic alterations commonly found in human lung adenocarcinomas (Wang et al., 2003b). The authors of this latter work suggest that, through alterations in p53 and Ink4a/Arf, the mutant A/J mice provide the best preclinical estimate of the effectiveness of chemopreventative agents (Wang et al., 2003b). There is one report of the use of A/J and *ras*H2 mice with MS, demonstrating small but significant increases in lung tumor incidence and multiplicity (Curtin et al., 2004). Another transgenic mouse approach uses the targeted overexpression of *c-myc* or *c-raf* in alveolar type II cells only under

the control of the surfactant C (SPC) promoter, resulting in invasive and metastasizing peripheral lung tumors that more closely resemble the human tumor pathology (Kerkhoff et al., 2000; Ehrhardt et al., 2001). Initial exposure studies have demonstrated a positive effect of NNK exposure on lung tumor progression in *c-myc*-SPC mice (Ehrhardt et al., 2003); however, it remains to be investigated how such a targeted transgenic model responds to MS.

33.4.6 DNA Repair

Identifying the role of MS in alterations in DNA-repair enzymes would be helpful in identifying the mechanism of the etiology of cancer (Hu et al., 2002) and could identify individuals at risk (Wei and Spitz, 1997; Spitz et al., 2003; Shen et al., 2003). The use of genetically modified mice has now been extended into the study of DNA repair, in that transgenic and knockout mice are available for studying DNA repair functions in both carcinogenesis and mutagenesis (Wijnhoven and vanSteeg, 2003). The *Xpa/p53* mutant mouse model is currently seen as being particularly promising (Wijnhoven and vanSteeg, 2003).

33.4.7 Tumor Angiogenesis

Most of the animal models for smoke-induced pulmonary carcinogenesis have focused on the initial, genotoxic, or epigenetic effects leading to the formation of an aggregate of autonomously proliferating, transformed cells. During the ongoing progression of growth, the tumor mass will organize its own vascularization. Angiogenesis is an important process that allows lung tumors to grow (Yano et al., 2003), with unknown links to components of MS.

Recent work with Lewis lung cancer cells injected subcutaneously into C57BL/6J mice exposed to ETSS (concentrations not given) indicated increased angiogenesis and tumor cell growth (Zhu et al., 2003). The authors hypothesized that nicotine may be involved in the angiogenesis, following other studies in wound healing (Heeschen et al., 2003). An alternative hypothesis is that MS interferes with hypoxia-driven angiogenesis, at least in normal tissue (Michaud et al., 2003).

33.4.8 Surrogate End Points and Models

In the absence of a reliable and reproducible experimental model for smoke-induced lung cancer, histopathological changes in the respiratory tract of rodents have traditionally been used as surrogates to evaluate the potential carcinogenic risk of cigarette smoke (Coggins et al., 1982b; Gaworski et al., 1997; Vanscheeuwijck et al., 2002; Terpstra et al., 2003). However, no direct link to smoke-induced neoplastic lesions can be made, because all the lesions observed are either adaptive and reversible (hyperplasia, metaplasia), regressive (atrophy), or inflammatory/ulcerative, but never progressive (dysplasia, carcinoma *in situ*, invasive carcinoma). Furthermore, the rodent models are generally more sensitive in the upper respiratory tract, whereas only weak histological changes can be observed in the lung. The fact that rodents as obligate nose breathers may retain certain smoke constituents in their nasal passages could result in a relatively low availability of such substances in the lung.

33.4.9 Mouse Skin Painting

In the context of this review, special attention will be given to the surrogate (dermal) model of MS lung carcinogenesis in mice.

The concept of initiation and promotion as essential steps in experimental carcinogenesis, reflecting the multistage aspect of human cancer etiology, has largely been built upon this reproducible tumor model, and also on the rat liver carcinogenesis model. Historically, the lack of an inhalation model for tobacco carcinogenesis has led researchers to use a technique termed "mouse skin painting." In this technique, tobacco smoke condensate (obtained by trapping the particulates of MS, usually in a cold trap) is applied to the backs of shaved mice, resulting in various types of

skin tumors (Flory, 1941; Wynder et al., 1953). The technique has been used to differentiate between different types of cigarettes (Coggins et al., 1982a; Brown et al., 1998; Gaworski et al., 1999). The technique was shortened from the original 2-year assay (Coggins et al., 1982a) to around 6 months (Gaworski et al., 1999), by the use of an inbred strain of mice ("SENCAR"), and work in this area continues (Stern et al., 1998; Watson et al., 2003). Finally, transgenic mice are also being used in the mouse skin painting assay (Stoll et al., 2001; Ohara et al., 2003), but no reports as yet with tobacco smoke condensate. Although a surrogate, this model is sensitive to an epigenetic, tumor-promoting activity of the particulate fraction of MS, an activity that might be missed in the available inhalation models.

33.4.10 Conclusion and Outlook

Despite the large relative risk for lung cancer in smokers and the variety of recent approaches, no reproducible model for lung tumorigenesis by inhalation of MS has been reported. The reasons for this apparent discrepancy are not known, but may be either host-specific or related to a lack of understanding of the pathogenesis of the disease and, hence, the lack of specific "biomarkers of effect" that would be required for model extrapolations. In particular, the role of epigenetic mechanisms might not have been considered appropriately in choosing the experimental models.

Therefore, the currently available models can only support a weight-of-evidence approach relying on surrogate markers and models when being applied to evaluate the lung cancer risk of cigarettes or PREPs (U.S. Institute of Medicine, 2001). The ongoing development of transgenic models and novel molecular approaches (genomics, proteomics, etc.) may hopefully give new insights into the complex pathogenesis of lung cancer and thus help to find improved models for risk evaluation.

33.5 GENERAL CONCLUSION

There are today no "adequate" animal models for the human cigarette-smoke-induced diseases reviewed here. Probably there will never be a model covering all aspects of a specific disease. However, progress has been made in developing models for certain mechanistic aspects of each disease, maybe with the exception of LC. Even with more models being eventually available, an overall weight-of-the-evidence evaluation will be necessary for a sound scientific assessment of risk.

Novel tools such as transgenic and/or "compromised" animal models (some of which are reviewed here), along with evolving *in vitro* technologies, may in the near future be combined with traditional techniques. Such models will be useful for the assessment of PREPs, but also for the investigation of diagnostic and therapeutic means for the respective diseases.

The recent work reviewed here clearly shows that immense progress has been made in the establishment of animal models to identify mechanisms. It is highly probable with evolving toxicological techniques such as transgenic and/or "compromised" animals that this rate of progress will continue to accelerate, even for LC.

REFERENCES

Adcock, I.M., Cosio, B., Tsaoprouni, L., Barnes, P.J., and Ito, K., *Antioxidants Redox Signaling*, 7, 144–152, 2005.

Agusti, A., MacNee, W., Donaldson, K., and Cosio, M.G., *Thorax,* 58, 832–834, 2003.

Ahrendt, S.A., Decker, P.A., Alawi, E.A., Zhu, Y.R., SanchezCespedes, M., Yang, S.C., Haasler, G.B., KajdacsyBalla, A., Demeure, M.J., and Sidransky, D., *Cancer*, 92, 1525–1530, 2001.

Anazawa, T., Dimayuga, P., Li, H., Tani, S., Bradfield, J., Chyu, K.-Y., Kaul, S., Shah, P.K., and Cercek, B., *Arterioscler. Thromb. Vasc. Biol.*, 24, 1652–1658, 2004.

Aoshiba, K., Koinuma, M., Yokohori, N., and Nagai, A., *Inhalation Toxicol.*, 15, 1029–1038, 2003a.

Aoshiba, K., Yokohori, N., and Nagai, A., *Am. J. Respir. Cell Mol. Biol.*, 28, 555–562, 2003b.

Auerbach, O., Hammond, E.C., and Garfinkel, L., *N. Engl. J. Med.*, 300, 381–386, 1979.
Ayres, P.H., Mosberg, A.T., Burger, G.T., Hayes, A.W., Sagartz, J.W., and Coggins, C.R.E., *Inhalation Toxicol.*, 1, 349–363, 1989.
Balmain, A. and Harris, C.C., *Carcinogenesis*, 21, 371–377, 2000.
Barnes, P.J., *Annu. Rev. Med. Select. Top. Clin. Sci.*, 54, 113–129, 2003.
Barnes, P.J., *Pharmacol. Res.*, 56, 515–548, 2004.
Barnes, P.J., and Cosio, M.G., *PLoS Med.*, 1, 25–27, 2004.
Bazzano, L.A., He, J., Muntner, P., Vupputuri, S., and Whelton, P.K., *Ann. Intern. Med.*, 138, 891–898, 2003.
Belvisi, M.G. and Bottomley, K.M., *Inflamm. Res.*, 52, 95–100, 2003.
Betsuyaku, T., Takeyabu, K., Tanino, M.. and Nishimura, M., *Eur. Respir. J.*, 19, 1051–1057, 2002.
Breslow, J.L., *Science*, 272, 685–688, 1996.
Brown, B., Kolesar, J., Lindberg, K., Meckley, D., Mosberg, A., and Doolittle, D., *Mutat. Res. Genet. Toxicol. Environ. Mutagen.*, 414, 21–30, 1998.
Burke, A. and FitzGerald, G.A., *Prog. Cardiovasc. Dis.*, 46, 79–90, 2003.
Burns, D.M., *Cancer*, 89, 2506–2509, 2000.
Burns, D.M., *Prog. Cardiovasc. Dis.*, 46, 11–30, 2003.
Campobasso, O., Andrion, A., Ribotta, M., and Ronco, G., *Int. J. Cancer*, 53, 205–208, 1993.
Cavarra, E., Bartelesi, B., Lucatelli, M., Fineschi, S., Lunghi, B., Gambelli, F., Ortiz, L.A., Martorana, P.A., and Lungarella, G., *Am. J. Respir. Crit. Care Med.*, 164, 886–890, 2001a.
Cavarra, E., Lucatelli, M., Gambelli, F., Bartelesi, B., Fineschi, S., Szarka, A., Giannerini, F., Martorana, P.A., and Lungarella, G., *Am. J. Physiol.*, 281, L412–L417, 2001b.
Charloux, A., Quoix, E., Wolkove, N., Small, D., Pauli, G., and Kreisman, H., *Int. J. Epidemiol.*, 26, 14–23, 1997.
Churg, A., Dai, J., Tai, H., Xie, C.S. and Wright, J.L., *Am. J. Respir. Crit. Care Med.*, 166, 849–854, 2002a.
Churg, A., Wang, R.D., Tai, H., Wang, X., Xie, C.S., Dai, J., Shapiro, S.D., and Wright, C.W., *Am. J. Respir. Crit. Care Med.*, 167, 1083–1089, 2003a.
Churg, A., Wang, R.D., Xie, C.S., and Wright, J.L., *Am. J. Respir. Crit. Care Med.*, 168, 199–207, 2003b.
Churg, A. and Wright, C.W., *Curr. Opin. Pulmon. Med.*, 11, 153–159, 2005.
Churg, A., Zay, K., Shay, S., Xie, C.S., Shapiro, S.D., Hendricks, R., and Wright, J.L., *Am. J. Respir. Cell. Mol. Biol.*, 27, 368–374, 2002b.
Coggins, C.R.E., *Toxicol. Pathol.*, 26, 307–314, 1998.
Coggins, C.R.E., *Toxicol. Pathol.*, 29, 550–557, 2001.
Coggins, C.R.E., *Inhalation Toxicol.*, 14, 991–1002, 2002.
Coggins, C.R.E., Haroz, R.K., Lam, R., and Morgan, K.T., *Toxicology*, 23, 177–185, 1982a.
Coggins, C.R.E., Lam, R., and Morgan, K.T., *Toxicology*, 22, 287–296, 1982b.
Cosio-Piqueras, M.G. and Cosio, M.G., *European Respiratory Journal*, 18, 41S-49S, 2001.
Curtin, G.M., Higuchi, M.A., Ayres, P.H., Swauger, J.E., and Mosberg, A.T., *Toxicol. Sci.*, 81, 26–34, 2004.
Czernin, J. and Waldherr, C., *Prog. Cardiovasc. Dis.*, 45, 395–404, 2003.
D' Agostini, F., Balansky, R.M., Bennicelli, C., Lubet, R.A., Kelloff, G.J., and deFlora, S., *Int. J. Oncol.*, 18, 607–615, 2001.
De Flora, S., Balansky, R.M., D' Agostini, F., Izzotti, A., Camoirano, A., Bennicelli, C., Zhang, Z.Q., Wang, Y., Lubet, R.A., and You, M., *Cancer Res.*, 63, 793–800, 2003a.
De Flora, S., D' Agostini, F., Balansky, R., Camoirano, A., Bennicelli, C., Bagnasco, M., Cartiglia, C., Tampa, E., Longobardi, M.G., Lubet, R.A., and Izzotti, A., *Mutat. Res. Fundam. Mol. Mech. Mutagen.*, 523, 237–252, 2003b.
Denson, KW.E., *Med. Sci. Res.*, 27, 75–82, 1999.
DeSanti, M.M., Martorana, P.A., and Cavarra, E., *Lab. Investig.*, 73, 40–47, 1995.
deSerres, F.T. *Environ. Health Perspect.*, 111, 1851–1854, 2003.
Devesa, S.S., Grauman, D.J., Blot, W.J., and Fraumeni, J.F., *J. Natl. Cancer Inst.*, 91, 1040–1050, 1999.
Di Stefano, A., Caramori, G., Oates, T., Capelli, A., Lusuardi, M., Gnemmi, I., Ioli, F., Chung, K.F., Donner, C.F., Barnes, P.J., and Adcock, I.M., *Eur. Respir. J.*, 20, 556–563, 2002.
Doll, R. and Peto, R., *Br. Med. J.*, 2, 1525–1536, 1976.
Ehrhardt, A., Bartels, T., Geick, A., Klocke, R., Paul, D., and Halter, R., *Br. J. Cancer*, 84, 813–818, 2001.
Ehrhardt, A., Bartels, T., Klocke, R., Paul, D., and Halter, R., *J. Cancer Res. Clin. Oncol.*, 129, 71–75, 2003.
Enstrom, J.E. and Heath, C.W., *Epidemiology*, 10, 500–512, 1999.
Finch, G.L., Nikula, K.J., Belinsky, S.A., Barr, E.B., Stoner, G.D., and Lechner, J.F., *Cancer Lett.*, 99, 161–167, 1996.

Flory, C.M., *Cancer Res.*, 1, 262–276, 1941.
Folts, J.D., Gering, S.A., Laibly, S.W., Bertha, B.G., Bonebrake, F.C., and Keller, J.W., *Adv. Exp. Med. Biol.*, 273, 339–358, 1990.
Gairola, C.G., Drawdy, M.L., Block, A.E., and Daugherty, A., *Atherosclerosis*, 156, 49–55, 2001.
Gaworski, C.L., Dozier, M.M., Gerhart, J.M., Rajendran, N., Brennecke, L.H., Aranyi, C., and Heck, J.D., *Food Chem. Toxicol.*, 35, 683–692, 1997.
Gaworski, C.L., Heck, J.D., Bennett, M.B., and Wenk, M.L., *Toxicology*, 139, 1–17, 1999.
Gering, S.A. and Folts, J.D., *J. Lab. Clin. Med.*, 116, 728–736, 1990.
Guerassimov, A., Hoshino, Y., Takubo, Y., Turcotte, A., Yamamoto, M., Ghezzo, H., Triantafillopoulos, A., Whittaker, K., Hoidal, J.R., and Cosio, M.G., *Am. J. Respir. Crit. Care Med.*, 170, 974–980, 2004.
Halbert, R.J., Isonaka, S., George, D., and Iqbal, A., *Chest*, 123, 1684–1692, 2003.
Halpern, M.T., Gillespie, B.W., and Warner, K.E., *J. Natl. Cancer Inst.*, 85, 422–423, 1993.
Hautamaki, R.D., Kobayashi, D.K., Senior, R.M., and Shapiro, S.D., *Science*, 277, 2002–2004, 1997.
Hazleton, Laboratories America Inc., Final Report, Inhalation Bioassay of Cigarette Smoke in Dogs, Effects of Nicotine and Carbon Monoxide on Atherogenesis, Project NCI-5HP-75-112, National Cancer Institute, Smoking and Health Program, Hazelton Laboratories America Inc., Vienna, VA, 1981, pp. 1–293.
Heeschen, C., Weis, M., and Cooke, J.P., *J. Am. Coll. Cardiol.*, 41, 489–496, 2003.
Hill, A.T., Bayley, D.L., Campbell, E.J., Hill, S.L., and Stockley, R.A., *Eur. Respir. J.*, 15, 886–890, 2000.
Hirsch, F.R., Franklin, W.A., Gazdar, A.F., and Bunn, P.A., *Clin. Cancer Res.*, 7, 5–22, 2001.
Hogg, J.C. and Senior, R.M., *Thorax*, 57, 830–834, 2002.
Hu, J.J., Mohrenweiser, H.W., Bell, D.A., Leadon, S.A., and Miller, M.S., *Toxicol. Appl. Pharmacol.*, 185, 64–73, 2002.
Hutchison, S., Sudhir, K., Sievers, R., Zhu, B.P., Sun, Y., Chou, T., Chatterjee, K., Deedwania, P., Cooke, J.P., Glantz, S.A., and Parmley, W.W., *Hypertension*, 34, 44–50, 1999.
Hutchison, S.J., Reitz, M.S., Sudhir, K., Sievers, R.E., Zhu, B.Q., Sun, Y.P., Chou, T.M., Deedwania, P.C., Chatterjee, K., Glantz, S.A., and Parmley, W.W., *Hypertension*, 29, 1186–1191, 1997a.
Hutchison, S.J., Sievers, R.E., Zhu, B.Q., Sun, Y.P., Stewart, D.J., Parmley, W.W., and Chatterjee, K., *Chest*, 120, 2004–2012, 2001.
Hutchison, S.J., Sudhir, K., Chou, T.M., Sievers, R.E., Zhu, B.Q., Sun, Y.P., Deedwania, P.C., Glantz, S.A., Parmley, W.W., and Chatterjee, K., *J. Am. Coll. Cardiol.*, 29, 800–807, 1997b.
Johnson, D., Freischlag, J.A., Coe, D.A., Mudaliar, J.H., Traul, D.K., Kelly, H., Hanson, L., Cambria, R.A., Seabrook, G.R., and Towne, J.B., *Ann. Vasc. Surg.*, 13, 480–483, 1999.
Kawano, R., Takeshima, Y., and Inai, K., *Jpn. J. Cancer Res.*, 87, 44–50, 1996.
Keller, J.W. and Folts, J.D., *Cardiovasc. Res.*, 22, 73–78, 1988.
Kerkhoff, E., Fedorov, L.M., Siefken, R., Walter, A.O., Papadopoulos, T., and Rapp, U.R., *Cell Growth Differ.*, 11, 185–190, 2000.
Khuder, S.A., *Lung Cancer*, 31, 139–148, 2001.
Kirkham, P.A., Spooner, G., FfoulkesJones, C., and Calvez, R., *Free Radic. Biol. Med.*, 35, 697–710, 2003.
Knight-Lozano, C.A., Young, C.G., Burrow, D.L., Hu, Z.Y., Uyeinami, D.L., Pinkerton, K.E., Ischiropoulos, H., and Ballinger, S.W., *Circulation*, 105, 849–854, 2002.
Knoerr, C., Stinn, W., Vanscheeuwijck, P., Kindt, R., and Haussmann, H.-J., in *The Toxicologist*, Vol. 72 (S-1), Society of Toxicology, Salt Lake City, UT, 2003, pp. 237.
Kodavanti, U.P. and Costa, D.L., *Respir. Physiol.*, 128, 57–70, 2001.
Kodavanti, U.P., Costa, D.L., and Bromberg, P.A., *Environ. Health Perspect.*, 106 (S1), 111–130, 1998.
Koul, A., Singh, A., and Sandhir, R., *Inhalation Toxicol.*, 15, 513–522, 2003.
Kuper, H., Boffetta, P., and Adami, H.O., *J. Intern. Med.*, 252, 206–224, 2002.
Kushi, A., Akiyama, K., Noguchi, M., Edamura, K., Yoshida, T., and Sasai, H., *Exp. Anim.*, 53, 437–443, 2004.
Law, M.M., Gelabert, H.A., Moore, W.S., Hajjar, G.E., Colburn, M.D., Petrik, P.V., and QuinonesBaldrich, W.J., *J. Vasc. Surg.*, 23, 401–409, 1996.
Law, M.R. and Wald, N.J., *Prog. Cardiovasc. Dis.*, 46, 31–38, 2003.
Lehr, H.A., *Microcirculation*, 7, 367–384, 2000.
Lehr, H.A., Kress, E., Menger, M.D., Friedl, H.P., Hubner, C., Arfors, K.E., and Messmer, K., *Free Radic. Biol. Med.*, 14, 573–581, 1993.
Lehr, H.A., Vollmar, B., Vajkoczy, P., and Menger, M.D., *Methods Enzymol.*, 300, 462–481, 1999.
Lehr, H.A., Weyrich, A.S., and Saetzler, R.K., *J. Clin. Investig.*, 99, 2358–2364, 1997.
Lough, J., *Arch. Pathol. Lab. Med.*, 102, 377–380, 1978.

Lovelace Respiratory Research Institute, Albuquerque, NM, 1995.
Lubet, R.A., Zhang, Z.Q., Wiseman, R.W., and You, M., *Exp. Lung Res.*, 26, 581–593, 2000.
Ma, L., Wang, W.P., Chow, J.Y.C., Yuen, S.T., and Cho, C.H., *Am. J. Physiol.*, 278, G10–G17, 2000.
MacNee, W. and Rahman, I., *Trends Mol. Med.*, 7, 55–62, 2001.
Magers, T., Talbot, P., DiCarlantonio, G., Knoll, M., Demers, D., Tsai, I., and Hoodbhoy, T., *Reprod. Toxicol.*, 9, 513–525, 1995.
Mahadeva, R. and Shapiro, S.D., *Thorax*, 57, 908–914, 2002.
March, T.H., Green, F.H.Y., Hahn, F.F., and Nikula, K.J., *Inhalation Toxicol.*, 12, 155–187, 2000.
Maronpot, R.R., in *Handbook of Toxicologic Pathology*, Haschek, W.M. and Rousseaux, C.G., (Eds., Academic Press, San Diego, CA, 1991, pp. 92–130.
Marshall, M., *Wien. Klin. Wochenschr.*, 98, 212–214, 1986.
Martorana, P.A., Brand, T., Gardi, C., vanEven, P., deSanti, M.M., Calzoni, P., Marcolongo, P., and Lungarella, G., *Lab. Investig.*, 68, 233–241, 1993.
Masui, T., Lechner, J.F., Yoakum, G.H., Willey, J.C., and Harris, C.C., *J. Cell. Physiol.*, 4, 73–81, 1986.
Matetzky, S., Tani, S., Kangavari, S., Dimayuga, P., Yano, J., Xu, H., Chyu, K.Y., Fishbein, M.C., Shah, P.K., and Cercek, B., *Circulation*, 102, 602–604, 2000.
Mauderly, J.L., Gigliotti, A.P., Barr, E.B., Bechtold, W.E., Belinsky, S.A., Hahn, F.F., Hobbs, C.A., March, T.H., Seilkop, S.K., and Finch, G.L., *Toxicol. Sci.*, 81, 280–292, 2004.
Mays, B.W., Freischlag, J.A., Eginton, M.T., Cambria, R.A., Seabrook, G/R., and Towne, J.B., *J. Surg. Res.*, 84, 35–39, 1999.
Melkonian, G., Le, C., Zheng, W., Talbot, P., and Martins-Green, M., *Toxicol. Appl. Pharmacol.*, 163, 26–37, 2000.
Michaud, S.E., Menard, C., Guy, L.G., Gennaro, G., and Rivard, A., *FASEB J.*, 17, U7–U23, 2003.
Mitsuuchi, Y. and Testa, J.R., *Am. J. Med. Genet.*, 115, 183–188, 2002.
Moolgavkar, S.H. and Knudson, A.G., *J. Natl. Cancer Inst.*, 66, 1037–1052, 1981.
Morton, D., Alden, C.L., Roth, A.J., and Usui, T., *Toxicol. Pathol.*, 30, 139–146, 2002.
Mudaliar, J.H., Freischlag, J.A., Johnson, D., Coe, D.A., Kelly, H., Hanson, L., Cambria, R.A., Seabrook, G.R., and Towne, J.B., *J. Vasc. Surg.*, 25, 884–889, 1997.
Nene, S., Gelabert, H., Moore, W., QuinonesBaldrich, W., SantibanezGallerani, A., and Ignarro, L., *J. Surg. Res.*, 71, 101–106, 1997.
Nikula, K.J. and Green, F.H.Y., *Inhalation Toxicol.*, 12, 123–153, 2000.
Obermueller-Jevic, U.C., Espiritu, I., Corbacho, A.M., Cross, C.E., and Witschi, H., *Toxicol. Sci.*, 69, 23–29, 2002.
Ofulue, A.F., Ko, M., and Abboud, R.T., *Am. J. Physiol. Lung Cell. Mol. Physiol.*, 19, L1134–L1144, 1998.
Ohara, M., Kawashima, Y., Kitajima, S., Mitsuoka, C., and Watanabe, H., *Cancer Sci.*, 94, 205–209, 2003.
Pauwels, R.A., Buist, A.S., and Calverley, P.M., *Am. J. Respir. Crit. Care Med.*, 163, 1256–1276, 2001.
Penn, A., Chen, L.-C., and Snyder, C.A., *Circulation*, 90, 1363–1367, 1994.
Penn, A., Keller, K., Snyder, C., Nadas, A., and Chen, L.C., *Environ. Health Perspect.*, 104, 1108–1113, 1996.
Penn, A. and Snyder, C.A., *Circulation*, 88, 1820–1825, 1993.
Petrik, P.V., Gelabert, H.A., Moore, W.S., Quinones-Baldrich, W., and Law, M.M., *Stroke*, 26, 1409–1414, 1995.
Puranik, R. and Celermajer, D.S., *Prog Cardiovasc. Dis.*, 45, 443–458, 2003.
Rangasamy, T., Cho, C,Y., Thimmulappa, R.K., Zhen, L., Srisuma, S.S., Kensler, T.W., Yamamoto, M., Petrache, I., Tuder, R.M., and Biswal, S., *J. Clin. Invest.*, 114, 1248–1259, 2004.
Raymond, T.L., Delucia, A.J., and Bryant, L.R., *Atherosclerosis*, 41, 27–33, 1982.
Rennard, S.I., *Chest*, 113, S235–S241, 1998.
Ribeiro-Jorge, P.A., QOzaki, M.R., and Almeida, E.A., *Clin. Exp. Pharmacol. Physiol.*, 22, 410–413, 1995.
Robles, A.I., Linke, S.P., and Harris, C.C., *Oncogene*, 21, 6898–6907, 2002.
Rogers, W.R., Carey, K.D., McMahan, C.A., Montiel, M.M., Mott, G.E., Wigodsky, H.S., and McGill, H.C., *Exp. Mol. Pathol.*, 48, 135–151, 1988.
Sarkar, R., Gelabert, H.A., Mohiuddin, K.R., Thakor, D.K., and Santibanez-Gallerani, A.S., *J. Surg. Res.*, 82, 43–47, 1999.
Seagrave, J., *J. Toxicol. Environ. Health Pt A*, 61, 69–78, 2000.
Sekhon, H.S., Wright, J.L., and Churg, A., *Am. J. Physiol.*, 267, L557–L563, 1994.
Sekido, Y., Fong, K.M., and Minna, J.D., *Ann. Rev. Med.*, 54, 73–87, 2003.

Selman, M., Cisneros-Lira, J., Gaxiola, M., Ramirez, R., Kudlacz, E.M., Mitchell, P.G., and Pardo, A., *Chest*, 123, 1633–1641, 2003.

Shen, H.B., Spitz, M.R., Qiao, Y.W., Guo, Z.Z., Wang, L.E., Bosken, C.H., Amos, C.I., and Wei, Q.Y., *Int. J. Cancer*, 107, 84–88, 2003.

Siafakas, N.M. and Tzortzaki, E.G., *Respir. Med.*, 96, 615–624, 2002.

Snider, G.L., *Am Rev. Respir. Dis.*, 146, 1334–1344, 1992a.

Snider, G.L., *Am Rev. Respir. Dis.*, 146, 1615–1622, 1992b.

Snider, G.L., *Am. J. Respir. Crit. Care Med.*, 167, 678–683, 2003.

Sobue, T., Yamamoto, S., Hara, M., Sasazuki, S., Sasaki, S., and Tsugane, S., *Int. J. Cancer*, 99, 245–251, 2002.

Spitz, M.R., Wei, Q.Y., Dong, Q., Amos, C.I., and Wu, X., *Cancer Epidemiol. Biomarkers Prev.*, 12, 689–698, 2003.

Stern, M.C., GimenezConti, I.B., Budunova, I., Coghlan, L., Fischer, S.M., DiGiovanni, J., Slaga, T.J., and Conti, C.J., *Carcinogenesis*, 19, 125–132, 1998.

Stinn, W., Kuhl, P., and Haussmann, H.-J., in *EUROTOX*Budapest, Hungary, pp. Poster presented at the 40th Congress of the European Congress of the European Societies of Toxicology. EUROTOX 2002, 2002.

Stinn, W., Teredesai, A., Kuhl, P., Knörr-Wittman, C., Kindt, R., Coggins, C.R.E., and Haussmann, H.-J., *Inhalation Toxicol.*, 17, 263–276, 2005.

Stoll, R.E., Furst, S.M., Stoltz, J.H., Lilly, P.D., and Mennear, J.H., *Toxicol. Pathol.*, 29, 535–540, 2001.

Storer, R.D., French, J.E., Donehower, L.A., Gulezian, D., Mitsumori, K., Recio, L., Schiestl, R.H., Sistare, F.D., Tamaoki, N., Usui, T., and vanSteeg, H., *Mutat. Res. Genet. Toxicol. Environ. Mutagen.*, 540, 165–176, 2003.

Sun, Y.P., Zhu, B.Q., Browne, A.E., Sievers, R.E., Bekker, J.M., Chatterjee, K., Parmley, W.W., and Glantz, S.A., *Circulation*, 104, 810–814, 2001.

Szmitko, P.E., Wang, C.H., Weisel, R.D., deAlmeida, J.R., Anderson, T.J., and Verma, S., *Circulation*, 108, 1917–1923, 2003a.

Szmitko, P.E., Wang, C.H., Weisel, R.D., Jeffries, G.A., Anderson, T.J., and Verma, S., *Circulation*, 108, 2041–2048, 2003b.

Takaoka, M., Sehata, S., Maejimi, T., Imai, T., Torii, M., Satoh, H., Toyosawa, K., Tanakamaru, Z.Y., Adachi, T., Hisada, S., Ueta, M., Ogasawara, H., Matsumoto, M., Kobayashi, K., Mutai, M., and Usui, T., *Toxicol. Pathol.*, 31, 191–199, 2003.

Takubo, Y., Guerassimov, A., Ghezzo, H., Triantafillopoulos, A., Bates, J.H.T., Hoidal, J.R., and Cosio, M.G., *Am. J. Respir. Crit. Care Med.*, 166, 1596–1603, 2002.

Tani, S., Dimayuga, P., Anazawa, T., Chyu, K.-Y., Li, H., Shah, P.K., and Cercek, B., *Atherosclerosis*, 175, 7–14, 2004.

Terpstra, P.M., Teredesai, A., Vanscheeuwijk, P.M., Verbeeck, J., Schepers, G., Radtke, F., Kuhl, P., Gomm, W., Anskeit, E., and Patskan, G., *J. Appl. Toxicol.*, 23, 349–362, 2003.

Tomisawa, M., Suemizu, H., Ohnishi, Y., Maruyama, C., Urano, K., Usui, T., Yasuhara, K., Tamaoki, N., and Mitsumori, K., *Toxicol. Lett.*, 142, 111–117, 2003.

Toyooka, S., Maruyama, R., Toyooka, K.O., McLerran, D., Feng, Z.D., Fukuyama, Y., Virmani, A.K., ZochbauerMuller, S., Tsukuda, K., Sugio, K., Shimizu, N., Shimizu, K., Lee, H., Chen, C.Y., Fong, K.M., Gilcrease, M., Roth, J.A., Minna, J.D., and Gazdar, A.F., *Int. J. Cancer*, 103, 153–160, 2003a.

Toyooka, S., Tsuda, T., and Gazdar, A.F., *Human Mutat.*, 21, 229–239, 2003b.

Traul, D.K., Freischlag, J.A., Chiang, P.C., Erickson, C.A., Cambria, R.A., Seabrook, G.R., and Towne, J.B., *Surg. Forum*, 46, 1995.

Trump, B.F., McDowell, E.M., Glavin, F., Barrett, L.A., Becci, P.J., Schurch, W., Kaiser, H.E., and Harris, C.C., *J. Natl. Cancer Inst.*, 61, 563–575, 1978.

Tuder, R.M., Petrache, I., Elias, J.A., Voelkel, N.F., and Henson, P.M., *Am. J. Respir. Cell Mol. Biol.*, 28, 551–554, 2003a.

Tuder, R.M., Zhen, L., Cho, C.Y., Taraseviciene-Stewart, L., Kasahara, Y., Salvemini, D., Voelkel, N.F., and Flores, S.C., *Am. J. Respir. Cell. Mol. Biol.*, 29, 88–97, 2003b.

U.S. Department of Health and Human Services, The Health Consequences of Smoking. Cancer. A Report of the Surgeon General, Public Health Service, Office on Smoking and Health., Rockville, MD, 1982.

U.S. Department of Health and Human Services, The Health Consequences of Smoking. Cardiovascular Disease. A report of the Surgeon General., Public Health Service. Office on Smoking and Health, Rockville, MD, 1983.

U.S. Department of Health and Human Services, The Health Consequences of Smoking. Chronic Obstructive Lung Disease, Public Health Service, Office on Smoking and Health, Rockville, MD, 1984.
U.S. Institute of Medicine, *Clearing the Smoke. Assessing the Science Base for Tobacco Harm Reduction*, National Academy Press, Washington, DC, 2001.
Valenca, S., Da Hora, K., Castro, P., Moraes, V.G., Carvalho, L., and Porto, L.C., *Toxicol. Pathol.*, 32, 351–356, 2004.
van Jaarsveld, H., Kuyl, J.M., and Alberts, D.W., *Free Radic. Res. Commun.*, 17, 263–299, 1992a.
van Jaarsveld, H., Kuyl, J.M., and Alberts, D.W., *Basic Res. Cardiol.*, 87, 393–399, 1992b.
van Jaarsveld, H., Kuyl, J.M., Strydom, M.S., and Alberts, D.W., *Res. Commun. Alcohol Subst. Abuse*, 15, 171–185, 1994.
Vanscheeuwijck, P.M., Teredesai, A., Terpstra, P.M., Verbeek, J., Kuhl, P., Gerstenberg, B., Gebel, S., and Carmines, E.L., *Food Chem. Toxicol.*, 40, 113–131, 2002.
Wang, X.L., Raveendran, M., and Wang, J., *Progr. Cardiovasc. Dis.*, 45, 361–382, 2003a.
Wang, Y., Zhang, Z., Kastens, E., Lubet, R.A., and You, M., *Cancer Res.*, 63, 4389–4395, 2003b.
Watson, R.E., Curtin, G.M., Doolittle, D.J., and Goodman, J.I., *Toxicol. Sci.*, 75, 289–299, 2003.
Wehner, A.P., in *Comparative Respiratory Tract Carcinogensis*, Vol. II. *Experimental Respiratory Tract Carcinogenesis*. Reznik-Schuller, H.M., Ed., CRC Press, Boca Raton, FL, 1983.
Wei, Q.Y. and Spitz, M.R., *Cancer Metastasis Rev.*, 16, 295–307, 1997.
Wijnhoven, S.W.P. and vanSteeg, H., *Toxicology*, 193, 171–187, 2003.
Wistuba, I.I., Gazdar, A.F., and Minna, J.D., *Semin. Oncol.*, 28, 3–13, 2001.
Witschi, H., Espiritu, I., Dance, S.T., and Miller, S.M., *Toxicol. Sci.*, 68, 322–330, 2002.
Witschi, H., Espiritu, I., Maronpot, R.R., Pinkerton, K.E., and Jones, A.D., *Carcinogenesis*, 18, 2035–2042, 1997a.
Witschi, H., Espiritu, I., Peake, J.L., Wu, K., Maronpot, R.R., and Pinkerton, K.E., *Carcinogenesis*, 18, 575–586, 1997b.
Witschi, H., Espiritu, I., Uyeminami, D., Suffia, M., and Pinkerton, K.E., *Inhalation Toxicol.*, 16, 27–32, 2004.
Witschi, H., Oreffo, V.I.C., and Pinkerton, K.E., *Fundam. Appl. Toxicol.*, 26, 32–40, 1995.
Wright, J.L. and Churg, A., *Chest*, 122, 301S–306S, 2002.
Wynder, E.L., Graham, E.A., and Croninger, A.B., *Cancer Res.*, 13, 855–864, 1953.
Wynder, E.L. and Hoffmann, D., *Cancer Res.*, 54, 5284–5295, 1994.
Xu, L., Cai, B.Q., and Zhu, Y.J., *Chinese Med. J.*, 117, 1611–1619, 2004.
Yano, S., Nishioka, Y., Goto, H., and Sone, S., *Cancer Sci.*, 94, 479–486, 2003.
Zhang, J., Jiang, S., and Watson, R.R., *Environ. Health Perspect.*, 109, 1007–1009, 2001.
Zhang, J., Liu, Y.L., Shi, J.Q., Larson, D.F., and Watson, R.R., *Exp. Biol. Med.*, 227, 823–829, 2002.
Zhao, B.H., Magdaleno, S., Chua, S., Wang, Y.L., Burcin, M., Elberg, D., Finegold, M., Tsai, S., and DeMayo, F.J., *Exp. Lung Res.*, 26, 567–579, 2000.
Zhu, B., Sun, Y., Sievers, R.E., Glantz, S.A., Parmley, W.W., and Wolfe, C.L., *Circulation*, 89, 1282–1290, 1994.
Zhu, B., Sun, Y., Sievers, R.E., Isenberg, W.M., Glantz, S.A., and Parmley, W.W., *J. Am. Coll. Cardiol.*, 21, 225–232, 1993.
Zhu, B.Q., Heeschen, C., Sievers, R., Karliner, J.S., Parmley, W.W., Glantz, S.A., and Cooke, J.P., *Cancer Cells*, 4, 191–196, 2003.
Zhu, B.Q., Sievers, R.E., Browne, A.E., Hillman, R.T., Chair, K., Lee, R.J., Chatterjee, K., Glantz, S.A., and Parmley, W.W., *J. Renin Angiotensin Aldosterone Syst.*, 3, 54–60, 2002.
Zhu, B.Q., Sun, Y.P., Sievers, R.E., Shuman, J.L., Glantz, S.A., Chatterjee, K., Parmley, W.W., and Wolfe, C.L., *Am. Heart J.*, 132, 91–100, 1996.
Zhu, B.Q., Sun, Y.P., Sudhir, K., Sievers, R.E., Browne, A.E.M., Gao, L.R., Hutchison, S.J., Chou, T.M., Deedwania, P.C., Chatterjee, K., Glantz, S.A., and Parmley, W.W., *J. Am. Coll. Cardiol.*, 30, 1878–1885, 1997.

34 The Impact of Active and Passive Exposure to Cigarette Smoke on Maternal and Fetal Health

Sher-Janel T. Todd and Stanley T. Omaye

CONTENTS

34.1 INTRODUCTION

Those who choose to engage in tobacco smoking subject themselves to air pollution and increase their risk for many chronic diseases. However, it is becoming increasingly apparent that nonsmokers exposed to environmental tobacco smoke (ETS) also are subject to the risks of such air pollution whether the exposure occurs indoors or outdoors. In addition, there is reason to believe that vulnerable subpopulations may be even more susceptible to the adverse health effects of ETS, such as, the elderly, the very young, those with compromised health, and pregnant women and their unborn children.

To understand the impact of cigarette smoking on pregnancy, it is important to consider a global and historical perspective of this serious health threat. This chapter reviews literature relating to tobacco use, health effects, reproductive issues, oxidative stress, biomarker research, and economics. Also addressed is ETS. ETS is a ubiquitous environmental pollutant found in highest concentrations in the homes of smokers and in work places where smoking is permitted (CA EPA 1997c; CDC 1993b; Nafstad et al., 1995a; Sterling et al., 1995). The Environmental Protection Agency (EPA) has defined ETS as a Class A carcinogen (Environmental Protection Agency, 1993), rich in carbon monoxide and nicotine, which has come under consideration by the EPA as an indoor air pollutant.

34.2 TOBACCO USE BY SELECTED DEMOGRAPHIC VARIABLES—UNITED STATES

34.2.1 Gender/Age

The Centers for Disease Control and Prevention (CDC) estimated that for the year 2000, 23.3% of American adults were active smokers (CDC, 2002c). The *Profile of General Demographic Characteristics* reported the total adult (18 years and older) population of the United States for the year 2000 was 209,128,094. Of this number, 108,133,727 were female and 100,994,367 were male (U.S. Census Bureau, 2002). This means there were an estimated 48.7 million adults who were active smokers. Approximately 26 million were men (25.7%) and more than 22.7 million were women (21%). The highest prevalence was in the age group 18 to 44, which includes the majority of the childbearing years (CDC, 2002c, 2002d).

34.2.2 Race/Ethnicity

The prevalence of cigarette smoking in racial/ethnic groups was highest among American Indians and Alaska Natives (36%). The lowest prevalence was in Asians (14.4%) and Hispanics (18.6%) (CDC, 2002c). See Table 34.1.

34.2.3 Income

The prevalence of cigarette smoking is generally inversely related to income level. Nationally, the median percent of smoking among individuals with an income of less than $15,000 is 29.7; whereas, for those with an income between $35,000 and $49,999, the median percent drops to 24.3. There is a dramatic drop to 17.2% for those with incomes greater than $50,000 (CDC, 2002d). See Table 34.2.

TABLE 34.1 Percentage of Cigarette Smoking by Race, 2000

Race	US
White	24.1
Black	23.2
Hispanic	18.6
American Indian/Alaskan Native	36.0
Asian/Pacific Islander	14.4
Other	24.4

Source: CDC (2002c).

TABLE 34.2 Percentage of Cigarette Smoking by Income, 2000

Income	US
< $15,000	29.7
$15,000–24,999	28.6
$25,000–34,999	26.1
$35,000–49,999	24.3
$50,000 +	17.2

Source: CDC (2002d).

TABLE 34.3 Percentage of Cigarette Smoking by Level of Education, 2000

Education	US
< HS	31.7
HS or GED	27.7
Some post HS	23.5
College graduate	12.3

Source: CDC (2002d).

34.2.4 Education

The prevalence of cigarette smoking is also inversely related to educational level. In 2000, smoking prevalence was highest among adults who had earned a General Education Development diploma (47.2%). Individuals with advanced degrees (e.g., Master's, Doctoral, and other professional degrees) had the lowest prevalence (8.4%) (CDC, 2002c, 2002d). The national Behavioral Risk Factor Surveillance System (BRFSS) data summarizing this relationship are presented in Table 34.3 (CDC, 2002d).

Kahn et al. (2002) studied the smoking behavior of a representative cohort of 8285 women before, during, and after pregnancy. The results of their study validated the premise that more educated women may quit smoking altogether during pregnancy and not relapse to smoking after their pregnancies, when compared with less educated women. Their recommendation is to target lower socioeconomic pregnant women with smoking interventions.

34.3 TOBACCO-RELATED MORTALITY

Tobacco products are the number one killer of the American public (CDC, 2002a). According to Dr. C. Everett Koop, former Surgeon General of the United States, tobacco is the only product which, when used exactly as intended by the manufacturer, causes the death of the consumer (Surgeon General, 1988). It is estimated that 430,000 to 450,000 Americans die of tobacco-related morbidity each year (CDC, 2002a). According to Hoppock and Houston (1990), the number of Americans who die each year of tobacco-related diseases is greater than the number of deaths from drug-related and alcohol-related causes, and more than the total American military fatalities in World Wars I and II, and Vietnam. Radecki and Zdunich (1993) characterized the number of smokers who die each year from tobacco-related diseases as greater than the deaths caused by accidents, suicides, homicides, AIDS, alcohol abuse, and illegal drug use combined. In 2000, CDC (CDC, 2002a) reported that the number of tobacco-related deaths is greater than the total of deaths from AIDS, alcohol, cocaine, heroin, homicide, suicide, motor vehicle accidents, and fires. In addition to mortality for the active smoker, there are about 3000 deaths per year for individuals who were passively exposed to cigarette smoke (CDC, 2002a, 2002c).

In the United States, smoking-related illnesses account for approximately 25% of total deaths and greater than 25% of deaths in the age group 35–64 years (Bartecchi et al., 1994; CDC, 2002a; McGinnis and Foege, 1993). Smoking contributes to the leading causes of death: cardiovascular disease, cerebrovascular accidents, lung cancer, and other lung diseases (CDC, 1992, 2002a, 2002c). Annually, from 1995 to 1999, there were approximately 124,813 deaths from lung cancer, 81,976 from ischemic heart disease, and 64,735 from chronic obstructive pulmonary disease (COPD) (CDC, 2002a).

According to former Surgeon General, Joycelyn Elders, "Cigarette smoking is still considered the chief preventable cause of premature disease and death in the United States" (CDC, 1994). CDC studies also point to the use of tobacco products as the leading preventable cause of premature morbidity and mortality (CDC, 2002a, 2002c). Although it is often difficult to place a monetary value on illness, and even more difficult to place a monetary value on human life, there are measurable economic impacts related to tobacco use.

34.4 TOBACCO-RELATED MORBIDITY

Tobacco use usually begins in early adolescence (Altman et al., 1989, 1991; Banspach et al., 1989; CDC, 1994, 2000; DiFranza, 1992; Elder and Stern, 1986). This behavior often leads to addiction (Benowitz, 1992; CDC, 1994, 1995, 2002d; DiFranza, 1992; Stolerman and Jarvis, 1995; Surgeon General, 1988) and significant adult pathology, including cancers, emphysema, asthma, chronic obstructive pulmonary disease, osteoporotic disease, reproductive disorders, periodontal disease, premature aging of the skin, and premature disability and death (CDC, 1989, 1991,1992, 1993a, 2000, 2002a; Office on Smoking and Health/National Center for Chronic Disease Prevention, 1995; Sims, 1994; Strategic Coalition of Girls & Women United Against Tobacco, 1995b; Surgeon General, 1988). These types of morbidity do not include those persons who are injured or killed in smoking-related fires.

Of the 170,000 new lung cancer cases diagnosed each year, only 13% are related to industrial exposure to carcinogens (e.g., asbestos fibers, radon gas). The remaining 87% are related to cigarette smoking (CDC, 2002a; Nadakavukaren, 2000a). Unfortunately, many persons do not understand the concept of synergism and how tobacco use can enhance the activity of other carcinogens, creating even greater risk to the individual (Eaton and Klaassen, 1996; Nadakavukaren, 2000b, 2000c; Timbrell, 1994).

There is also morbidity for the passive smoker. There are many studies that correlate passive exposure and adverse health effects (Byrd, 1992; California EPA, 1997a, 1997b; Council on Scientific Affairs, 1994; Glantz and Parmley, 1995; Kritz et al., 1995; Pacifici et al., 1995; Witschi et al., 1995, 1997).

The exposure of the U.S. population to ETS was investigated using participants from the Third National Health and Nutrition Examination Survey (NHANES III), 1988–1991 (Pirkle et al., 1996). The purpose of the study was to estimate how much the workplace and home environments contributed to passive exposure. This study used a nationally representative cross-sectional survey with a population total of 16,818, and an age range of two months and older. Cotinine measurements were performed on 10,642 of the participants four years of age and older. The cotinine levels reflected exposure to ETS from the previous one to two days. Based on the survey, questionnaire information, and serum cotinine levels, it was determined a significant proportion of the population was exposed to ETS at home and at the workplace. From the NHANES III questionnaire data for ages two months to 11 years, the prevalence of reported ETS exposure at home was 43%. Of adult nontobacco users aged 17 years and older, 37.4% reported exposure to ETS at home or at work with the percentage for males (43.5%) being higher than for females (32.9%). Approximately 47.7% of working adults reported ETS exposure at home or at work with an average exposure duration of 4.4 h. However, when examining the population of persons tested for serum cotinine levels, of the nontobacco users aged four years and older, 87.9% had detectable cotinine levels in their serum. This argues that there is much greater exposure to ETS than the self-report by interview would indicate. Therefore, in addition to the morbidity among the 25–30% of tobacco users, there is reason to be concerned about the health of the nontobacco-using, but ETS-exposed, population as well.

Some of the same diseases that affect the active smoker also affect the passive smoker. Glantz and Parmley (1995) studied peer-reviewed literature from 1986 to 1994 with most of the investigation focusing on the years 1990 to 1994. Their review supported an estimation that ETS contributed to 37,000 heart disease deaths, making passive exposure to smoking the third leading cause of preventable death in the United States.

34.5 WOMEN AND TOBACCO

34.5.1 Addiction

CDC (1995) analyzed data from 1991 and 1992 to assess nicotine addiction in American women. Their analysis showed that in females 12 years and older, 75% of the interviewees described feelings of dependency on cigarettes and an inability to quit smoking. This study did not look at all the symptoms of addiction and is not considered comprehensive. Nevertheless, this information has ramifications for pregnancy because more women are initiating smoking behavior in adolescence, becoming pregnant at

younger ages, and smoking more heavily than in the past. Further, women have greater difficulty than men in quitting (CDC, 1991, 1993a, 1993b, 1995, 2002d; Fortmann and Killen, 1994).

The 2001 Report of the Surgeon General gave similar findings. Greater than 75% of the women interviewed wanted to quit smoking and more than 46% had tried to quit, unsuccessfully, during the previous year. These women all demonstrated indicators of addiction as measured by time of first cigarette after waking, withdrawal symptoms, using cigarettes as a calming agent, and feelings of dependency on cigarettes (Surgeon General, 2002).

Selby et al. (2001) conducted an investigation of 19 white pregnant women who smoked heavily and could not quit. On average, they had begun smoking at 14.5 years and had smoked for 17 ± 6 years. Nicotine and cotinine levels were assessed and nicotine levels were lower than expected. The conclusion of the study was that heavily smoking women may be rapid metabolizers of nicotine to cotinine and therefore smoke more to maintain nicotine levels.

34.6 TOBACCO AND PREGNANCY

34.6.1 Prevalence of Smoking during Pregnancy

More than 22 million American women were smoking in 2000 (CDC, 2002c). It is estimated that 12 to 30% of women smoke during pregnancy (CDC, 2002b; Surgeon General, 2002). During the year 2000, there were 4.3 million births in the United States and there were approximately 62 million women of childbearing age. Therefore, there was a pregnancy rate of about 7%. There is an incongruence in the reported data regarding the percent of pregnant women who smoke and the number of low-birthweight (LBW) babies. On one hand, reported smoking rates among pregnant women are falling, while the rate of LBW has not fallen but has risen slightly (Surgeon General, 2002). This may be, in part, a result of difficulty in assessing the actual number and percent of women who smoked during pregnancy. Many states do not record this information in the birth record. Nevertheless, it can be estimated that there are 48,000 to 1.2 million women per year who smoke during pregnancy. In a year-long survey in the low-income prenatal clinic used for the present study, the rate of smoking was approximately 30% (unpublished data).

Ross et al. (2002) reported the prevalence of smoking in pregnant women in Women, Infants, and Children's Clinics (WIC) in Minneapolis and Saint Paul, Minnesota, as 21%. Ninety-eight women were interviewed and did not know their self-reported smoking status would be biologically confirmed. Five women who denied smoking had cotinine levels consistent with active smoking (mean = 64 ng/ml). The deception rate was 25%.

Tappin et al. (1997) collected anonymous antenatal blood specimens from the first two prenatal visits over a six-month period. Samples from pregnancies that were confirmed to have ended in a live birth were analyzed for cotinine. Slightly more than 35% of the samples were positive for cotinine levels consistent with active smoking.

34.6.2 Reproductive Morbidity and Mortality

34.6.2.1 Maternal Active Smoking

It is hypothesized that 25% of pregnancies end in spontaneous abortion and it is rarely possible to determine an exact cause. Spontaneous abortion is more common in cigarette smokers than in nonsmokers. A carefully controlled study suggested an 80% increase in the rate of spontaneous abortion in smokers when compared with nonsmokers (Kline et al., 1977).

In 1982, Weinberger and Weiss described cigarette smoking as one of the risk factors which can (1) reduce fertility and (2) increase the incidence of spontaneous abortion, abruptio placentae, placenta previa, bleeding during pregnancy, and premature rupture of the placental membranes. Thus, smoking can prevent pregnancy from occurring, cause serious complications during the pregnancy, and may prevent the pregnancy from going to term (Windham et al., 1992).

McDonald et al. (2002) investigated the effect of smoking on homocysteine and folate levels in 40 actively smoking and 40 nonsmoking pregnant women. Inadequate folate levels have been linked to several negative reproductive outcomes, including neural tube defects in the fetus, abruptio placentae, miscarriage, and stillbirth. Homocysteine levels should decrease in healthy pregnant women. Although there was no difference in homocysteine levels between the two groups, there was a difference in folate levels. Serum folate was significantly lower in pregnant smokers than in pregnant nonsmokers.

34.6.2.2 ETS Exposure and Pregnancy

Environmental tobacco smoke, as stated previously, is a ubiquitous environmental pollutant. Approximately 65–85% of the nonsmoking American public, including pregnant women, fetuses, neonates, infants, and children, show detectable levels of cotinine (the nicotine biomarker) in serum and plasma samples, as well as other body fluids and tissues (Matt et al., 1999; Pirkle et al., 1996). For the past several years, concern has been mounting about the deleterious health effects caused by ETS, especially in vulnerable populations (Ahluwalia et al., 1997; Kaufman et al., 2002; Mannino et al., 2001).

In the first of two studies, Nelson et al. (1999b) passively exposed pregnant Wistar rats to varying numbers of cigarettes (1–4) during the first, second, or third week of gestation. They investigated the carboxyhemoglobin (COHb) levels pre- and postexposure, as well as the birthweight of the pups. Although no macroscopically gross abnormalities were seen, upon autopsy, widespread skeletal retardation was seen in all exposed groups regardless of the dose. Further, the COHb levels rose from 1.2% preexposure to 6–12% postexposure depending on the number of cigarettes to which those pregnant rats had been exposed.

In a subsequent study, Nelson et al. (1999a) again passively exposed pregnant Wistar rats to varying numbers of cigarettes (1–4) during the first, second, or third week of gestation. They autopsied the animals and examined them for histological changes in lung, liver, renal, gastric, and intestinal tissues. One of the frequent changes they found was an increased incidence of apoptosis (controlled cell death). Therefore, they concluded exposure to ETS may cause adverse tissue changes in the fetus.

34.6.2.3 Infant Morbidity and Adverse Birth Outcomes

The following studies show an epidemiological association between active and passive exposure to tobacco and adverse birth outcomes. These outcomes often continue into childhood and may lead to an increase in morbidity and mortality among the neonates, infants, and children exposed to tobacco. There may also be an association between *in utero* tobacco exposure and childhood cancer, leading to premature death.

34.6.3 Low Birthweight (LBW), Intrauterine Growth Restriction/ Retardation (IUGR), and Small for Gestational Age (SGA)

34.6.3.1 Active Smoking

Restriction of fetal growth is a primary finding in studies of fetuses and neonates of smoking women. Smoking is related, in a dose–response manner, to the delivery of LBW babies. Analyses of several studies showed that small for gestational age (SGA) births and intrauterine growth restriction or retardation (IUGR) were higher in a smoking group than in a nonsmoking group (CDC, 1990; Miyao et al., 1995; Spinillo et al., 1994b, 1994c).

Goldenberg (1994) investigated the historical trends in LBW. It is not clear if he did this by a review of the literature or by studying the randomized trials funded by the National Institutes of Health. However, he found that despite the research dollars spent and the clinical care resources utilized, there has been only limited success in reducing the incidence of fetal growth restriction. He

also concluded that, although there has been success in keeping LBW babies alive, there are many variables related to fetal growth retardation. At the top of the list is cigarette smoking. Actively smoking mothers, more than any other risk factor, account for the preponderance of LBW infants.

England et al. (2001) investigated maternal smoking at enrollment in their study and during the third trimester of pregnancy to ascertain if there was a decrease in neonatal birthweight. Although they collected self-reported smoking behavior from 1583 subjects, they also analyzed urine cotinine. They discovered that third-trimester smoking increases resulted in a sharp decrease in birthweight. This decrease leveled off at greater than eight cigarettes per day. Their recommendation is that women who cannot quit smoking during pregnancy limit their cigarette consumption to fewer than eight cigarettes per day.

Spinillo et al. (1994b), in a case-control study of prospectively recorded data, examined the risk of fetal growth retardation from prenatal smoking when compared with other risk factors. The other variables included maternal age, nulliparity, low maternal prepregnancy weight, previous LBW infant, female fetal sex, maternal prenatal hypertension, low socioeconomic conditions, and caffeine consumption. The cases included 347 singleton pregnancies with a diagnosis of IUGR, and 694 controls with appropriate growth for gestational age fetuses. The database was collected during a five-year period from 1988 to 1992 at the Department of Obstetrics and Gynaecology of the University of Pavia, Italy. Growth restriction was studied using a series of sonogram measurements. The results of this study showed that in actively smoking nulliparous women, the risk of IUGR was significant and increased by a factor of three. Additionally, IUGR was increased 5.4 times in actively smoking women 20 years of age or younger when compared to older women. For actively smoking women with low prepregnancy weight, IUGR increased fourfold. The researchers concluded that there is a cumulative effect when there are multiple risk factors and they recommend that prenatal counseling include a discussion of the effects of smoking on pregnancy outcome.

In a case-control study, Spinillo et al. (1994c) evaluated the interactions between smoking in pregnancy and fetal growth and gender. All the patients were delivered at Policlinico San Matteo Hospital in Pavia, Italy. Cases were 530 singleton neonates, with both a sonographic diagnosis of IUGR and a birthweight below the 10th percentile, who were delivered between 1987 and 1992. The controls were 782 singleton pregnancies with fetuses of appropriate growth delivered from September through November 1989 and January through April 1991. Information related to maternal sociodemographics and clinical history was collected at delivery. After controlling for the variables of social class, gestational hypertension, and preeclampsia, the results showed that fetal growth retardation was significantly greater in male fetuses than in female fetuses. The results of this study also validated other studies showing that the factor which best correlates with decreased birthweight is maternal smoking during pregnancy. This study also validates a previous study by Spinillo et al. (1994b) that showed there is a synergistic effect of smoking when combined with any other risk factor.

Miyao et al. (1995) used a matched-pair longitudinal method to study the relationship between maternal prenatal smoking and neonatal head circumference. From April 1990 to March 1992, these investigators surveyed 508 primiparas in 11 hospitals in Aichi, Japan. All of the 508 newborns were measured for birthweight and head circumference. For this case-control study, 47 smokers and their neonates and 47 nonsmokers and their neonates were chosen in random order from a computer database. The results of the study showed that head circumference was significantly smaller in neonates of smoking mothers when compared with nonsmoking mothers. The researchers expressed concern because microcephaly has been associated with mental retardation and the rate of smoking among Japanese females is increasing.

Milner et al. (1999) studied maternal smoking and its effect on neonatal lung function. The study included 189 pregnant smokers and 100 pregnant nonsmokers and their neonates. Although their study did not find an adverse effect on fetal lung growth, they did note a statistically significant reduction in birthweight and length for the infants of smokers. This finding is not surprising because neonates are exposed to nicotine through placental circulation rather than respiration.

Horta et al. (1997) investigated 5166 births, maternal cigarette smoking, and the frequency of LBW, premature delivery, and IUGR. While they found no association between maternal smoking

and preterm birth, they did note a direct dose–response relationship between the number of cigarettes smoked and IUGR. Because of the IUGR, there was an increase in LBW. Their study also demonstrated that ETS-exposed pregnant women are at risk for selected adverse pregnancy outcomes.

34.6.3.2 Active Smoking or Passive Exposure

Lieberman et al. (1994) evaluated the risk of SGA infants born to women who stopped smoking or began smoking during their pregnancies. This cohort study was conducted at Boston Hospital from 1977 to 1980 and examined a variety of exposure levels and birth outcomes. The final study population was 11,177 women and their infants. Data were collected from interviews and medical record reviews. The data included medical and obstetric history, course of the current pregnancy, and infant outcome. The researchers discovered that the number of cigarettes smoked and how long the mother smoked during the pregnancy greatly influenced the effect of LBW when compared to women who quit smoking early in the pregnancy or cut down significantly. Women who initiated smoking in the third trimester had the same risk as those who smoked continuously throughout the pregnancy. These results present a compelling reason to provide targeted smoking cessation interventions for all pregnant women, especially in their third trimester (Kendrick et al., 1995; Windsor et al., 1998).

Mainous and Hueston (1994) looked for a threshold effect when studying LBW and passive smoke exposure. They analyzed the data from the 1988 National Health Interview Survey. The information was collected from family members residing in the household. The sample was limited to information supplied by the biological mother of children less than six years old. Data were taken only from mothers who had not smoked in the year prior to their pregnancy and who did not smoke throughout their pregnancy. The study results showed that mothers with a high exposure to ETS were 2.31 times more likely to have a LBW infant ($<$2500g) when compared with mothers with a low exposure. The investigators concluded that prenatal counseling needs to include not only advice for the active smoker to quit smoking, but also advice to nonsmokers to avoid exposure to ETS.

Roquer et al. (1995) studied the influence of various degrees of active and passive smoking exposure on 129 term neonates. After delivery, the smoking habits of the mother were surveyed and newborns were classified in one of four exposure groups: (1) nonsmoking and nonexposed mother, (2) nonsmoking but exposed mother at home or at work; (3) mother smoking fewer than 10 cigarettes/day; and (4) mother smoking 10 cigarettes/day or more. After controlling for work environment and parity, this epidemiological study showed that active smoking and exposure to a significant level of passive smoking had the same effect. Further, all growth parameters in the infant were reduced, and the results suggested that these reductions in length may persist throughout childhood. These investigators recommend that in any study of the effect of smoking on the fetus and infant, the effect of passive smoking should be studied as well.

34.6.3.3 Passive Exposure

Martinez et al. (1994) studied the effect of paternal smoking on the birthweight of neonates whose mothers were nonsmokers. They also assessed paternal smoking by self-report questionnaire and neonatal serum cotinine levels from cord blood for 175 newborns. Of the 138 mothers who reported that they had not smoked during pregnancy, only two of these had newborns with cotinine levels indicating that their mothers were, in fact, active smokers. Of the 136 nonsmoking mothers, their infants' cord blood cotinine level was significantly correlated with the number of cigarettes smoked per day by the father. A mean birthweight deficit of 88 grams was found in newborns whose fathers smoked more than 20 cigarettes per day and whose mothers were nonsmokers. Their study showed no significant relationship between maternal exposure to ETS and duration of pregnancy. However, the reduction in birthweight, which is caused by active and passive exposure to ETS, may lead to an increased risk of infant mortality during the first year of life.

Rebagliato et al. (1995) conducted a prospective cohort study in Valencia, Spain with a population of 710 nonsmoking pregnant women in their third trimester. This study examined the exposure of these women to ETS and the relationship to low infant birthweight. Each woman was interviewed by using a structured questionnaire eliciting information related to ETS exposure during the previous two days and throughout the entire pregnancy. The questionnaire assessed the woman's exposure from four potential sources: (1) partner smoking at home; (2) other family members smoking at home; (3) exposure at the worksite, and (4) exposure in indoor and outdoor public and private places and in transportation. Saliva was collected from the participants to assess their cotinine levels by gas chromatography. Data were also collected on pregnancy outcome and neonatal condition from both the mothers and the hospital records. Birthweight was the outcome measured. This investigation suggested a weak correlation between passive ETS exposure and LBW. However, the cotinine level in the maternal saliva was significant and could only have come from very high exposure to ETS. To reduce the risk of IUGR and LBW, these researchers recommend a smoking restriction policy in public and enclosed workplaces where nonsmoking pregnant women are present.

34.6.4 Preterm Birth

In 1957, there was still controversy in the medical community about whether smoking was harmful to the pregnant woman and the developing fetus. Simpson (1957) studied active cigarette smoking during pregnancy and gave the first report about a possible relationship between active smoking and premature delivery. Approximately 47% of the study participants admitted smoking. Prematurity rates were compared among three groups: nonsmokers, light smokers, and heavy smokers, as well as number of cigarettes smoked per day. This preliminary study was based on information from greater than 7000 patients and demonstrated an increased incidence of prematurity for active smokers at twice the rate for nonsmokers.

Simpson also reported that as early as 1935, approximately 48% of pregnant women in the United States and Canada were smoking. This information came from a survey sent to leading obstetricians in those two countries by Dr. A.M. Campbell (results reported in the *J. Mich. Med. Soc.,* 34, 146, 1935; article no longer available). She cited another study by Dr. P. Bernard (*Medizinische,* 3, 58, 1949; article no longer available) based on 20 years of observations from his practice comparing women who smoked and women who did not smoke. He related that health disorders, including problems during pregnancy, were more frequent in smokers.

Spinillo et al. (1994a) evaluated several risk factors (lack of adequate prenatal care, intravenous drug use, abdominal trauma, preexisting or gestational diabetes, preeclampsia and chronic hypertension) associated with abruptio placentae and preterm deliveries. In a prospective study conducted from 1985 to1991, they investigated all of the 24- to 36-week gestation deliveries in the Department of Obstetrics and Gynaecology of the University of Pavia, Italy. Their case-control investigation of 55 consecutive index cases and 726 control cases supported the fact that smoking during pregnancy was a significant risk factor for abruption, low gestational age, and preterm delivery. They recommend intensive surveillance for pregnant women with high-risk factors, including smoking.

Peacock et al. (1995), in a prospective study of several risk factors and pregnancy outcome, examined the relationship between several variables and preterm birth. In addition to smoking, these investigators studied the effect of socioeconomic status, education, alcohol and caffeine consumption, and psychological stress. The study was conducted in a district general hospital in inner London with a population of 1513 women. While they found no apparent affect of smoking on the overall length of gestation, they did show a relationship between delivery prior to 32 weeks and smoking. Their study also supported the finding that lack of fetal growth is strongly associated with smoking during pregnancy.

There may be other adverse effects on birth outcomes. The following studies describe some of these effects, which are related to the mother's active or passive smoking.

34.6.5 Pulmonary and Respiratory Function

34.6.5.1 Active Smoking

Tager et al. (1993) prospectively followed 97 newborns for whom they had obtained pulmonary function data prior to six months of age. The purpose of the study was twofold: (1) to determine whether a relationship exists between lung function and subsequent lower respiratory infections (LRI); and (2) to determine whether there is a correlation between maternal smoking and lung function and LRI. Infant pulmonary function was assessed by partial respiratory flow volume curves and helium-dilution measurement of functional residual capacity. Maternal prenatal smoking was assessed by urine cotinine measurements and standard questionnaire. Occurrence of LRI was evaluated using standardized questionnaires at each well-baby visit, making weekly telephone calls to mothers, and reviewing all medical records. Infants who developed LRI during the first year of life had lower preillness length-corrected forced expiratory flow than those who did not experience LRI. Maternal prenatal smoking may contribute independently to reduced airway size and/or changes in respiratory mechanics especially in female infants.

Tager et al. (1995) prospectively studied 159 infants during the first 18 months of life. Infant pulmonary function was assessed at two to six weeks of age and at four to six months, nine to 12 months, and 15–18 months of age by partial expiratory flow-volume curves and helium-dilution. Maternal smoking was assessed by standard questionnaire and urine cotinine measurements. After controlling for the effects of growth (length), maternal smoking during pregnancy was associated with a significant reduction in infant lung function.

34.6.5.2 Active Smoking or Passive Exposure

Researchers in Boston conducted a longitudinal investigation of the effects of prenatal smoking and postnatal passive smoking on pulmonary function and infant respiratory illness (Hanrahan et al., 1992; Tager et al., 1993, 1995). These studies were conducted at East Boston Neighborhood Health Center. The researchers concluded that there are deficits in children that are the residual effects of prenatal smoking and are present at birth; further, postnatal exposure cannot completely account for lower flow levels found in infants of smoking mothers. The first published study was conducted on women seeking prenatal care from March 25, 1986 to May 1, 1989. The pulmonary function of eighty healthy infants was tested shortly after birth. The mothers' prenatal smoking was measured at each prenatal visit by a questionnaire and by urine cotinine measurements. The results of the study showed that in young infants there were significant reductions in forced expiratory flow rates. The researchers hypothesize that exposure to tobacco smoke may impair fetal airway development and/or alter the elastic properties of the lung.

34.6.6 Germinal Matrix and Intracranial Hemorrhage

Studies by Spinillo et al. (1995a, 1995b) support the hypotheses that heavy maternal smoking may be associated with an increased risk of germinal matrix and intracranial hemorrhage, as well as influence the progression of intraventricular hemorrhage in preterm infants. As many as 50% of infants delivered prior to 34 weeks gestation may experience germinal matrix or intraventricular hemorrhage, which may lead to cerebral palsy and neurodevelopmental delays.

Spinillo et al. (1995a) evaluated the effect of prenatal risk factors in 302 singleton preterm infants delivered between 24 and 33 weeks gestation. In addition to cigarette smoking, the investigators looked at maternal social class, nulliparity, oral tocolysis (used to treat preterm labor), and use of low-dose aspirin. They used sonograms for all infants to detect the presence of intracranial hemorrhage. For the population studied, 9.6% of infants (29 of 302) experienced germinal matrix hemorrhage and 11.6% (35 of 302) experienced intraventricular hemorrhage. The risk factor of significance was cigarette smoking, with the number of cigarettes smoked increasing the risk for both types of hemorrhage. The other variables outlined above did not seem to influence the prevalence of this neonatal problem. However,

gestational age was an influencing factor whereas birthweight was not. These researchers recommend reducing the antenatal risk factors that lead to prematurity and either germinal matrix or intraventricular hemorrhage. Once again it is noted that cigarette smoking is a preventable cause of prematurity.

Spinillo et al. (1995b) conducted a prospective observational case-control study of infants delivered from 1987 to1993 at the University of Pavia, Italy, Department of Obstetrics and Gynaecology. This study was designed to evaluate the effect of prenatal maternal smoking during the second and third trimester and risk factors for fetal and neonatal intracranial hemorrhage. Information about maternal history, pregnancy, and delivery was collected. In addition to a detailed smoking history, the data included maternal sociodemographic variables. Sonograms were performed to determine fetal growth retardation based on abdominal or cephalic circumferences at less than the 10th percentile. The prenatal and postnatal characteristics of infants born at 24–33 weeks gestation with intracranial hemorrhage were compared with selected age-matched control infants. A crude analysis of the data showed a prevalence of intracranial hemorrhage of 20.7% (96 of 463). The research team also studied postnatal risk factors in the neonate, including low five-minute Apgar scores, respiratory distress syndrome, acidosis, and bronchopulmonary dysplasia. All of these factors were significantly associated with intracranial hemorrhage; therefore, the investigators had to examine these associations independently to evaluate the risk from maternal smoking. They cross-tabulated all variables and the multivariate analysis showed that the risk of intracranial hemorrhage in infants was confined to those born to heavy smokers.

34.6.7 Craniosynostosis

Studies by Alderman et al. (1994) and Beeram et al. (1993) suggest an association between fetal tobacco exposure and the occurrence of craniosynostosis, usually a rare condition. Their research suggests a link between intrauterine tobacco exposure and premature closure of the sutures. Alderman et al. (1994) analyzed data from a population-based case-control study to evaluate the relationship between maternal prenatal smoking and alcohol consumption and the risk of craniosynostosis. They used data obtained from the Colorado Craniosynostosis Registry from 1986 to1989. Two hundred thirty-three children had a confirmed diagnosis of craniosynostosis by an independent radiologist. Two hundred twelve children (91%) participated in the study as cases. A random sampling of birth records provided 367 eligible controls, and 291 (79%) of these participated in the study. Interviews were conducted with the mothers of the children and a regression technique was devised to estimate the odds ratio of craniosynostosis for smoking and alcohol consumption. The relative odds ratio (ROR) for craniosynostosis for an infant born to a smoker was 1.7. The ROR increased to 3.5 for an infant born to a mother who smoked more than one pack per day. No strong association was found for alcohol consumption and craniosynostosis. The researchers concluded that maternal prenatal smoking may increase the risk for craniosynostosis in infants.

Beeram et al. (1993) studied the clinical data for three infants with craniosynostosis born to African-American mothers in Washington, DC, who used crack cocaine and tobacco throughout their pregnancies. The usual incidence of craniosynostosis has been reported to be 3.1 to 5 per 10,000 live births, and is usually lower in blacks, singleton, and female births. These three infants were the only reported cases of this condition during the past 10 years at the DC General Hospital. The investigators ruled out other hypothesized causes of craniosynostosis such as family history, hyperthyroidism, disturbances of calcium and phosphate metabolism, and exposure to teratogens. The researchers point out that three cases do not prove a cause-and-effect relationship. However, they argue there is an association and base this hypothesis on the fact that fetal prenatal exposure to tobacco has been associated with fetal growth retardation and this may be a predisposing factor for craniosynostosis. They recommend that all infants born with this condition be assessed for exposure to tobacco and cocaine.

34.6.8 Congenital Urinary Tract Anomaly

There appears to be an association between active maternal smoking and other adverse birth outcomes. Li et al. (1996) conducted a case-control study of infants born in western Washington in

1990 and 1991. They interviewed 117 of 187 mothers of infants with a confirmed congenital urinary tract anomaly. Also interviewed were 369 mothers of controls who were randomly selected from all singleton live births delivered in five major hospitals in western Washington for the same period. Mothers were interviewed in person and a detailed smoking history was elicited. The data were analyzed, controlling for confounding variables such as maternal age, socioeconomic demographics, parity, previous prenatal history, family medical history, infant gender, and county of residence. The data suggest an association between maternal smoking and the risk of congenital urinary tract anomalies. This study also suggests an inverse dose–response relationship with a stronger association between light smoking and these anomalies.

Studies demonstrating an epidemiological relationship are strengthened when basic scientific research can find a biologically plausible mechanism to explain the epidemiological association (Fushimi et al., 1992; Haak et al., 1994; Haddow et al., 1991; Joschko et al., 1991; Ong et al., 1994; Reiben, 1992; Schuller et al., 1993). The following studies suggest some mechanisms whereby the insult to the fetus may occur.

34.6.9 Childhood Brain Tumors

There may also be a link between fetal exposure to nicotine and the risk of brain tumors in children. A European study of mothers' active smoking and passive exposure during pregnancy supported a possible association between *in utero* exposure to tobacco smoke and an increased risk of brain tumor development (Filippini et al., 1994). These researchers conducted a retrospective case-control study of 91 (of a possible 103) cases and 321 population controls from northern Italy. The participants were matched for age, gender, and residence. The cases were children up to 15 years of age first diagnosed with a primary brain tumor between January 1, 1985 and December 31, 1988. Controls were randomly selected from the computerized records of the Regional Health Service. Information was collected by direct interview and questionnaire. Specific questions covered parental smoking habits, the child's exposure to the active smoking of the mother prenatally, and the child's passive exposure to parental smoking postnatally. The investigators identify that the sample size was small and the study was conducted in a single geographic area. However, the odds ratio (OR) appears to be elevated with smoking, although not significantly. The OR increases when both the smoking of the mother and her passive exposure to other smokers is considered. There seems to be a greater association between paternal smoking and the risk of development of childhood brain tumor. The authors also identify that self-report bias may affect accuracy in retrospective studies. They believe there is cause for concern for prenatal and postnatal exposure of children to cigarette smoke.

34.6.10 Genotoxicity and Carcinogenesis

34.6.10.1 Active Smoking

A study by Lahdetie et al. (1993) looked at the relationship between maternal smoking and genotoxicity in second trimester amniotic fluid. Cotinine concentrations were measured in amniotic fluid samples from 22 smoking and 37 nonsmoking pregnant women. In addition, 15 samples from the smokers and nonsmokers were studied by induction of sister-chromatid exchanges in Chinese hamster ovary cells as an indicator of exposure to potential genotoxic activity during pregnancy. In the metabolic tests, there were significantly increased sister-chromatid exchanges in the amniotic fluid of smokers when compared with nonsmokers. This is an indication of potential genotoxicity to the fetus.

Coghlin et al. (1991) studied the maternal–fetal exchange of a potent tobacco-related human carcinogen (4-aminobyphenyl) in 14 smoking and 38 nonsmoking pregnant women. Carcinogen-hemoglobin adducts were detected in all maternal and fetal blood samples with significantly higher levels in maternal and fetal blood samples from smokers. This study showed that this particular xenobiotic crosses the placenta and binds to fetal hemoglobin. Further research to examine the "fingerprint" of childhood cancers to see if there is a match is suggested.

34.6.10.2 Active Smoking or Passive Exposure

Ammenheuser et al. (1994) studied cord blood samples from 10 newborns whose mothers smoked during pregnancy and 10 newborns of nonsmoking mothers. In addition, blood samples were obtained from five of the smoking and five of the nonsmoking mothers. In an assay for somatic cell mutation, investigators found that there were elevated frequencies of mutant lymphocytes at the hprt locus in the blood samples of smoking mothers and their newborns. This study suggests there is a potential for carcinogenic agents to cross the placenta when an actively or passively exposed mother exposes the fetus to cigarette smoke. This study adds to the growing evidence of the hazard of maternal smoking and maternal ETS exposure to the health of the fetus and neonate.

Lackmann and coworkers (1999) studied the exposure carcinogens transferred from the mother to the fetus. Until their study, there was little information regarding fetal uptake of these compounds. They analyzed the first urine output from neonates of 17 nonsmokers and 31 smokers for several carcinogenic compounds. Their results demonstrated that the urine samples from neonates of mothers who smoked had 5–10% of the levels of adult smokers of two metabolites of NNK, the tobacco-specific transplacental carcinogen.

34.6.11 Placental Insufficiency and Fetal Hypoxia

Bush et al. (2000) studied the effect of maternal cigarette smoking and oxygen diffusion across the placenta. They used randomly selected placental tissue sections from two groups, nonsmokers and smokers. Although they could not demonstrate a conductance problem when comparing nonsmokers and smokers, their results did show that the fetus experiences chronic hypoxia, which implies a reduced transplacental partial pressure gradient. This was evidenced by elevated hematocrits and suggests a causal mechanism for LBW babies.

34.6.12 Fetal Heart Rate and Activity Problems

Coppens et al. (2001) investigated maternal smoking and the effect on the fetus by monitoring fetal heart rate and activity with an ultrasound transducer. There were 13 nonsmokers and 13 smokers for comparison and parameters were analyzed by computer. They also assessed changes, by Doppler, in the umbilical artery. The results showed that fetuses of smoking mothers had significantly more time with a low fetal heart rate than those of nonsmoking mothers. Fetal activity was reduced at both low and high heart rates. The umbilical artery showed acute and transient increases in the pulsatility index. The conclusion, based on the computer findings, was that these alterations may be indicative of a problem in neurodevelopmental maturation from chronic fetal hypoxemia.

34.6.12.1 Infant Mortality

Wise et al. (1995) studied the 1988 national linked birth/infant death data. Their analysis showed that very LBW infants (<1500 g) were only 1.2% of all births but represented a disproportionate 64.2% of all neonatal deaths. LBW is causally linked to fetal tobacco exposure. The studies cited provide evidence of a relationship between fetal *in utero* exposure to tobacco smoke and thousands of preventable fetal, neonatal, and infant deaths. In addition to the financial impact, for the families who lose a pregnancy, a newborn, or infant, there is a human cost in feelings of guilt, anger, fear, and grieving.

Andres and Day (2000) conducted a meta-analysis of greater than 30 studies investigating the effects of maternal tobacco use and perinatal complications. Their estimation, after this analysis, is that 15–20% of pregnant women smoke. They specifically looked at studies related to perinatal mortality, preterm birth, premature rupture of the membranes, and abnormalities of fetal growth. They also looked at placental complications, such as abruptio placentae and placenta previa. Finally, they

looked at studies regarding smoking and sudden infant death syndrome (SIDS). The results of the meta-analysis suggest that tobacco use in pregnancy is related to the following:

- 20–30% of all LBW neonates
- 15% of all preterm births
- 150% increase in overall perinatal mortality, including SIDS

34.6.13 Sudden Infant Death Syndrome (SIDS)

Smoking has also been implicated in numerous studies of sudden infant death syndrome (SIDS) (DiFranza and Lew, 1995; Haglund, 1993; Haglund et al., 1995; Klonoff-Cohen et al., 1995; Milerad et al., 1993, 1994; Reid, 1991; Schoendorf and Kiely, 1992). An association between maternal smoking and SIDS has been postulated since 1966 (Haglund 1993).

34.6.13.1 Active Smoking

DiFranza and Lew (1995), in a review of the literature, found 20 studies related to the effect of maternal cigarette smoking on pregnancy complications and sudden infant death syndrome. Their meta-analyses showed that 13 of the studies supported an association between spontaneous abortion and maternal smoking. They estimated that 19,000 to 141,000 tobacco-induced abortions occur each year. This association remained even after controlling for other parameters such as age, race, ethnicity, education, and employment status. They concluded that cigarette smoking is a preventable cause of pregnancy complications and that all women should be advised to quit smoking. They also recommended that effort should be expended to prevent nicotine addiction in young girls, given that smoking cessation has a low success rate.

DiFranza and Lew (1995) conducted a review of the literature related to the annual morbidity and mortality among fetuses and infants that can be associated with tobacco use by pregnant women. Their meta-analysis, described earlier, demonstrated a threefold increased risk of SIDS related to smoking during and after pregnancy. This relationship is still apparent after controlling for other variables such as age, race, ethnicity, education, and employment status. The risk of SIDS is increased when there is exposure to ETS.

Haglund (1993) reviewed literature from 1959 to1992. At the time of his review there were only 43 articles where SIDS and smoking were the main topic. The OR for SIDS ranges between 1.5 and 5. The author discovered a dose–response relationship between different levels of smoking and the OR, sometimes as great as 2.7. Haglund concluded that "maternal smoking is one of the most important preventable risk factors for SIDS" (p. 38).

A cohort study by Haglund et al. (1995) determined the relative risk for SIDS based on smoking behavior and seasonality. This investigation supported their previous studies that linked smoking and an increase in SIDS cases. Of a possible 831,000 survivors of the neonatal period, the study included 813,000 infants born in Sweden between 1983 and 1990 who survived the first week of life. Also included were all SIDS cases from 1973 to 1990 when the time trend seasonal pattern was evaluated. Haglund et al. found the risk for SIDS in winter to be 3.5 times greater than the risk in summer. When taken together, maternal smoking and winter season showed the highest incidence rate of SIDS, and nonsmoking and summer the lowest incidence rate. Additionally, the effect of smoking is more pronounced for early (7–90 days) SIDS deaths (relative rate, 3.5), than for late (91–180 days) SIDS deaths (relative rate, 2.5). The authors acknowledge that maternal self-report may have affected the validity of the results and increasing antismoking publicity could also create bias. If this is true, the risk estimates would have been even higher than observed.

34.6.13.2 Active Smoking and Passive Exposure

Klonoff-Cohen et al. (1995) studied the relationship between SIDS and smoking during pregnancy, postnatal smoke exposure from either parent, other live-in adults, daycare providers, and exposure

from breast-feeding. In this case-control study, conducted in five counties in southern California, there were 200 families of various racial and ethnic groups with infants who died of SIDS. Investigators obtained the prenatal and postnatal smoking histories of 200 women and all members of their households who had apparently healthy infants die of SIDS. These were compared with the prenatal and postnatal smoking histories of 200 women and all members of their households delivering healthy infants who are living. The case-control selection process and details related to the interview process were not included in this article. The investigators examined the dose–response effect by looking at: (1) the total number of adults smoking; (2) the total number of adults smoking in the same room as the infant; and (3) the total number of cigarettes the infant was exposed to per day. Breast-feeding factors were recorded especially with regard to the number of cigarettes smoked by the mother while breast-feeding. This study is the first of its type in the United States to review passive smoking and passive smoking during breast-feeding. This study showed that infants who died of SIDS had more exposure to tobacco smoke. This effect was seen after controlling for variables such as prenatal exposure to tobacco smoke, parental age and educational status, maternal recreational drug use, prenatal care, medical conditions at birth, birthweight, infant's routine sleep position, and breast-feeding status. Although breast-feeding is usually protective against SIDS and other childhood illnesses, analysis of this study's data showed that infant exposure to maternal cigarette smoke negated this protective function.

Lewis and Bosque (1995) studied the infants of 13 pregnant smokers and 34 pregnant nonsmokers. Serum samples were analyzed for cotinine and the infants were assessed at two and three months of age for ventilatory and awakening responses to hypoxia. Their study demonstrated that cotinine levels were higher in maternal smokers and their infants had lower birthweights. Further, more infants of smokers had a deficient hypoxia awakening response, which may be a risk for SIDS.

Milerad et al. (1993) reviewed epidemiological data from a previous study in Sweden conducted by Norvenius. They compared birth records and circumstances of death for infants who died between 1975 and 1977 with the same information for infants who died between 1985 and 1987. The data were collected from autopsy records and matched to the birth certificates with attention being given to any risk factors associated with the increasing incidence of SIDS. The SIDS death rate for the years 1975 to 1977 was 0.51 per 1000 live births based on a total of 298,010 live births and 151 deaths from SIDS. The SIDS death rate for the years 1985 to 1987 was 0.93 per 1000 live births based on a total of 304,860 live births and 283 SIDS deaths. After controlling for preterm birth and LBW, maternal smoking was found to be one of the major risk factors. Elevated levels of nicotine metabolites have been found in the pericardial fluid of infants who died of SIDS, pointing to significant tobacco exposure just prior to death. Tobacco use in Sweden has increased among young women and the incidence of SIDS has increased twofold when comparing the 1970s data with the 1980s data. These investigators also confirmed the increased incidence in the winter and suggest the increase is a multifactorial problem related to exposure to tobacco smoke, unfavorable thermal environment, prone sleeping position, and increased respiratory infections. These authors recommend prospective case-control studies be conducted to determine the contribution of each of the risk factors to SIDS.

Milerad et al. (1994) analyzed the nicotine and cotinine levels in the pericardial fluid of 24 infants who died between the fall of 1988 and the spring of 1989. The pericardial fluid was obtained during autopsy. Sixteen of the cases fit the classic definition of SIDS: the unexpected death of an infant where the medical history gives no explanation and a postmortem examination fails to demonstrate an adequate cause of death. Eight victims had other contributing causes of death. Seven of the 16 SIDS cases and five non-SIDS cases had moderate to marked exposure to nicotine as evidenced by cotinine levels. Two SIDS and two non-SIDS cases had been heavily exposed. The study results showed that the level of cotinine in the pericardial fluid of almost half of the infants demonstrated moderate exposure to nicotine near the time of death. The pericardial fluid of one-third of the infants showed heavy exposure to nicotine at the time of death. Approximately 43% of the women who returned the questionnaire classified themselves as smokers or heavy smokers. The autopsies

showed that 71% of the infants had detectable cotinine levels. Milerad et al. concluded that ETS was present in most cases of sudden death, both SIDS and non-SIDS cases.

Schoendorf and Kiely (1992) performed a case-control analysis on the 1988 National Maternal and Infant Health Survey data. This database included 10,000 live births, 4000 fetal deaths, and 6000 infant deaths. The study included only infants born to African-American, non-Hispanic, or white non-Hispanic mothers. Retrospective data were collected for all infants by a survey questionnaire that included 200 questions related to pregnancy and infancy, parental and family characteristics, and maternal and infant health. Birth certificate information was available for all participants and death certificate information was available for infants in the death cohort. The final population consisted of 201 black infants and 234 white infants who died of SIDS, and a control group of approximately 3000 infants of each race. Analysis of the data showed that infants who died of SIDS had greater exposure to tobacco smoke than the control group infants. The data also suggested that there is an independent association between both prenatal exposure and postnatal exposure to tobacco smoke and an increased risk of SIDS. However, prenatal exposure to tobacco smoke has also been associated with LBW, which can lead to infant mortality. These authors also point out that self-report of smoking habit may be problematic, especially if the mother has had an infant die of SIDS.

Kinney et al. (1993) have shown, in baseline studies on the brains of aborted fetuses and infants who had died of SIDS, that there are brain stem changes in infants exposed to cigarette smoke *in utero* that may lead to SIDS. They analyzed brain stem sections from five fetuses with a median gestational age of 22 weeks and five infants with a median age of 47 weeks postconception. In this experimental design study they documented the marked (qualitative) decline in [^{3}H]nicotine binding in the developing brain stem, which may provide a neurochemical basis for SIDS, and neurological deficits in the infants of actively smoking mothers.

34.6.14 Oxidative Stress

Over 4000 components are found in the gas-phase and particulate-phase mixture of cigarette smoke including noxious chemicals such as reactive oxygen species (ROS), reactive nitrogen species (RNS), polycyclic hydrocarbons, heterocyclic nitrosamines, redox compounds, aldehydes, and acrolein (Pryor, 1997; Cross et al., 1999; Traber et al., 2000). Many of these chemicals have been implicated in cigarette-smoke-related diseases, atherosclerosis, and cancer, and likely involve facets of oxidative stress. Thus, there is an accumulation of studies targeting the interactions between cigarette smoke, oxidative stress, and the potential role that antioxidants perform in the modulation of such diseases. However, because the molecular mechanisms involved between cigarette smoke and disease are far from clear, the role played by antioxidant therapy in cigarette-smoke-induced diseases remains poorly understood. Coincidentally, ETS is also associated with atherosclerotic vascular disease (Glantz and Parmley, 1991; Steenland, 1992) and cancer (Wald, 1986). Passive smoking may be involved in atherogenesis by altering lipoproteins, increasing oxidized low-density lipoproteins, and decreasing plasma antioxidants. ETS, like cigarette smoke, may play a role in carcinogenesis because it contains a number of cocarcinogens, which have been found to increase in the blood and urine of ETS-exposed individuals.

ROS include oxygen radicals and other oxidants that do not contain unpaired electron species. The primary oxygen-derived free radical produced by biological systems is the superoxide anion (O_2^-) (Reddy and Omaye, 1987; Elsayed, 2001). Biological systems include activated phagocytes and the mitochondria of all cells (by-products of leaky aerobic respiration). Depending on concentration amounts and cellular location of O_2^- generation, oxidative damage may be non- or consequential resulting in cellular damage. RNS refers to the family of metabolites derived from NO•, such as nitrogen dioxide and peroxynitrite, which are potential mediators of cellular damage. Oxidative/nitrosative stress can have diverse effects such as the induction of defense systems, which attempt to protect cells from damage. Moreover, if prooxidant effects exceed defense effects, damage can occur to essential proteins, lipids, carbohydrates, and DNA/RNA bases leading to abnormal

increases in intracellular "free" calcium and perhaps induction of apoptosis or necrosis (Halliwell and Gutteridge, 1999; van der Vliet and Cross, 2000).

Antioxidant defense systems are broadly divided into exogenous and endogenous parallel subsystems (Reddy and Omaye, 1987; Halliwell and Gutteridge, 1999; Elsayed and Bendich, 2001). The endogenous system is composed of enzyme systems, specific synthesized compounds, and other compounds that exhibit antioxidant activity but usually serve other biological purposes. The glutathione (GSH) peroxidase system and the superoxide dismutase system are two examples of enzyme systems that play very important roles in protecting against oxidative stress or minimizing the oxidation damage in lung and other tissues. Endogenous antioxidants include melatonin, lipoic acid, coenzyme Q, vitamin E, and vitamin C. Endogenous compounds that act as antioxidants include bilirubin, uric acid, and many proteins, particularly those that contain reactive sulfur moieties. Exogenous antioxidants are those compounds derived from the diet or provided as therapeutic agents, *e.g.*, vitamin E, vitamin C, various iron-chelating compounds, and probucol (Halliwell and Gutteridge, 1999; Weisburger and Chung, 2002).

Like tobacco smoke, ETS can aggravate and likely initiate health problems associated with oxidative stress; however, the differences being quantitatively less pronounced (Faruque et al., 1995). Plasma vitamin C concentrations are found to be intermediate, between active smokers and nonsmokers not exposed to ETS (Tribble et al., 1993; Jacob, 2000; Alberg, 2002). In ETS-exposed individuals, serum lipid peroxidation, assessed by the thiobarbituric acid and conjugated dienes indices were increased and serum total antioxidant defense and vitamin C concentrations were decreased. In addition, the ability of low-density lipoproteins to resist *ex vivo* copper-initiated oxidation was reduced, along with an increased uptake of LDL cholesterol into isolated cultured macrophages (Valkonen and Kuusi,1998). However, ironically, ETS does not affect fat-soluble antioxidants, such as vitamin E and beta-carotene. Also, when healthy Japanese males were subjected to smoking, no differences in lipid peroxidation were found between active smokers, ETS-exposed, and nonsmokers, but the ratio of oxidized to reduced vitamin C in plasma was greater in both the active and ETS groups than in the nonsmokers (Ayaori et al., 2000). Such studies emphasize the crucial role for the water-soluble antioxidant, vitamin C, in the dynamics of protecting blood components from oxidative stress. Also, such findings are consistent with our recent suggestion that inadequate plasma vitamin C may compromise the ability of blood vitamin E to regenerate (recycle) back to the protective reduced form of vitamin E, thereby promoting more oxidative stress due to the prooxidant vitamin E radical (Nakamura and Omaye, unpublished results, 2003). Prior ingestion of vitamin C prevents smoke-induced decrease in plasma antioxidant defense, decrease in resistance of LDL to oxidation, and increase in plasma lipid peroxidation (Valkonen and Kuusi, 2000). Thus, antioxidants, particularly, aqueous soluble antioxidants like vitamin C, are crucial in the protection of tissue against oxidative damage.

34.6.15 Adult

Zhou et al. (2000) studied oxidative stress, cigarette smoking, smoking cessation, and the effects on plasma constituents and enzymes in 1255 smokers and 524 nonsmokers. Among the many parameters investigated were vitamin E, vitamin C, and lipoperoxides. As expected, in the smokers, lipoperoxide levels were increased and antioxidants were decreased. For the group of smokers who quit, after one year their values were not significantly different from the matched control group. The results of this study support the theory that smoking increases oxidative stress and that many months of cessation have to pass before plasma levels of lipoperoxides decrease and antioxidant status returns to normal.

34.6.16 Neonatal

Schwarz et al. (1997) investigated the effects of maternal smoking by studying 26 neonates of nonsmoking mothers and 19 neonates of smoking mothers. Their primary goal was to ascertain if formula was prooxidant compared with colostrum by measuring breath ethane. When the initial breath ethane

test was conducted on all the neonates, it was shown that infants of smokers had higher ethane levels than nonsmokers, thus demonstrating that smoking is prooxidant for the fetus.

One of the risks to the fetus of a smoking mother is prematurity. Robles et al. (2001) studied oxidative stress in 10 newborns delivered at term (group A) and 10 newborns who were premature (group B). In group A, they analyzed cord blood samples for α-tocopherol, Coenzyme Q10, and hydroperoxides, and venous samples at 3 and 72 h. In group B, they analyzed in the same manner and added a sample at 24 h and 7 d. Hydroperoxides were increased in both groups at birth; however, they were higher in premature neonates. In term newborns, levels of vitamin E increased significantly by 72 h; however, in premature newborns vitamin E remained two to three times lower. All neonates demonstrate an increase in oxidative stress and a decrease in antioxidants at birth; however, the increase in oxidative stress and the decrease in antioxidant protection are greater in premature infants. The study results did not identify if any of the mothers were smokers.

34.6.17 Thiobarbituric Acid (TBARS) and Lipid Peroxidation

One of the indicators of oxidative stress is an increase in lipid peroxidation above what occurs from normal metabolic processes and aging (Halliwell and Gutteridge, 1999; Jahn et al., 1997). Excessive oxidative stress with concomitant lipid peroxidation and a decrease in vitamin E status leads to several disease states including atherosclerosis. Thiobarbituric acid reacts with lipoperoxidation aldehydes, such as malondialdehyde. The TBARS assay is one of the most common methods used to assess lipid peroxidation. Unfortunately, although this is a simple and inexpensive assay, it does not have good specificity (Agarwal and Chase, 2002; Hong et al., 2000; Jardine et al., 2002; LaPenna et al., 2001).

Little and Gladen (1999) conducted a review of 48 studies to determine the levels of lipid peroxidation in a normal pregnancy. They cited 19 studies that found higher lipid peroxide levels in healthy pregnant women than in healthy, nonpregnant women. These higher levels begin in the second trimester and taper off during the third trimester. Studies were cited that suggest that problems during a pregnancy such as preeclampsia may increase lipid peroxidation even further. Uncontrolled oxidative stress may lead to maternal and fetal morbidity.

Mihailovic et al. (2000) compared blood samples and plasma samples from 20 healthy nonpregnant women and 115 pregnant women. Samples were taken during the second and third trimesters and within 2 d of delivery. The samples consisted of amniotic fluid and umbilical cord blood and were analyzed by glutathione peroxidase and malondialdehyde (MDA). The results of the study demonstrated an increase in MDA, probably related to lipid peroxidation, and a decrease in total antioxidant status.

34.6.18 Nutrition and Tobacco Exposure

In discussing oxidative stress, it is important to take into consideration the impact that tobacco use has on nutritional state. In general, it is assumed that a smoker has a less healthy nutritional pattern than a nonsmoker. Dallongeville et al. (1998) conducted a meta-analysis of 51 studies from 15 countries, involving 47,250 nonsmokers and 35,870 smokers. They assessed both the size of the sample and the nutrient intake of the participants. While smokers had greater energy intakes, the energy was in the form of total fat, saturated fat, cholesterol, and alcohol, with less intake of polyunsaturated fat. There was no difference in nutrient intake between the two groups for protein and carbohydrate. The conclusion of this analysis was that there are substantial differences between the nutrition of smokers and nonsmokers. For the smoker, these differences may lead to oxidative stress and an increased incidence of cancer and coronary heart disease.

34.6.18.1 Vitamin C

Vitamin C is also known as the water-soluble ascorbic acid. Humans cannot synthesize this vitamin, yet it must be present in the diet to prevent the disease scurvy. Vitamin C is an essential cofactor

for at least eight enzymes. According to Halliwell and Gutteridge (1999), it is unknown whether vitamin C has antioxidant properties *in vivo* (p. 203). However, *in vitro* studies have demonstrated the ability of vitamin C to act as a reducing agent, to scavenge free radicals such as the superoxide and hydroxyl radicals, O_3, and the nitrite ion, and to protect membranes and lipoproteins against peroxidation induced by the toxic compounds in cigarette smoke. Vitamin C also has an essential function in the recycling of the vitamin E radical to vitamin E. This system of recycling vitamin E is considered to be the most important function of vitamin C *in vivo.*

Zhang et al. (1999) studied 24 subjects with varying smoking habits. They hypothesized that the velocity of blood flow is reduced because of oxidative stress caused by smoking. They further hypothesized that this effect could be counteracted by vitamin C. They tested the nail-fold circulation prior to, and one and 30 minutes after, smoking. The results of this test demonstrated a dramatically reduced microcirculatory blood flow (by 40–50%) in 23 of 24 subjects. The same test was performed twice more with the same subjects. Prior to the second test, the subjects were given a 1-g dose of vitamin C with no effect. However, when a 2-g dose of vitamin C was given 2 h prior to smoking, the blood flow was decreased by only about 20–25%. The conclusion was that pretreatment with a single high dose of vitamin C can markedly reduce the microcirculatory effect caused by smoking.

Valkonen and Kuusi (2000) investigated vitamin C and the atherogenic effects of exposure to ETS. Blood samples were taken from 10 nonsmoking individuals (five women and five men) who were apparently healthy. All of the subjects lived and worked in smoke-free environments. After two days of making certain they were not exposed to any ETS, the participants were exposed to either normal air or ETS (to act as their own controls). On the first day, the subjects breathed normal air; on day 2 they breathed ETS for 30 min; on day 3 ETS for 30 min; and on day 4 normal air. On days 3 and 4, 2 h prior to exposure, the participants were given 3 g, orally, of vitamin C. Blood samples were drawn prior to and 1.5 h after the exposure periods. On day 2, when there was no vitamin C supplementation prior to ETS exposure, there was a significant decrease in vitamin C 1.5 h after the exposure, but no change in vitamin E. There was also a significant increase in TBARS. On day 3, when vitamin C supplementation was given, the decrease in vitamin C was more apparent; however, the formation of TBARS was significantly lower. One of the findings of the study is that 30 min of ETS exposure causes depletions in the antioxidant defense system and could lead to atherosclerosis.

34.6.18.2 Vitamin E

Vitamin E is also known as the fat-soluble vitamin E. The most important function of vitamin E is to inhibit lipid peroxidation. During this process, vitamin E is converted to the vitamin E radical. Although there are other potential outcomes for the vitamin E radical, such as conversion to vitamin E-quinone or other metabolites excreted in the urine, with sufficient vitamin C the vitamin E radical is regenerated to vitamin E.

34.6.18.3 Total Antioxidant Defense

Kim and Lee (2001) investigated the potential for antioxidant protection in individuals who smoked 20 or more cigarettes per day. They assessed vitamin E, beta-carotene, vitamin C, and red ginseng influence on lipid peroxidation as measured by plasma MDA concentrations. At baseline, smokers had significantly elevated levels of total cholesterol, triacylglycerols, and MDA. At baseline, smokers also had significantly lower levels of vitamin C, beta-carotene, and vitamin E. Study subjects were supplemented with antioxidants for 4 wk. When reevaluated, smokers had significantly higher levels of antioxidants, high-density lipoprotein, and lower MDA values. These authors concluded that smokers have inadequate antioxidant defense systems and supplementation may help to alleviate oxidative stress.

Wei et al. (2001) used NHANES III data to investigate the relationship between smoking and serum levels of antioxidants in 7873 subjects. Cotinine assay was used to determine actual smoking status. Their study confirmed that smokers have reduced dietary intakes of antioxidants and the effects of smoking further reduce their serum antioxidant levels.

34.6.19 Nutrition and Pregnancy

During pregnancy, it is necessary to augment almost all nutrients. It is especially important to increase folic acid (Pitkin, 1977) to prevent neural tube defects. Many, but not all, of the nutrients can be obtained in the diet provided the pregnant woman has sufficient intake. However, in the case of a smoker, the normal nutrition may not be adequate. Therefore, supplementation with folic acid and prenatal vitamins is essential.

34.6.19.1 Vitamin C

Barrett et al. (1991) analyzed serum and amniotic fluid samples from 34 pregnant women, 15 smokers, and 19 nonsmokers. The primary purpose of the study was to ascertain if vitamin C levels were lower in women experiencing premature rupture of the membranes (PROM) when compared with women without PROM. Although they did not find a relationship between PROM and lower vitamin C levels, these researchers did find lower vitamin C levels in smokers (41.9 μm/l) than in nonsmokers (64.7 μm/l).

Based on a study comparing levels of vitamin C and E in maternal and fetal plasma, amniotic fluid, and chorioamnion, Woods et al. (2002) concluded that the process of labor causes oxidative stress. They studied five healthy women undergoing a repeat cesarean section and five women having an uncomplicated vaginal delivery. The study did not specify whether all the women were nonsmokers. The nutrient intakes for vitamins were similar in both groups of women. In general, maternal and neonatal vitamin E levels were the same. However, in the women undergoing labor, vitamin C levels in all three measured fluids were lower. It was surmised that vitamin C is depleted during labor while it scavenges ROS and recycles the lipid-soluble vitamin E to stop ROS tissue damage. This is an important finding because women who smoke may not have sufficient vitamin C to adequately withstand the stress of labor.

On the other hand, there can also be a prooxidant effect of vitamin C. Many pregnant women are given iron supplementation in combination with vitamin C, which can promote the generation of free radicals. Lachili et al. (2001) studied the effect of this type of supplementation in 27 third-trimester pregnant women. The matched control group also numbered 27 third-trimester pregnant women who did not receive supplementation. They analyzed plasma levels of TBARS, antioxidants, including vitamin E, and iron. As expected, in the supplemented group, iron levels were higher. However, TBARS were also elevated and vitamin E was lower. Therefore, iron and elevated vitamin C intake can cause oxidative stress during the pregnancy for the mother and the fetus.

34.6.19.2 Vitamin E

Bolisetty et al. (2002) collected maternal and cord blood samples on the day of delivery and again on day 4 from 14 smokers and 18 nonsmokers. The purpose of their investigation was to assess the influence of smoking on plasma antioxidant vitamins, including vitamin E. Unfortunately, they did not biologically confirm smoking status with a cotinine assay. There was no difference in MDA levels; however, the analysis did demonstrate a significant difference in vitamin E concentration between the two groups.

34.6.19.3 Total Antioxidant Defense

Steuerer et al. (1999) investigated birthweight, antioxidant vitamin concentrations, and the effects of maternal smoking. Over a four-year period, 222 pregnant women participated in the study. There were 58 active smokers, 107 ETS exposed, and 57 nonsmokers not exposed. Their study established a significant correlation between maternal and umbilical cotinine levels. Babies born to smoking mothers who were less than the 25th percentile had significantly lower vitamin E levels. Because smoking causes oxidative stress and affects placental perfusion, this was suggested as a possible reason for the birthweight reduction in children of smoking mothers.

34.7 BIOMARKERS FOR TOBACCO EXPOSURE

As previously stated, epidemiological studies have documented that maternal cigarette smoking is associated with an increased incidence of infant morbidity and mortality (Ahlsten et al., 1993; Lahdetie et al., 1993; Reid 1991; Whincup et al., 1994; Wise et al., 1995). One problem in conducting research designed to assess fetal exposure to tobacco smoke is the inaccuracy of self-reported exposures from mothers (Apseloff et al., 1994; Emmons et al., 1994; Etzel et al., 1985; Giovino et al., 1995; Kintz et al., 1993; Koren 1995; Perez-Stable et al., 1995). Self-reporting is not considered reliable for assessing either smokers' or nonsmokers' levels of exposure (Apseloff et al., 1994; Emmons et al., 1994; Nafstad et al., 1996; Perez-Stable et al., 1995; Windsor, 1998).

34.7.1 Nicotine

It is estimated that for each cigarette smoked, the smoker receives about 1 mg of nicotine (Benowitz, 1992). There has been some research documenting biological markers for this exposure in both the mother and the neonate (Eliopoulos et al., 1994; Etzel et al., 1985; Jordanov, 1990; Kintz et al., 1992b, 1993; Klein et al., 1994; Koren, 1995; Luck and Nau, 1985; Luck et al., 1985; Nafstad et al., 1995b; Oryszczyn et al., 1991; Ostrea et al., 1994). Attempts have been made to measure serum levels of nicotine; however, the half-life of nicotine in humans is quite short, ranging from 1 to 3 h (Etzel et al., 1985; Rama Sastry and Janson, 1995; Schulte-Hobein et al., 1992).

34.7.2 Cotinine

Cotinine is the major metabolite of nicotine and is only found *in vivo* in individuals who have been, actively or passively, exposed to tobacco smoke (Ahlsten et al., 1993; Ammenheuser et al., 1994; Benowitz and Jacob, 1994; Deutsch et al., 1992; Eskenazi and Bergmann, 1995; Kintz, 1992; Koren, 1995; Li and Gorrod, 1994; Manchester et al., 1992; Takeda et al., 1993; Urakawa et al., 1994). The half-life of cotinine is approximately 19–39 h, making it a more useful biomarker than nicotine (Apseloff et al., 1994; Bardy et al., 1993; Rama Sastry and Janson, 1995). There is a dose–response relationship between serum cotinine and the level of active exposure to tobacco smoke (Rosa et al., 1992). Therefore, active smokers demonstrate a positive correlation between their serum cotinine level and the number of cigarettes smoked per day (Benowitz, 1992; Kintz, 1992; Perez-Stable et al., 1995; Rosa et al., 1992). When conducting clinical trials, because of deception or underestimation, it is considered important to measure cotinine levels to identify smokers and to biologically confirm active and passive exposure (Apseloff et al., 1994; Nafstad et al., 1996; Roussel et al., 1991).

Mathews et al. (1999) questioned whether cotinine levels were of value in predicting adverse birth outcomes in nulliparous women. Subjects were recruited and randomly selected from antenatal clinics. Cotinine levels were analyzed for 845 women at 16 wks gestation and compared with self-reported smoking status. Both measures of smoking status were used to predict premature delivery and SGA neonates. The results of this study confirm that 15–35% of pregnant women have cotinine levels inconsistent with their reported smoking behavior. Therefore, it is more accurate to use cotinine levels to determine actual smoking status, especially if one is interested in predicting a poor pregnancy outcome.

34.7.3 Biomarker Compartments

Nicotine and cotinine have been found in many bodily fluids and tissues in both animal and human studies. Electron-impact and chemical ionization were used in 1992 to study both nicotine and cotinine in rat plasma and brain (Deutsch et al., 1992). Rat hair studies were conducted in 1995 to elucidate differences in uptake of nicotine and cotinine based on pigmentation, using capillary gas chromatography (Gerstenberg et al., 1995).

In humans, studies have included investigating the concentrations of nicotine and its metabolite, cotinine, in saliva and urine (Jordanov, 1990; Luck and Nau, 1985; Takeda et al., 1995); serum and

plasma (English et al., 1994; Eskenazi and Bergmann, 1995; Eskenazi and Trupin, 1995; Luck and Nau, 1985; Rosa et al., 1992); the fetus and placenta (Luck et al., 1985); amniotic fluid (Jordanov, 1990; Luck et al., 1985; Ruhle et al., 1995); meconium and cord blood (Nafstad et al., 1995b; Oryszczyn et al., 1991); breast milk (Luck and Nau, 1985; Schulte-Hobein et al., 1992); and hair (Gwent et al., 1995; Kintz, 1992; Kintz et al., 1992a; Koren, 1995; Uematsu, 1993). Nicotine and cotinine studies were conducted on liver, kidney, spleen, lung, skeletal, and adipose tissue, from 10 human autopsies, using capillary gas chromatography/mass spectrometry (GC/MS) (Urakawa et al., 1994). Concentrations of nicotine and cotinine in hair have been studied for several years in forensic science and toxicology (Kintz, 1992; Kintz et al., 1992a).

34.7.4 Fetal and Neonatal Biomarker Research

The clinical significance of cotinine in researching the adverse effects of fetal exposure to cigarette smoke has come to the forefront during the last five years with nicotine and cotinine being studied in women and their newborns (Eliopoulos et al., 1994; English et al., 1994; Jordanov, 1990; Koren, 1995; Luck and Nau, 1985; Nafstad et al., 1995b; Oryszczyn et al., 1991; Ruhle et al., 1995; Schulte-Hobein et al., 1992). For the most part, these studies were done using maternal and neonatal urine, serum, and hair. It is sometimes difficult to obtain neonatal urine samples; therefore, a good alternative is to use the discarded cord blood to obtain a serum sample. When studying infant cotinine levels, it is better to use maternal serum or plasma to compare results with matched infant cord blood. Cotinine in hair gives the most definitive results, but it is not always possible to obtain a sufficient sample as some infants have very little hair at birth. Also, about 10% of mothers who participated in these studies preferred not to have their baby's hair cut (Eliopoulos et al., 1994).

34.7.5 Biomarkers and Prenatal ETS Exposure

Although there is considerable research outlining the relationships among active smoking, cotinine levels, and adverse birth outcomes, until recently there had been little published research on the associations among the following factors: prenatal maternal exposure to ETS, cotinine levels, and adverse birth outcomes (CA EPA, 1997b, 1997c; Emmons et al., 1994; Ey et al., 1995; Jordanov, 1990; Luck and Nau, 1985; Mainous and Hueston, 1994; Ostrea et al., 1994; Strategic Coalition of Girls & Women United Against Tobacco, 1995a; Witschi et al., 1995, 1997). As with active smoking, it is also difficult to gauge ETS exposure levels through self-report of nonsmokers (Apseloff et al., 1994; Eskenazi and Bergmann, 1995; Eskenazi and Trupin, 1995; Nafstad et al., 1995a). It has been documented that cotinine crosses the placental barrier and can be measured in fetal blood (Ammenheuser et al., 1994; Etzel et al., 1985; Kintz et al., 1992b, 1993; Koren, 1995; Lahdetie et al., 1993; Manchester et al., 1992; Milart et al., 1994; Nafstad et al., 1995b; Olsen, 1992; Rama Sastry and Janson, 1995). Therefore, the neonate of a mother who has been exposed to ETS may have detectable cotinine in the umbilical cord blood.

34.7.6 Bioaccumulation of Cotinine in Hair

Unfortunately, because of its half-life, cotinine is a suitable biomarker only for tobacco exposure that occurred within the last 19 to 40 h. When measured at delivery, cotinine assays will not detect tobacco exposures that occurred greater than two days prior. Cotinine testing may also miss intermittent ETS exposure in nonsmoking mothers. One method of overcoming these shortcomings is to measure cotinine levels in the hair of the maternal-infant dyad.

During the last decade, the use of hair has emerged for estimation of long-term exposure to drugs or chemicals (Eliopoulos et al., 1994, 1996; Gerstenberg et al., 1995; Ishiyama et al., 1983; Kintz, 1992; Kintz et al., 1992a, 1992b, 1993; Klein et al., 1994; Koren, 1995; Uematsu, 1993). According to Eliopoulos et al. (1996), bioaccumulation of cotinine in hair reflects long-term systemic exposure

to cigarette smoke. Evaluation of cotinine in hair does not present the same half-life problem as evaluating cotinine in other body fluids and tissues, e.g., serum, blood, and plasma. Cotinine is taken up in the hair shaft and remains firmly sequestered (Kintz et al., 1993). Many infants begin hair growth during the seventh month of gestation and it is estimated that each centimeter of growth represents one month of exposure (Kintz, 1992; Uematsu, 1993). Analysis of hair samples may detect not just the presence and level of exposure but also time-dependent changes in exposure to xenobiotic agents. These factors may be the key to determining prenatal and perinatal risk.

34.7.7 Laboratory Methods for Biomarker Detection

There are other chemicals from tobacco smoke that can be investigated in both the mother and the neonate, such as carbon monoxide and thiocyanate. However, the methods for detecting and the detection limits for cotinine have proven to be the most accurate and cost-effective. Minimum detection limits (MDL) of 5 ng/ml are considered good and detection limits below that are considered excellent (Bernert et al., 2000).

Several methods have been utilized to effectively detect both nicotine and cotinine. Cotinine is generated by nicotine after metabolism in the liver and, on average, 70% of nicotine is metabolized by C-oxidation to cotinine. There is individual variation in how much nicotine is actually metabolized to cotinine, with a range of 55–92%, and there are persons who convert less nicotine to cotinine. A dual-isotope method to study the metabolism of nicotine to cotinine in serum samples has provided information on the conversion of nicotine to cotinine, and the clearance of cotinine from the body (Benowitz and Jacob, 1994). The MDL was approximately 24.5 ng/ml and a positive correlation was established in the Benowitz and Jacob study for the clearance of both nicotine and cotinine. This is significant when looking at individual differences in the clearance of the toxic nicotine.

In a study using saliva, GC/MS was used to measure cotinine concentrations (Emmons et al., 1994). The MDL was <0.5 ng/ml. To obtain a saliva sample, wax is chewed and the saliva spit into a container. While this is a cost-effective method for detecting nicotine and cotinine, it is not possible to use saliva testing in the study of infants since infants cannot chew and spit.

Radioimmunoassay (RIA) was used to determine cotinine levels in a study investigating Black-White differences in serum cotinine levels among pregnant women and the birthweight of their infants. The MDL was 2 ng/ml (English et al., 1994). Van Vunakis et al. (1993) used RIA to determine nicotine and cotinine levels in various biological tissues such as urine, serum, saliva, and cerebrospinal fluid. The MDL was approximately 0.5 ng/ml for cotinine in serum or plasma and 10–15 ng/ml for cotinine in saliva or urine. No levels of detection were given for nicotine.

Solid-phase extraction, with high-performance liquid chromatography (HPLC) and HPLC-particle beam mass spectrometry, has been used to detect nicotine and cotinine concentrations in the serum of cigarette smokers. Nonsmokers with no exposure to ETS for one week were used as controls. The mean concentration of cotinine was 133.5 ± 75.0 ng/ml of serum (Pacifici et al., 1993). Pichini et al. (1992), and Zuccaro et al. (1993) used HPLC with ultraviolet (UV) photometric detection for quantification of cotinine and trans-3′-hydroxycotinine in human serum. These investigators studied smokers' daily intake and showed a correlation between nicotine intake and the serum levels of the two metabolites. Rop et al. (1993) used HPLC to determine nicotine and several of its metabolites in the urine of smokers and nonsmokers. They recommend that all biomarkers, not only nicotine and cotinine, be assessed when trying to determine the exposure of smokers and nonsmokers.

Cotinine has been measured in amniotic fluid by direct colorimetric method with an MDL of 0.65 μmol/l (Jordanov, 1990). This method involves an invasive procedure to obtain the amniotic fluid. For this reason, and also for economic considerations, this test would not ordinarily be done unless the pregnant woman was having amniocentesis for a medically necessary purpose.

Luck and Nau (1985) studied urine and serum samples from infants and breast milk and serum samples from smoking mothers. Nicotine and cotinine measurements in milk and urine samples were done by GC with a nitrogen-selective detector (PN-detector). The MDL for nicotine was 0.2 ng/ml and for cotinine was 5 ng/ml.

Nafstad et al. (1995b) studied thiocyanate and cotinine in maternal serum and infant cord serum. Thiocyanate was measured by colorimetric method and cotinine by double antibody RIA. The MDL for thiocyanate was 5 μmol/l and for cotinine 5 ng/ml.

Oryszczyn et al. (1991) studied cord blood immunoglobulin E (IgE) and cotinine to compare infants of smokers and nonsmokers. Cotinine was measured by competitive RIA. The MDL in cord blood was 0.5 ng/ml.

Hair concentrations of nicotine and cotinine have been measured using RIA with an MDL of 0.25 ng/ml for nicotine and 0.1 ng/ml for cotinine (Eliopoulos et al., 1994, 1996). Kintz (1992) used GC/MS to analyze for nicotine and cotinine to differentiate between smokers and nonsmokers. An MDL of 0.005 ng/mg for nicotine and 0.01 ng/mg for cotinine was achieved. A value of 2 ng/mg of hair was determined to distinguish between smokers and nonsmokers. In a related study, Kintz et al. (1992a) used GC/MS to analyze nicotine and cotinine in human hair. In the hair of nonsmokers, the MDL for nicotine was 0.06 to 1.82 ng/mg, and 0.01 to 0.13 ng/mg for cotinine. There was a significant difference in the MDL of the two analytes in the hair of smokers. Nicotine ranged from 0.91 to 33.89 and cotinine ranged from 0.09 to 4.99.

A method called isotope dilution-liquid chromatography-tandem mass spectrometry has been developed for the detection of cotinine in serum. This method has a reported MDL as low as 0.030 ng/ml (Bernert et al., 1997; CDC, 1993b). All of the analytical methods and instrumentation are very costly. A simple cotinine analysis by GC can cost $37/test, and hair analysis for cotinine starts at $123/test. Although this type of testing is usually done in research facilities, some insurance companies are now testing employees of private companies to make certain they are nonsmokers and/or do not use illegal drugs.

34.8 HEALTH ISSUES AND NATIONAL OBJECTIVES

In light of the health-related issues discussed above, the federal government has established health objectives for the nation. These have been established for each decade since 1990. The year 2000 objectives were only partially met. The current objectives for 2010 that relate to tobacco and pregnancy issues include:

- Reduce cigarette smoking from 24 to 12% among people aged 18 and older.
- Reduce tobacco use by adolescents from 40 to 21%.
- Reduce the initiation of tobacco use among children and adolescents.
- Increase from 41 to 75% the smoking cessation attempts by adult smokers.
- Increase from 14 to 40% smoking cessation during pregnancy.
- Increase tobacco cessation attempts from 76 to 84% among adolescents.
- Reduce from 27 to 10% the proportion of children who are regularly exposed to tobacco smoke at home.
- Reduce from 65 to 45% the proportion of nonsmokers exposed to ETS.

These objectives are of great importance in addressing some of the causative factors related to poor pregnancy outcomes in the United States. Achieving these objectives will reduce the human and economic cost of tobacco use.

34.9 TOBACCO AND ECONOMIC IMPACT

34.9.1 National Morbidity

The costs of tobacco-related morbidity to society are about $100 billion each year in lost productivity and $50 billion in direct medical costs (Bartecchi et al., 1994; CDC, 2002a; MacKenzie et al., 1994). Miller et al. (1999) used an econometric statistical model to determine the "smoking attributable fraction" (p. 1) for individual annual U.S. medical care costs attributable to smoking. They specifically

investigated four types of medical services: hospital, ambulatory, prescription drug, and other (including home healthcare and durable medical equipment). The major conclusion of this economic study is that cigarette use "... affects medical expenditures to a significant and substantial degree" (p. 390). This study validates studies by Bartecchi et al. (1994) and MacKenzie et al. (1994) that suggested smoking costs society over $50 billion per year in direct medical costs. These costs are not exclusively borne by the smokers. Individuals who smoke may be receiving services through Medicare and Medicaid. Therefore, the cost is spread over most of the taxpaying public, 75% of whom do not smoke.

Persons who smoke, and their dependents, have more healthcare office visits than do nonsmokers. On average, smokers visit healthcare practitioners six times more per year than nonsmokers, and dependents of smokers average four visits more per year than those of nonsmokers (DiFranza, 1996; Lesmes and Donofrio, 1992; MacKenzie et al., 1994). Smokers may have higher premiums for health insurance (Wallace, 2002). Unfortunately, this cost is spread over the group being insured because premiums are based on the number of persons in the group (Lesmes, 1992; St. Mary's HealthFirst Insurance Underwriter, personal communication, March 26, 1997).

Smokers may also pay higher premiums for term life insurance because they die prematurely at twice the rate of nonsmokers (MacKenzie et al., 1994). Insurance companies may give as much as a 15% discount on policies written for nonsmoking drivers and 10% for nonsmoking homeowners (Action on Smoking and Health, 1998; Finance Centers, 2002; Horowitz, 2002; Western Insurance Information Service, 2002; WorldNow and KRQE, 2002).

Smokers may also have lost workdays, which cost their employers in terms of decreased productivity. The U.S. Office of Technology Assessment (as cited in MacKenzie et al., 1994) estimates that lost productivity related to disability and premature death from smoking cost $47 billion annually (1990 dollars) for smokers and $8.6 billion for passive smokers. These statistics represent: (1) loss of workdays, as cigarette smokers, on average, lose 6.5 days more per year than nonsmokers (CDC, 2000a; MacKenzie et al., 1994); (2) premature morbidity and mortality (an average 11.5 years of potential life lost [YPLL] for an approximate total of 5 million YPLL); and (3) adverse financial effects for the families of smokers.

The latest CDC (2002a) report on annual smoking-attributable mortality, YPLL, and economic costs for the years 1995 to 1999, confirm the number of premature deaths to be about 440,000. CDC estimates a staggering $157 billion loss in annual health-related costs. For men and women who smoked, the YPLL was 13.2 and 14.5, respectively. In reality, these figures may be underestimations because smoking may not be given as the actual cause of death.

34.9.2 National Mortality

The economic impact related to victims of smoking-related fires comes from the National Fire Protection Association (as cited in MacKenzie et al., 1994). Cigarette smoking caused 187,000 fires in the United States in 1991, which cost approximately $552 million in direct property damage. This figure does not include the medical care costs or funeral costs for victims of the fires.

These morbidity and mortality statistics and costs to society do not include the worldwide statistics. Nor do they include the fetuses, neonates, and infants who die of a cause related to a pregnant woman's active smoking (CDC, 1990, 1991, 1993a; DiFranza and Lew, 1995) or a pregnant woman's passive exposure to mainstream and sidestream smoke, which together comprise what is known as ETS (Byrd, 1992; CA EPA, 1997a, 1997d; CDC, 1993b; Chen and Petitti, 1995).

34.10 SUMMARY

For many years it was believed that the uterine environment provided total protection to the developing fetus. Three decades of research has shown that this environment is, in fact, impacted by a number of external variables. Many of these factors are under the mother's direct control, whereas some are the result of her passive interaction with environmental toxins. Much of the literature

suggesting a correlation between maternal smoking and birth outcomes is based on epidemiological association. This literature review examined the more global perspective of the environment of tobacco use. It then focused on various aspects of basic science and epidemiological research to provide scientific information on fetal tobacco exposure, nicotine, the cotinine biomarker, and adverse birth outcomes.

This chapter points to the importance of identifying successful efforts to reduce or eliminate smoking during pregnancy. Some of the biomarker information can be used to form the basis for additional research exploring the relationship between various levels of ETS exposure and birth outcomes.

Much of the literature on adverse birth outcomes is related to LBW and SGA babies. More research is needed, examining *in utero* tobacco exposure and its effect on head circumference, birth height, and Apgar scores.

Research also shows that maternal self-reporting is not a reliable indicator of actual smoking behavior, nor of passive exposure to ETS. Cotinine is a useful biomarker for determining this exposure, and although this assay represents an expense, it may be of great practical value for determining effectiveness of clinic-based tobacco cessation / reduction interventions. The purpose of this review was to report and critique literature related to tobacco smoke exposure *in utero,* biomarkers for this exposure, and adverse pregnancy and birth outcomes.

REFERENCES

Agarwal, R. and Chase, S.D., Rapid, fluorimetric-liquid chromatographic determination of malondialdehyde in biological samples, *J Chromatogr B*, 775, 121–126, 2002.

Ahlsten, G., Cnattingius, S., and Lindmark, G., Cessation of smoking during pregnancy improves foetal growth and reduces infant morbidity in the neonatal period. A population-based prospective study, *Acta Paediatr.*, 82, 177–181, 1993.

Ahluwalia, I.B., Grummer-Strawn, L., and Scanlon, K.S., Exposure to environmental tobacco smoke and birth outcome: increased effects on pregnant women aged 30 years or older, *Am. J. Epidemiol.*, 146, 42–47, 1997.

Alberg, A.J., The influence of cigarette smoking on circulating concentrations of antioxidant micronutrients, *Toxicology*, 180, 121–137, 2002.

Alderman, B.W., Bradley, C.M., Greene, C., Fernbach, S.K., and Baron, A.E., Increased risk of craniosynostosis with maternal cigarette smoking during pregnancy, *Teratology*, 50,13–18, 1994.

Altman, D.G., Foster, V., Rasenick-Douss, L., and Tye, J.B., Reducing the illegal sale of cigarettes to minors, *JAMA*, 261, 80–83, 1989.

Altman, D.G., Rasenick-Douss, L., Foster, V., and Tye, J.B., Sustained effects of an educational program to reduce sales of cigarettes to minors, *Am. J. Public Health*, 81, 891–893, 1991.

Ammenheuser, M.M., Berenson, A.B., Stiglich, N.J., Whorton, E.B., and Ward, J.B., Elevated frequencies of hprt mutant lymphocytes in cigarette-smoking mothers and their newborns, *Mutat. Res.*, 304, 285–294, 1994.

Andres, R.L. and Day, M.C., Perinatal complications associated with maternal tobacco use, *Semin. Neonatol.*, 5, 231–241, 2000.

Apseloff, G., Ashton, H.M., Friedman, H., and Gerber, N., The importance of measuring cotinine levels to identify smokers in clinical trials, *Clin. Pharmacol. Ther.*, 56, 460–461, 1994.

ASH (Action on Smoking and Health), Double discounts for non-smokers, 1998. Available at http://www.no-smoking.org/oct98/10–0l-98–1.html.

Ayaori, M., Hisada, T., Suzukawa, M., Yoshida, H., Nishiwaki, M., Ito, T., Nakajima, K., Higashi, K., Yonemura, A., Ishikawa, T., Ohsuzu, F., Nakamura, H., Plasma levels and redox status of ascorbic acid and levels of lipid peroxidation products in active and passive smokers, *Environ. Health Perspect.*, 108, 105–108, 2000.

Banspach, S.W., Lefebvre, R.C., and Carleton, R.A., Increasing awareness of the pressures related to smoking: An analysis of two models of anti-smoking curriculum in the classroom, *Health Educ. Res.*, 4, 69–78, 1989.

Bardy, A.H., Seppala, T., Lillsunde, P., Kataja, J.M., Koskela, P., Pikkarinen, J., and Hiilesmaa, V.K., Objectively measured tobacco exposure during pregnancy: Neonatal effects and relation to maternal smoking, *Br. J. Obstet. Gynaecol.*, 100, 721–726, 1993.

Barrett, B., Gunter, E., Jenkins, J., and Wang, M., Ascorbic acid concentration in amniotic fluid in late pregnancy, *Biol. Neonate*, 60, 333–335, 1991.

Bartecchi, C.E., MacKenzie, T.D., and Schrier, R.W., The human costs of tobacco use, Part 1, *N. Engl. J. Med.*, 330, 907–912, 1994.

Beeram, M.R., Abedin, M., Shoroye, A., Jayam-Trouth, A., Young, M., and Reid, Y., Occurrence of craniosynostosis in neonates exposed to cocaine and tobacco in utero, *J. Natl. Med. Assoc.*, 85, 865–868, 1993.

Benowitz, N.L., Cigarette smoking and nicotine addiction, *Med. Clin. North Am.*, 76, 415–437, 1992.

Benowitz, N.L. and Jacob, P., Metabolism of nicotine to cotinine studied by a dual stable isotope method, *Clin. Pharmacol. Ther.*, 56, 484–493, 1994.

Bernert, J.T., Jr., McGuffey, J.E., Morrison, M.A., and Pirkle, J.L., Comparison of serum and salivary cotinine measurements by a sensitive high-performance liquid chromatography-tandem mass spectrometry method as an indicator of exposure to tobacco smoke among smokers and nonsmokers, *J. Anal. Toxicol.*, 24, 333–339, 2000.

Bernert, J.T., Jr., Turner, W.E., Pirkle, J.L., Sosnoff, C.S., Akins, J.R., Waldrep, M.K., Ann, Q., Covey, T.R., Whitfield, W.E., Gunter, E.W., Miller, B.B., Patterson, D.G., Jr., Needham, L.L., Hannon, W.H., Sampson, E.J., Development and validation of sensitive method for determination of serum cotinine in smokers and nonsmokers by liquid chromatography/atmospheric pressure ionization tandem mass spectrometry, *Clin. Chem.*, 43, 2281–2291, 1997.

Bolisetty, S., Naidoo, D., Lui, K., Koh, T.H., Watson, D., Montgomery, R., and Whitehall, J., Postnatal changes in maternal and neonatal plasma antioxidant vitamins and the influence of smoking, *Arch. Dis. Child. Fetal Neonatal. Ed.*, 86, F36–F40, 2002.

Bush, P.G., Mayhew, T.M., Abramovich, D.R., Aggett, P.J., Burke, M.D., and Page, K.R., Maternal cigarette smoking and oxygen diffusion across the placenta, *Placenta*, 21, 824–833, 2000.

Byrd, J.C., Environmental tobacco smoke. Medical and legal issues, *Med. Clin. North Am.*, 76, 377–398, 1992.

California Environmental Protection Agency, Executive Summary, Health Effects of Exposure to Environmental Tobacco Smoke: Final Report., Office of Environmental Health Hazard Assessment, 1997a, pp. ES-5–6.

California Environmental Protection Agency, Exposure Measurement and Prevalence, Health Effects of Exposure to Environmental Tobacco Smoke: Final Report, Office of Environmental Health Hazard Assessment, 1997b, pp. 2-1–2-74.

California Environmental Protection Agency, Exposure measurement and prevalence, Health Effects of Exposure to Environmental Tobacco Smoke: Final Report, Office of Environmental Health Hazard Assessment, 1997c, pp. 2-1–2-74.

Centers for Disease Control, Reducing the Health Consequences of Smoking, 25 Years of Progress: A Report of the Surgeon General, DHHS Publication (CDC) 89–8411. U.S. Dept. of Health & Human Services, Public Health Service, Rockville, MD, 1989.

Centers for Disease Control, Effects of maternal cigarette smoking on birthweight & preterm birth—Ohio—1989, *Morb. Mortal. Wkly. Rep.*, 39, 662–665, 1990.

Centers for Disease Control, Cigarette smoking among reproductive-aged women-Behavioral Risk Factor Surveillance System-1989, *Morb. Mortal. Wkly. Rep.*, 40, 719–723, 1991.

Centers for Disease Control and Prevention. Year 2000: National Health Objectives, 1992. Appendix M, in *Community Health Nursing: Process and Practice for Promoting Health,* 3rd ed., Stanhope, M. and Lancaster, J., Eds., Mosby Year Book, St. Louis,. pp. 918–928.

Centers for Disease Control and Prevention, Special focus: surveillance for reproductive health, *Morb. Mortal. Wkly. Rep.*, 42 (SS-6), 1–85, 1993a.

Centers for Disease Control and Prevention, Preliminary data: Exposure of persons aged greater than 4 years to tobacco smoke—United States, 1988–1991. *Morb. Mortal. Wkly. Rep.*, 42, 37–39, 1993b.

Centers for Disease Control and Prevention, Preventing tobacco use among young people: a report of the Surgeon General (Executive Summary), *Morb. Mortal. Wkly. Rep.*, 43 (RR-4), 1–10, 1994.

Centers for Disease Control and Prevention, Indicators of nicotine addiction among women—United States, 1991–1992, *Morb. Mortal. Wkly. Rep.*, 44, 102–105, 1995.

Centers for Disease Control and Prevention, Tobacco use, in *Healthy People 2010: National Healthy People 2010 Objectives*, 27, 3–36, 2000.

Centers for Disease Control and Prevention, Annual smoking-attributable mortality, years of potential life lost, and economic costs—United States, 1995–1999, *Morb. Mortal. Wkly. Rep.*, 51, 300–303, 2002a.

Centers for Disease Control and Prevention, Births: Final data for 2001, *National Vital Statistics Reports*, 51, 1–104, 2002b.

Centers for Disease Control and Prevention, Cigarette smoking among adults—United States, 2000, *Morb. Mortal. Wkly. Rep.*, 51, 642–645, 2002c.

Centers for Disease Control and Prevention, Prevalence data—2000, *Behavioral Risk Factor Surveillance System*, 2002d. Available at http://www.cdc.gov/brfss.

Chen, L.H. and Petitti, D.B., Case-control study of passive smoking and the risk of small-for-gestational age at term, *Am. J. Epidemiol.*, 142,158–165, 1995.

Coghlin, J., Gann, P.H., Hammond, S.K., Skipper, P.L., Taghizadeh, K., Paul, M., and Tannenbaum, S.R., 4-Aminobiphenyl hemoglobin adducts in fetuses exposed to the tobacco smoke carcinogen *in utero, J. Natl. Cancer Inst.*, 83, 274–280, 1991.

Coppens, M., Vindla, S., James, D.K., and Sahota, D.S., Computerized analysis of acute and chronic changes in fetal heart rate variation and fetal activity in association with maternal smoking, *Am. J. Obstet. Gynecol.*, 185, 421–426, 2001.

Council on Scientific Affairs, American Medical Association, Environmental tobacco smoke. Health effects and prevention policies, *Arch. Fam. Med.*, 3, 865–871, 1994.

Cross, C.E., Traber, M., Eiserich, J., and van der Vliet, A., Micronutrient antioxidant and smoking, *Br. Med. Bull.*, 55, 691–704, 1999.

Dallongeville, J., Marecaux, N., Fruchart, J.C., and Amouyel, P., Cigarette smoking is associated with unhealthy patterns of nutrient intake: a meta-analysis, *J. Nutr.*, 128, 1450–1457, 1998.

Deutsch, J., Hegedus, L., Greig, N.H., Rapoport, S.L., and Soncrant, T.T., Electron-impact and chemical ionization detection of nicotine and cotinine by gas chromatography-mass spectrometry in rat plasma and brain, *J. Chromatogr.*, 579, 93–98, 1992.

DiFranza, J.R., Preventing teenage tobacco addiction, *J. Fam. Pract.*, 34, 753–756, 1992.

DiFranza, J.R., How many children are harmed by tobacco smoke: Morbidity & mortality in children associated with the use of tobacco products by other people, *Pediatrics*, 97, 560–568, 1996.

DiFranza, J.R. and Lew, R.A., Effect of maternal cigarette smoking on pregnancy complications and sudden infant death syndrome, *J. Fam. Pract.*, 40, 385–394, 1995.

Eaton, D.L. and Klaassen, C.D., *Casarett and Doull's Toxicology: The Basic Science of Poisons,* 5th ed., McGraw-Hill Health Professions Division, New York, 1996. pp. 13–33.

Elder, J.P. and Stern, R.A., The ABCs of adolescent smoking prevention: an environment and skills model, *Health Educ. Q.*, 13, 181–191, 1986.

Eliopoulos, C., Klein, J., and Koren, G., Validation of self-reported smoking by analysis of hair for nicotine and cotinine, *Ther. Drug Monit.*, 18, 532–536, 1996.

Eliopoulos, C., Klein, J., Phan, M.K., Knie, B., Greenwald, M., Chitayat, D., and Koren, G., Hair concentrations of nicotine and cotinine in women and their newborn infants, *JAMA*, 271, 621–623, 1994.

Elsayed, N.M., Antioxidant mobilization in response to oxidative stress: a dynamic environmental-nutritional interaction, *Nutrition*, 17, 828–834, 2001.

Elsayed, N.M. and Bendich, A., Dietary antioxidants: potential effects on oxidative products in cigarette smoke, *Nutr. Res.*, 21, 551–567, 2001.

Emmons, K.M., Abrams, D.B., Marshal, R., Marcus, B.H., Kane, M., Novotny, T.E., and Etzel, R.A., An evaluation of the relationship between self-report and biochemical measures of environmental tobacco smoke exposure, *Prev. Med.*, 23, 35–39, 1994.

England, L.J., Kendrick, J.S., Wilson, H.G., Merritt, R.K., Gargiullo, P.M., and Zahniser, S.C., Effects of smoking reduction during pregnancy on the birth weight of term infants, *Am. J. Epidemiol.*, 154, 694–701, 2001.

English, P.B., Eskenazi, B., and Christianson, R.E., Black-White differences in serum cotinine levels among pregnant women and subsequent effects on infant birthweight, *Am. J. Public Health*, 84, 1439–1443, 1994.

Environmental Protection Agency, Respiratory Health Effects of Passive Smoking: Lung Cancer and Other Disorders, EPA/600/6–90/006F, 1993.

Eskenazi, B. and Bergmann, J.J., Passive and active maternal smoking during pregnancy, as measured by serum cotinine, and postnatal smoke exposure. I. Effects on physical growth at age 5 years, *Am. J. Epidemiol.*, 142, S10–S18, 1995.

Eskenazi, B. and Trupin, L.S., Passive and active maternal smoking during pregnancy, as measured by serum cotinine, and postnatal smoke exposure. II. Effects on neurodevelopment at age 5 years, *Am. J. Epidemiol.*, 142, S19–S29, 1995.

Etzel, R.A., Greenberg, R.A., Haley, N.J., and Loda, F.A., Urine cotinine excretion in neonates exposed to tobacco smoke products *in utero, J. Pediatr.*, 107, 146–148, 1985.

Ey, J.L., Holberg, C.J., Aldous, M.B., Wright, A.L., Martinez, F.D, and Taussig, L.M., Passive smoke exposure and otitis media in the first year of life, *Pediatrics*, 95, 670–677, 1995.

Faruque, M.D.O., Khan, M.R., Rahman, M.D.M., and Ahmed, F., Relationship between smoking and antioxidant nutrient status, *Br. J. Nutr.*, 73, 625–632, 1995.

Filippini, G., Farinotti, M., Lovicu, G., Maisonneuve, P., and Boyle, P., Mothers' active and passive smoking during pregnancy and risk of brain tumours in children, *Int. J. Cancer*, 57, 769–774, 1994.

Finance Centers, Homeowner's insurance: reducing premiums, 2002. Available at http://partners.financenter.com/abcnews/learn/guides/homeinsure/hinsprem.fcs.

Fortmann, S.P. and Killen, J.D., Who shall quit? Comparison of volunteer and population-based recruitment in two minimal-contact smoking cessation studies, *Am. J. Epidemiol.*, 140, 39–51, 1994.

Fushimi, H., Inoue, T., Yamada, Y., Matsuyama, Y., and Kameyama, M., Profound vasoconstrictive effect of cigarette smoking in diabetics with autonomic neuropathy, *Diabetes Res. Clin. Pract.*, 16, 191–195, 1992.

Gerstenberg, B., Schepers, G., Voncken, P., and Voelkel, H., Nicotine and cotinine accumulation in pigmented and unpigmented rat hair, *Drug Metab. Dispos.*, 23, 143–148, 1995.

Giovino, G.A., Henningfield, J.E., Tomar, S.L., Escobedo, L.G., and Slade, J., Epidemiology of tobacco use and dependence, *Epidemiol. Rev.*, 17, 48–65, 1995.

Glantz, S.A. and Parmley, W.W., Passive smoking and heart disease, *Circulation*, 83, 1–12, 1991.

Glantz, S.A. and Parmley, W.W., Passive smoking and heart disease: mechanisms and risk, *JAMA*, 273, 1047–1053, 1995.

Goldenberg, R.L., The prevention of low birthweight and its sequelae, *Prev. Med.*, 23, 627–631, 1994.

Gwent, S.H., Wilson, J.F., Tsanaclis, L.M., and Wicks, J.F., Time course of appearance of cotinine in human beard hair after a single dose of nicotine, *Ther. Drug Monit.*, 17, 195–198, 1995.

Haak, T., Jungmann, E., Raab, C., and Usadel, K.H., Elevated endothelin-1 levels after cigarette smoking, *Metabolism*, 43, 267–269, 1994.

Haddow, J.E., Knight,G.J., Kloza, E.M., Palomaki, G.E., and Wald, N.J., Cotinine-assisted intervention in pregnancy to reduce smoking and low birthweight delivery, *Br. J. Obstet. Gynaecol.*, 98, 859–865, 1991.

Haglund, B., Cigarette smoking and sudden infant death syndrome: some salient points in the debate, *Acta Paediatr.*, 82 (Suppl.), 37–39, 1993.

Haglund, B., Cnattingius, S., and Otterblad-Olausson, P., Sudden infant death syndrome in Sweden, 1983–1990: Season at death, age at death, and maternal smoking, *Am. J. Epidemiol.*, 142, 619–624, 1995.

Halliwell, B. and Gutteridge, J.M.C., *Free Radicals in Biology and Medicine*. 3rd ed., Oxford University Press, Oxford, 1999, pp. 247–264, 291–306, 393–400, 407–410, 422–425, 584–590.

Hanrahan, J.P., Tager, I.B., Segal, M.R., Tosteson, T.D., Castile, R.G., Van Vunakis, H., Weiss, S.T., and Speizer, F.E., The effect of maternal smoking during pregnancy on early infant lung function, *Am. Rev. Respir. Dis.*, 145, 1129–1135, 1992.

Hong, Y.L., Yeh, S.L., Chang, C.Y., and Hu, M.L., Total plasma malondialdehyde levels in 16 Taiwanese college students determined by various thiobarbituric acid tests and an improved high-performance liquid chromatography-based method, *Clin. Biochem.*, 33, 619–625, 2000.

Hoppock, K.C. and Houston, T.P., Availability of tobacco products to minors, *J. Fam. Pract.*, 30, 174–176, 1990.

Horowitz, D., What insurance can mean to you. Save on car insurance, 2002. Available at http://www.debtfree-benefits.com/ACCESS/Wealth/Article46.17.html.

Horta, B.L., Victora, C.G., Menezes, A.M., Halpern, R., and Barros, F.C., Low birthweight, preterm births and intrauterine growth retardation in relation to maternal smoking, *Paediatr. Perinat. Epidemiol.*, 11, 140–151, 1997.

Ishiyama, I., Nagai, T., and Toshida, S., Detection of basic drugs (methamphetamine, antidepressants, and nicotine) from human hair, *J. Forensic Sci.*, 28, 380–385, 1983.

Jacob, R.A., Passive smoking induces oxidant damage preventable by vitamin C, *Nutr. Rev.*, 58, 239–241, 2000.

Jahn, K., Peiker, G., and Winnefeld, K., Behavior of thiobarbituric acid reactive substances, alpha-tocopherol, glutathione and selenium during hypertension in pregnancy, *Med. Klin.*, 92 (Suppl. 3), 38–40, 1997.

Jardine, D., Antolovich, M., Prenzler, P.D., and Robards, K., Liquid chromatography-mass spectrometry (LC-MS) investigation of the thiobarbituric acid reaction substances (TBARS) reaction, *J. Agric. Food Chem.*, 50, 1720–1724, 2002.

Jordanov, J., Cotinine concentrations in amniotic fluid and urine of smoking, passive smoking and nonsmoking pregnant women at term and in the urine of their neonates on 1st day of life, *Eur. J. Pediatr.*, 149, 734–737, 1990.

Joschko, M.A., Dreosti, E.I., and Tulsi, R.S., The teratogenic effects of nicotine in vitro in rats, *Neurotoxicol. Teratol.*, 13, 307–316, 1991.

Kahn, R.S., Certain, L., and Whitaker, R.C., A reexamination of smoking before, during, and after pregnancy, *Am. J. Public Health*, 92, 1801–1808, 2002.

Kaufman, F.L., Kharrazi, M., DeLorenze, G.N., Eskenazi, B., and Bernert, J.T., Estimation of environmental tobacco smoke exposure during pregnancy using a single question on household smokers versus serum cotinine, *J. Expo. Anal. Environ. Epidemiol.*, 12, 286–295, 2002.

Kendrick, J.S., Zahniser, S.C., Miller, N., Salas, N., Stine, J., Gargiullo, P.M., Floyd, R.L., Spierto, F.W., Sexton, M., and Metzger, R.W., Integrating smoking cessation into routine public prenatal care: the Smoking Cessation in Pregnancy project, *Am. J. Public Health*, 85, 217–222, 1995.

Kim, H.S. and Lee, B.M., Protective effects of antioxidant supplementation on plasma lipid peroxidation in smokers, *J. Toxicol. Environ. Health A*, 63, 583–598, 2001.

Kinney, H., O'Donnell, T., Kriger, P., and White, W.F., Early developmental changes in [^{3}H]nicotine binding in the human brainstem, *Neuroscience*, 55, 1127–1138, 1993.

Kintz, P., Gas chromatographic analysis of nicotine and cotinine in hair, *J. Chromatogr.*, 580, 347–353, 1992.

Kintz, P., Kieffer, I., Messer, J., and Mangin, P., Nicotine analysis in neonates' hair for measuring gestational exposure to tobacco, *J. Forensic Sci.*, 38, 119–123, 1993.

Kintz, P., Ludes, B., and Mangin, P., Evaluation of nicotine and cotinine in human hair, *J. Forensic Sci.*, 37, 72–76, 1992a.

Kintz, P., Tracqui, A., and Mangin, P., Tobacco, drug and narcotic abuse during pregnancy. Evaluation of *in utero* exposure by analysis of hair of the neonate, *Presse Med.*, 21, 2139–2141, 1992b.

Klein, J., Forman, R., Eliopoulos, C., and Koren, G., A method for simultaneous measurement of cocaine and nicotine in neonatal hair, *Ther. Drug Monit.*, 16, 67–70, 1994.

Kline, J., Stein, Z.A., Susser, M., and Warburton, D., Smoking: a risk factor for spontaneous abortion, *N. Engl. J. Med.*, 297, 793–796, 1977.

Klonoff-Cohen, H., Edelstein, S.L., Lefkowitz, E.S., Srinivasan, I.P., Kaegi, D., Chang, J.C., and Wiley, K.J., The effect of passive smoking and tobacco exposure through breast milk on sudden infant death syndrome, *JAMA*, 273, 795–798,1995.

Koren, G., Measurement of drugs in neonatal hair; a window to fetal exposure, *Forensic Sci. Int.*, 70, 77–82, 1995.

Kritz, H., Schmid, P., and Sinzinger, H., Passive smoking and cardiovascular risk, *Arch. Intern. Med.*, 155, 1942–1948, 1995.

Lachili, B., Hininger, I., Faure, H., Arnaud, J., Richard, M.J., Favier, A., and Roussel, A.M., Increased lipid peroxidation in pregnant women after iron and vitamin C supplementation, *Biol. Trace Elem. Res.*, 83, 103–110, 2001.

Lackmann, G.M., Salzberger, U., Tollner, U., Chen, M., Carmella, S.G., and Hecht, S.S., Metabolites of a tobacco-specific carcinogen in urine from newborns, *J. Natl. Cancer Inst.* 91, 459–465, 1999.

Lahdetie, J., Engström, K., Husgafvel-Pursiainen, K., Nylund, L., Vainio, H., and Sorsa, M., Maternal smoking induced cotinine levels and genotoxicity in second trimester amniotic fluid, *Mutat. Res.*, 300, 37–43, 1993.

LaPenna, D., Ciofani, G., Pierdomenico, S.D., Giamberardino, M.A., and Cuccurullo, F., Reaction conditions affecting the relationship between thiobarbituric acid reactivity and lipid peroxides in human plasma, *Free Radic. Biol. Med.*, 31, 331–335, 2001.

Lesmes, G.R., Corporate healthcare costs and smoke-free environments, *Am. J. Med.*, 93 (Suppl 1A), 48S–54S, 1992.

Lesmes, G.R. and Donofrio, K.H., Passive smoking; the medical and economic issues, *Am. J. Med.*, 93 (Suppl 1A), 38S–42S, 1992.

Lewis, K.W. and Bosque, E.M., Deficient hypoxia awakening response in infants of smoking mothers: Possible relationship to sudden infant death syndrome, *J. Pediatr.*, 127, 691–699, 1995.

Li, D.-K., Mueller, B.A., Hickok, D.E., Daling, J.R., Fantel, A.G., Checkoway, H., and Weiss, N.S., Maternal smoking during pregnancy and the risk of congenital urinary tract anomalies, *Am. J. Public Health*, 86, 249–253, 1996.

Li, Y. and Gorrod, J.W., N-hydroxymethylnorcotinine, a new primary in vitro metabolite of cotinine, *Xenobiotica*, 24, 409–415, 1994.

Lieberman, E., Gremy, I., Lang, J.M., and Cohen, A.P., Low birthweight at term and the timing of fetal exposure to maternal smoking, *Am. J. Public Health*, 84, 1127–1131, 1994.

Little, R.E. and Gladen, B.C., Levels of lipid peroxides in uncomplicated pregnancy: a review of the literature, *Reprod. Toxicol.*, 13, 347–352, 1999.

Luck, W. and Nau, H., Nicotine and cotinine concentrations in serum and urine of infants exposed via passive smoking or milk from smoking mothers, *J. Pediatr.*, 107, 816–820, 1985.

Luck, W., Nau, H., Hansen, R., and Steldinger, R., Extent of nicotine and cotinine transfer to the human fetus, placenta and amniotic fluid of smoking mothers, *Dev. Pharmacol. Ther.*, 8, 384–395, 1985.

MacKenzie, T.D., Bartecchi, C.E., and Schrier, R.W., The human costs of tobacco use, Part 2, *N. Engl. J. Med.*, 330, 975–980, 1994.

Mainous, A.G. and Hueston, W.J., Passive smoke and low birth weight. Evidence of a threshold effect, *Arch. Fam. Med.*, 3, 875–878, 1994.

Manchester, D.K., Bowman, E.D., Parker, N.B., Caporaso, N.E., and Weston, A., Determinants of polycyclic aromatic hydrocarbon-DNA adducts in human placenta, *Cancer Res.*, 52, 1499–1503, 1992.

Mannino, D.M., Caraballo, R., Benowitz, N., and Repace, J., Predictors of cotinine levels in US children: data from the Third National Health and Nutrition Examination Survey, *Chest*, 120, 718–724, 2001.

Martinez, F.D., Wright, A.L., Taussig, L.M., Group Health Medical Association, The effect of paternal smoking on the birthweight of newborns whose mothers did not smoke, *Am. J. Public Health*, 84, 1489–1491, 1994.

Mathews, F., Smith, R., Yukdin, P., and Neil, A., Are cotinine assays of value in predicting adverse pregnancy outcome? *Ann. Clin. Biochem.*, 36, 468–476, 1999.

Matt, G.E., Wahlgren, D.R., Hovell, M.F., Zakarian, J.M., Bernert, J.T., Meltzer, S.B., Pirkle, J.L., and Caudill, S., Measuring environmental tobacco smoke exposure in infants and young children through urine cotinine and memory-based parental reports: empirical findings and discussion, *Tob. Control*, 8, 282–289, 1999.

McDonald, S.D., Perkins, S.L., Jodouin, C.A., and Walker, M.C., Folate levels in pregnant women who smoke: an important gene/environment interaction, *Am. J. Obstet. Gynecol.*, 187, 620–625, 2002.

McGinnis, J.M. and Foege, W.H., Actual causes of death in the United States, *JAMA*, 270, 2207–2212, 1993.

Mihailovic, M., Cvetkovic, M., Ljubic, A., Kosanovic, M., Nedeljkovic. S., Jovanovic. I., and Pesut. O., Selenium and malondialdehyde content and glutathione peroxidase activity in maternal and umbilical cord blood and amniotic fluid, *Biol. Trace Elem. Res.*, 73, 47–54, 2000.

Milart, P., Kauffels, W., and Schneider, J., Vasoactive effects of nicotine in human umbilical arteries, *Zentralbl. Gynakol.*, 116, 217–219, 1994.

Milerad, J., Norvenius, G., and Wennergren, G., SIDS outdoors and seasonality in Sweden 1975–1987, *Acta Paediatr.*, 82, 1039–1042, 1993.

Milerad, J., Rajs, J., and Gidlund, E., Nicotine and cotinine levels in pericardial fluid in victims of SIDS, *Acta Paediatr.*, 83, 59–62, 1994.

Miller, V.P., Ernst, C., and Collin, F., Smoking-attributable medical care costs in the USA, *Soc. Sci. Med.*, 48, 375–391, 1999.

Milner, A.D., Marsh, M.J., Ingram, D.M., Fox, G.F., Susiva, C., Effects of smoking in pregnancy on neonatal lung function, *Arch. Dis. Child. Fetal Neonatal Ed.*, 80, F8–F14, 1999.

Miyao, M., Furuta, M., Matsushita, Y., Ogiso, M., Ishihara, S., and Teo, P.C., A matched-pair longitudinal study on the relationship between maternal smoking and head circumference of newborns., *Tohoku J. Exp. Med.*, 175, 135–137, 1995.

Nadakavukaren, A., Environmental disease: environmental carcinogens: smoking, in *Our Global Environment: A Health Perspective*, 5th ed., Waveland Press Inc., Prospect Heights, IL, 2000a, pp. 210–211, 214.

Nadakavukaren, A., Public outcast #1, in *Our Global Environment: A Health Perspective*, 5th ed., Waveland Press Inc., Prospect Heights, IL, 2000b, pp. 218–220.

Nadakavukaren, A., Synergism 1 + 1 = 5, in *Our Global Environment: A Health Perspective*, 5th ed., Waveland Press Inc., Prospect Heights, IL, 2000c, pp. 216.

Nafstad, P., Botten, G., Hagen, J.A., Zahlsen, K., Nilsen, O.G., Silsand, T., and Kongerud, J., Comparison of three methods for estimating environmental tobacco smoke exposure among children aged between 12 and 36 months, *Int. J. Epidemiol.*, 42, 88–94, 1995.

Nafstad, P., Kongerud, J., Botten, G., Urdal, P., Silsand, T., Pedersen, B.S., and Jaakkola, J.J.K., Fetal exposure to tobacco smoke products: A comparison between self-reported maternal smoking and concentrations of cotinine and thiocyanate in cord serum, *Acta Obstet. Gynecol. Scand.*, 75, 902–907, 1996.

Nafstad, P., Magnus, P., and Stray-Pedersen, B., Opposing placental gradients for thiocyanate and cotinine at birth, *Early Hum. Dev.*, 42, 73–79, 1995b.

Nakamura, Y.K. and Omaye, S.T., Serum lipoprotein oxidation in young and old rats, unpublished results, 2003.
Nelson, E., Goubet-Wiemers, C., Guo, Y., Jodscheit, K., Maternal passive smoking during pregnancy and foetal developmental toxicity. Part 2: histological changes, *Hum. Exp. Toxicol.*, 18, 257–264, 1999a.
Nelson, E., Jodshceit, K., and Guo, Y., Maternal passive smoking during pregnancy and fetal developmental toxicity. Part 1: gross morphological effects, *Hum. Exp. Toxicol.*, 18, 252–256, 1999b.
Office on Smoking and Health/National Center for Chronic Disease Prevention and Health Promotion, *Tobacco Use in Nevada*, Centers for Disease Control and Prevention, Washington, DC, 1995.
Olsen, J., Cigarette smoking in pregnancy and fetal growth: does the type of tobacco play a role, *Int. J. Epidemiol.*, 21, 279–284, 1992.
Ong, C.N., Lee, B.L., Shi, C.Y., Ong, H.Y., and Lee, H.P., Elevated levels of benzene-related compounds in the urine of cigarette smokers, *Int. J. Cancer*, 59, 177–180, 1994.
Oryszczyn., M.P., Godin, J., Annesi, I., Hellier, G., and Kauffmann, F., In utero exposure to parental smoking, cotinine measurements, and cord blood IgE*, J. Allergy Clin. Immunol.*, 87, 1169–1174, 1991.
Ostrea, E.M., Knapp, D.K., Romero, A., Montes, M., and Ostrea, A.R., Meconium analysis to assess fetal exposure to nicotine by active and passive maternal smoking*, J. Pediatr.*, 124, 471–476, 1994.
Pacifici, R., Altieri, I., Gandini, L., Lenzi, A., Passa, A.R., Pichini, S., Rosa, M., Zuccaro, P., and Dondero, F., Environmental tobacco smoke: Nicotine and cotinine concentration in sperm, *Environ. Res.*, 68, 68–72, 1995.
Pacifici, R., Pichini, S., Altieri, I., Rosa, M., Bacosi, A., Caronna, A., and Zuccaro, P., Determination of nicotine and two major metabolites in serum by solid-phase extraction and high-performance liquid chromatography, and high-performance liquid chromatography-particle beam mass spectrometry*, J. Chromatogr.*, 612, 209–213, 1993.
Peacock, J.L., Bland, J.M., and Anderson, H.R., Preterm delivery: Effects of socioeconomic factors, psychological stress, smoking, alcohol, and caffeine, *BMJ*, 26, 531–535, 1995.
Perez-Stable, E.J., Benowitz, N.L., and Marin, G., Is serum cotinine a better measure of cigarette smoking than self-report? *Prev. Med.*, 24, 171–179, 1995.
Pichini, S., Altieri, I., Pacifici, R., Rosa, M., Ottaviani, G., and Zuccaro, P., Simultaneous determination of cotinine and trans-3'-hydroxycotinine in human serum by high-performance liquid chromatography, *J. Chromatogr.*, 577, 350–361, 1992.
Pirkle, J.L., Flegal, K.M., Bernert, J.T., Brody, D.J., Etzel, R.A., and Maurer, K.R., Exposure of the US population to environmental tobacco smoke: the third national health and nutrition examination survey, 1988 to 1991, *JAMA*, 275, 1233–1240, 1996.
Pitkin, R.M., Nutritional influences during pregnancy, *Med. Clin. North Am.*, 61, 3–15, 1977.
Pryor, W., Cigarette smoke radicals and the role of free radicals in chemical carcinogenicity*, Environ. Health Perspect.*, 95, 875–882, 1997.
Radecki, T.E. and Zdunich, C.D., Tobacco sales to minors in 97 US and Canadian communities, *Tob. Control*, 2*,* 300–305, 1993.
Rama Sastry, B.V. and Janson, V.E., Smoking, placental function, and fetal growth, in *Placental Toxicology*, Rama Sastry, B.V., Ed., CRC Press, Boca Raton, FL, 1995. pp. 45–81.
Rebagliato, M., Florey, Cd.V., and Bolumar, F., Exposure to environmental tobacco smoke in nonsmoking pregnant women in relation to birth weight, *Am. J. Epidemiol.*, 142, 531–537, 1995.
Reddy, A.K. and Omaye, S.T., Target organ toxicity: Metabolic and biochemical responses following lung exposure, in *Inhalation Toxicology*, Salem, H., Ed., Marcel Dekker, New York, 1987. pp. 223–253.
Reiben, F.W., Smoking behaviour and increase in nicotine and carboxymhaemoglobin in venous blood, *Clin. Investig.*, 70, 335–342, 1992.
Reid, G., Sudden infant death syndrome (SIDS) and disordered blood flow, *Med. Hypotheses*, 36, 295–299, 1991.
Robles, R., Palomino, N., and Robles, A., Oxidative stress in the neonate, *Early Hum. Dev.*, 65 (Suppl), S75–S81, 2001.
Rop, P.P., Grimaldi, F., Oddoze, C., and Viala, A., Determination of nicotine and its main metabolites in urine by high-performance liquid chromatography, *J. Chromatogr.*, 612, 302–309, 1993.
Roquer, J.M., Figueras, J., Botet, F., and Jiminez, R., Influence on fetal growth of exposure to tobacco smoke during pregnancy, *Acta Paediatr.*, 84, 118–121, 1995.
Rosa, M., Pacifici, R., Altieri, I., Pichini, S., Ottaviani, G., and Zuccaro, P., How the steady-state cotinine concentration in cigarette smokers is directly related to nicotine intake, *Clin. Pharmacol. Ther.*, 52, 321–329, 1992.

Ross, J.A., Swensen, A.R., and Murphy, S.E., Prevalence of cigarette smoking in pregnant women participating in the special supplemental nutrition programme for Women, Infants and Children (WIC) in Minneapolis and Saint Paul, Minnesota, USA, *Paediatr. Perinat. Epidemiol.*, 16, 246–248, 2002.

Roussel, G., Le-Quang, N.T., Migueres, M.L., Roche, D., Mongin-Charpin, D., Chretien, J., and Ekindjian, O.G., An interpretation of urinary cotinine values in smokers and nonsmokers, *Rev. Malad. Respir.*, 8, 225–232, 1991.

Ruhle, W., Graf von Ballestrem, C.L., Pult, H.M., and Gnirs, J., Correlation of cotinine level in amniotic fluid, umbilical artery blood and maternal blood, *Geburtsh. Frauenheilkd.*, 55, 156–159, 1995.

Schoendorf, K.C. and Kiely, J.L., Relationship of sudden infant death syndrome to maternal smoking during and after pregnancy, *Pediatrics*, 90, 905–908, 1992.

Schuller, H.M., Jorquera, R., Reichert, A., and Castonguay, A., Transplacental induction of pancreas tumors in hamsters by ethanol and the tobacco-specific nitrosamine, *Cancer Res.*, 53, 1498–1501, 1993.

Schulte-Hobein, B., Schwartz-Bickenbach, D., Abt, S., Plum, C., and Nau, H., Cigarette smoke exposure and development of infants throughout the first year of life: Influence of passive smoking and nursing on cotinine levels in breast milk and infant's urine, *Acta Paediatr.*, 81, 550–557, 1992.

Schwarz, K.B., Cox, J.M., Sharma, S., Clement, L., Witter, F., Abbey, H., Sehnert, S.S., Risby, T.H., Prooxidant effects of maternal smoking and formula in newborn infants, *J. Pediatr. Gastroenterol. Nutr.*, 24, 68–74, 1997.

Selby, P., Hackman, R., Kapur, B., Klein, J., and Koren, G., Heavily smoking women who cannot quit in pregnancy: evidence of pharmacokinetic predisposition, *Ther. Drug Monit.*, 23, 189–191, 2001.

Simpson, W.J., A preliminary report on cigarette smoking and the incidence of prematurity, *Am. J. Obstet. Gynecol.*, 73, 808–815, 1957.

Sims, J., Tobacco. The leading cause of death,. *J. Fla. Med. Assoc.*, 81, 818–821, 1994.

Spinillo, A., Capuzzo, E., Colonna, L., Solerte, L., Nicola, S., and Guaschino, S. Factors associated with abruptio placentae in preterm deliveries, *Acta Obstet. Gynecol. Scand.*, 73, 307–312, 1994a.

Spinillo, A., Capuzzo, E., Nicola, S.E., Colonna, L., Egbe, T.O., and Zara, C., Factors potentiating the smoking-related risk of fetal growth retardation, *BJOG*, 101, 954–958, 1994b.

Spinillo, A., Capuzzo, E., Nicola, S., Colonna, L., Iasci, A., and Zara, C., Interaction between fetal gender and risk factors for fetal growth retardation, *Am. J. Obstet. Gynecol.*, 171, 1273–1277, 1994c.

Spinillo, A., Ometto, A. Bottino, R. Piazzi, G., Iasci, A., and Rondini, G., Antenatal risk factors for germinal matrix hemorrhage and intraventricular hemorrhage in preterm infants, *Eur. J. Obstet. Gynecol. Reprod. Biol.*, 61, 13–19, 1995a.

Spinillo, A., Ometto, A., Stronati, M., Piazzi, G., Iasci, A., and Rondini, G., Epidemiologic association between maternal smoking during pregnancy and intracranial hemorrhage in preterm infants, *J. Pediat.*, 127, 472–478, 1995b.

Steenland, K., Passive smoking and the risk of heart disease, *JAMA*, 267, 94–99, 1992.

Sterling, T.D., Rosenbaum, W.L., and Weinkam, J.J., Concentration of indoor particulate matter as a determinant of respiratory health in children, *Am. J. Epidemiol.*, 141, 581–582, 1995.

Steuerer, A., Rosenbaum, P., Heller, W.D., Scherer, G., Sennewald, E., Funk, B., and Schmidt, W., Influence of maternal smoking behaviour and antioxidant vitamin concentrations on the birth weight of new born infants, *Z. Geburtsh. Neonatol.*, 203, 110–114, 1999.

Stolerman, I.P. and Jarvis, M.J., The scientific case that nicotine is addictive, *Psychopharmacology (Berl.)*, 117, 2–10, 1995.

Strategic Coalition of Girls & Women United Against Tobacco, Passive smoking linked with SIDS, *The Newsletter of the Strategic Coalition of Girls & Women United Against Tobacco*, May 3, 1995a.

Strategic Coalition of Girls & Women United Against Tobacco, Tobacco kills half of long-term smokers, *The Newsletter of the Strategic Coalition of Girls & Women United Against Tobacco*, May 5, 1995b.

Surgeon General, The Health Consequences of Smoking: Nicotine Addiction. A Report of the Surgeon General, DHHS (CDC) Publication 88-8406, U.S. Department of Health and Human Services, Public Health Service, U.S. Government Printing Office, Rockville, MD, 1988.

Surgeon General, Pattern of Tobacco Use Among Women and Girls—Fact Sheet, *Women and Smoking: A Report of the Surgeon General-2001*, U.S. Government Printing Office, Rockville, MD, 2002, pp. 1–5.

Tager, I.B., Hanrahan, J.P., Tosteson, T.D., Castile, R.G., Brown, R.W., Weiss, S.T., and Speizer, F.E., Lung function, pre- and post-natal smoke exposure, and wheezing in the first year of life, *Am. Rev. Respir. Dis.*, 147, 811–817, 1993.

Tager, I.B., Ngo, L., and Hanrahan, J.P., Maternal smoking during pregnancy: Effects of lung function during the first 18 months of life, *Am. J. Respir. Crit. Care Med.*, 152, 977–983, 1995.

Takeda, N., Jitsunari, F., Asakawa, F., Suna, S., Manabe, Y., Fukunaga, I., and Gotoh, A., Simultaneous determination of cotinine and creatinine by high performance liquid chromatography, *Sangyo Igaku*, 35, 198–199, 1993.

Takeda, N., Jitsunari, F., Asakawa, F., Suna, S., Manabe, Y., and Goda, K., A study on urine cotinine for the evaluation of smoking cessation, *Nippon Eiseigaku Zasshi*, 50, 637–651, 1995.

Tappin, D.M., Ford, R.P.K., and Schluter, P.J., Smoking during pregnancy measured by population cotinine testing, *N. Z. Med. J.*, 110, 311–314, 1997.

Timbrell, J.A., *Principles of Biochemical Toxicology*, 2nd ed., Taylor & Francis, London, 1994. p. 1.

Traber, M.G., van der Vliet, A., Reznick, A.Z., and Cross, C.E., Tobacco-related diseases: Is there a role for antioxidant supplementation? *Clin. Chest Med.*, 21, 173–187, 2000.

Tribble, D.L., Giuliano, L.J., and Fortmann, S.P., Reduced plasma ascorbic acid concentration in nonsmokers regularly exposed to environmental tobacco smoke, *Am. J. Clin. Nutr.*, 58, 886–890, 1993.

Uematsu, T., Utilization of hair analysis for therapeutic drug monitoring with a special reference to ofloxacin and to nicotine, *Forensic Sci. Int.*, 63, 261–268, 1993.

Urakawa, N., Nagata, T., Kudo, K., Kimura, K., and Imamura, T., Simultaneous determination of nicotine and cotinine in various human tissues using capillary gas chromatography/mass spectrometry, *Int. J. Legal Med.*, 106, 232–236, 1994.

U.S. Census Bureau, DP-1. Profile of General Demographic Characteristics: 2000, *Data Set: Census 2000 Summary File 1 (SF) 100-Percent Data*, 2002. Available at http://factfinder.census.gov.

Valkonen, M. and Kuusi, T., Passive smoking induces atherogenic changes in low-density lipoprotein, *Circulation*, 97, 3012–3016, 1998.

Valkonen, M.M. and Kuusi, T., Vitamin C prevents the acute atherogenic effects of passive smoking, *Free Radic. Biol. Med.*, 28, 428–436, 2000.

van der Vliet, A. and Cross, C.E., Oxidants, nitrosants and the lung, *Am. J. Med.,* 109, 398–421, 2000.

Van Vunakis, H., Gijka, H.B., and Langone, H.B., Radioimmunoassay for nicotine and cotinine, *IARC Sci. Publ.*, 109, 293–299, 1993.

Wald, N.J., Does breathing other people's tobacco smoke cause lung cancer? *Br. Med. J.*, 293, 1503–1504, 1986.

Wallace, J., WV state workers who smoke will pay more for insurance, *Daily Mail*, May 10, 2002, p. 1.

Wei, W., Kim, Y., and Boudreau, N., Association of smoking with serum and dietary levels of antioxidants in adults: NHANES III, 1988–1994, *Am. J. Public Health*, 91, 258–264 2001.

Weinberger, S.E. and Weiss, S.T., Pulmonary diseases: Smoking and pregnancy, in *Medical Complications During Pregnancy*, 2nd ed., Burrow, G.N. and Ferris, T.F., Eds., W.B. Saunders Company, Philadelphia, 1982. pp. 405–434.

Weisburger, J.H. and Chung, F-L., Mechanisms of chronic disease causation by nutritional factors and tobacco products and their prevention by tea polyphenols, *Food Chem. Toxicol.*, 40, 1145–1154, 2002.

Western Insurance Information Service, Car insurance—lowering your premiums, *Woman Motorist*, 2002. Available at http://www.womanmotorist.com/ftrs/carinsprem 1.shtml.

Whincup, P., Cook, D., Papacosta, O., Walker, M., and Perry, I., Maternal factors and development of cardiovascular risk: Evidence from a study of blood pressure in children, *J. Hum. Hypertens.*, 8, 337–343, 1994.

Windham, G.C., Swan, S.H., and Fenster, L., Parental cigarette smoking and the risk of spontaneous abortion, *Am. J. Epidemiol.*, 135, 1394–1403, 1992.

Windsor, R.A., Boyd, N.R., Orleans, and C.T., A meta-evaluation of smoking cessation intervention research among pregnant women: improving the science and art, *Health Educ. Res.*, 13, 419–438, 1998.

Wise, P.H., Wampler, N., and Barfield, W., The importance of extreme prematurity and low birthweight to US neonatal and mortality patterns: Implications for prenatal care and women's health, *J. Am. Med. Womens Assoc.*, 50, 152–155, 1995.

Witschi, H., Joad, J.P., and Pinkerton, K.E., The toxicology of environmental tobacco smoke, *Annu. Rev. Pharmacol. Toxicol.*, 37, 29–52, 1997.

Witschi, H., Pinkerton, K.E., Coggins, C.R.E., Penn, A., and Gori, G.B., Environmental tobacco smoke: Experimental facts and societal issues, *Fundam. Appl. Toxicol.*, 24, 3–12, 1995.

Woods, J.R., Jr., Cavanaugh, J.L., Norkus, E.P., Plessinger, M.A., and Miller, R.K., The effect of labor on maternal and fetal vitamins C and E, *Am. J. Obstet. Gynecol.*, 187, 1179–1183, 2002.

WorldNow and KRQE, Did you say discounts? 2002. Available at http://www.krqe.com.

Zhang, J., Ying, X., Lu, Q., Kallner, A., Xiu, R.J., Henriksson, P., and Bjorkhem, I., A single high dose of vitamin C counteracts the acute negative effect on microcirculation induced by smoking a cigarette, *Microvasc. Res.*, 58, 305–311, 1999.

Zhou, J.F., Yan, X.F., Guo, F.Z., Sun, N.Y., Qian, Z.J., and Ding, D.Y., Effects of cigarette smoking and smoking cessation on plasma constituents and enzyme activities related to oxidative stress, *Biomed. Environ. Sci.*, 13, 44–55, 2000.

Zuccaro, P., Altieri, I., Rosa, M. Passa, A.R., Pichini, S., Ricciarello, G., and Pacifici, R., Determination of nicotine and four metabolites in the serum of smokers by high-performance liquid chromatography with ultraviolet detection, *J. Chromatogr.*, 621, 257–261, 1993.

Part IV

Inhalation Toxicology of Bioaerosols

35 Health Aspects of Bioaerosols

Kevin P. O'Connell and Harry Salem

CONTENTS

35.1 INTRODUCTION

Bioaerosols are colloidal suspensions in air of liquid droplets or solid particles that contain, or have adsorbed to them, biologically active materials. These materials include living organisms (bacteria, fungi, viruses, algae, protozoa) and the products of living organisms (such as pollen, dander, fecal material, toxins, etc.). The particles in a bioaerosol can range in size from a single virus or bacterial spore (less than one micrometer), to particles or liquid droplets that are visible to the eye. Particles in a bioaerosol may also be "compound" in nature (for example, a particle composed of microorganisms clinging to larger biological particles such as pollen or skin flakes).

A review of the inhalation toxicology literature confirms what most urban dwellers know: we live in a sea of airborne chemicals. Synthetic and naturally occurring compounds enter the lungs and may affect the respiratory system directly or, by using this route as a portal of entry, may adversely affect some other part of the body (Salem, 1987). The chemicals potentially inhaled by people in industrialized urban environments number in the tens to hundreds of thousands. Similarly, bacteria, viruses, pollen, and mold abound in ambient air. However, unlike many chemicals, most microorganisms do not survive the desiccation that results from being suspended in air, being separated from a suitable host, or from exposure to the ambient UV radiation in sunlight. Free-living microorganisms that remain viable are further assumed not to be able to propagate, due to the lack of growth

substrate in air, but can be transmitted over long distances, and some can be transmitted from person to person. Different microorganisms are found at various heights from the ground, and the size and diversity of microbial populations in air are dependent on geographic location, the population density and activity of humans, and environmental factors such as temperature, humidity, and the intensity of UV radiation in sunlight (Al-Dagal and Fung, 1990). Viable microorganisms are known to occur at altitudes of 20 miles, and fungal spores have been isolated from air over the North Pole.

Bioaerosols affect the health of plants and the state of the environment. Plant pathogenic bacteria, fungi, and viruses can be aerosolized by the impact of rain droplets on diseased leaves or plant litter, carried by wind and deposited on healthy but susceptible host plants. Some plant pathogens, such as *Pseudomonas syringae,* inflict their damage in part by seeding the formation of ice crystals on the surface of host plant leaves (reviewed by Hirano and Upper, 2000); this activity, which is likely present in other organisms as well, almost certainly affects the small-scale spatial distribution of snowfall. The ice-nucleating properties of *P. syringae* are now exploited by the ski resort industry. Cells of *P. syringae* are grown in large batches, killed, and added to water in snowmaking systems to facilitate the formation of ice crystals. These systems spray a fine mist of water into cold air. The addition of ice crystal-nucleating bacteria raises the mean temperature at which ice crystals will form, resulting in snowmaking that is more efficient at higher temperatures. Commercial formulations of *P. syringae,* such as "Snomax®" (www.snomax.com; York International, Victor, NY) are sold worldwide.

The presence of biological materials in aerosols likewise has a dramatic influence on human and animal health. We are all familiar with the effects of pollen on hay fever sufferers. Likewise, the outcome of having a sneezing victim of the common cold show up for work is well-understood: aerosolized rhinoviruses infect coworkers, who later become ill and can transmit the illness to others. More seriously, the inhalation of aerosolized *Mycobacterium tuberculosus* cells is the primary means of acquiring tuberculosis (Beggs et al., 2003) (http://www.emedicine.com/med/ topic1103.htm). So-called "sick buildings" disseminate microorganisms from damp, dusty ventilation systems, resulting in workers with a variety of symptoms (Manuel, 2003). A severe example of infections originating in a ventilation system was the outbreak of Legionnaires' disease in Philadelphia in 1976 (Shelton et al., 2000), which sickened over 200 convention goers and killed 34. This proven ability for pathogens to be distributed over wide areas by air has made aerosolization the dissemination method of choice by workers in biological weapons (BW) research efforts, both in the United States (until the end of the offensive effort in 1969; Regis, 1999) and abroad. The aim of this chapter is to review the sources of bioaerosols and basic aerobiology as it pertains to disease.

35.1.1 Airborne Contagion

Air has long been recognized as a vehicle for dissemination of microorganisms, whether dynamic contagion is involved with a geometric increase in cases, or static infection arising from a single source, and whether humans, animals, or plants are implicated as hosts (Kundsin, 1980). Examples of microorganisms causing respiratory infection or sensitization when inhaled are presented in Table 35.1 Air itself has even been mistakenly blamed for the spread of disease; nighttime air historically was blamed as the cause of malaria ("mal-aria," or bad air), rather than *Plasmodium* spp. (vectored by mosquitoes), the actual causative agent. Wells (1955) originally described airborne contagion as the chain reaction indoors resulting from person-to-person transfer of droplet nuclei implicated in respiratory tract infection. At a conference on Airborne Contagion sponsored in 1979 by the New York Academy of Sciences, the concept of airborne contagion was expanded to include aerosols from intermediary or inanimate sources such as air conditioners, dental drills, fungal spores from soil, and insulating materials, as well as long-range outdoor transfer of microorganisms. Many environmental and physiological factors may play a significant role in the pathogenesis of infectious diseases. Some bacteria (especially members of the genuses *Bacillus* and *Clostridium*) and fungi produce spores that are hardy and may persist in the environment for many years. Microbial spores

are easily transported through the air, and their concentrations in the air are subject to seasonal, diurnal, and geographic variations. The outdoors are abundant with microbial spores which freely penetrate indoors by many routes, including open windows and mechanical air intakes. Mannis et al. (1986) reported that protozoa (unicellular, often motile organisms) also grow in indoor reservoirs and can cause severe infections as well as producing antigenic or toxic metabolites. These may contribute to hypersensitivity pneumonitis and "humidifier fever" (Edwards et al., 1976).

Naturally occurring airborne organisms comprise the "seed cultures" that begin the process of "sick building syndrome" (SBS), a set of illnesses (rhinitis, asthma, other allergic and systemic reactions) that result from blooms of microbial growth in damp air-handling systems (Burge, 2004; Fung and Hughson, 2003). Buildings and building materials are colonized by naturally occurring microflora during construction, early occupation, and throughout the life span of the building. In ventilated buildings, the indoor air concentrations are directly correlated with the outdoor concentrations. Additionally, many thousands of spores are present per gram of surface dust in most enclosed spaces (Spendlove and Fannin, 1983). "Tight" buildings have been associated with SBS; the emphasis on energy conservation requires the recirculation of air in such buildings, whether heated or air-conditioned. In buildings with accumulations of water in walls, carpeting, or ventilation systems, less make-up outdoor air from the outside results in an accumulation of significant concentrations of mold or other allergens. Making buildings more airtight can result in higher exposures to more recirculated air with an increased potential for exposure to more airborne microorganisms.

35.1.2 Sources of Bioaerosols

Microorganisms (viruses, bacteria, protozoa, and fungi) require a growth medium (or host) for growth and reproduction. Although some of these organisms may survive on nutrient-poor environmental surfaces for various durations of time, they only very rarely survive to cause disease when they reenter the air (Burge, 1990). Exceptions to this are found among organisms that produce environmentally hardy structures such as spores. Microorganisms, although frequently found in air, are not known to multiply there, again largely because of a lack of nutrients. Outdoor air rarely contains pathogens, in part, because of the bactericidal effects of drying, ozone, and ultraviolet irradiation. Indoor air, however, may contain pathogenic organisms that are shed from the skin, hands, and respiratory tract of humans as well as from their clothing. Talking, coughing, and sneezing can produce respiratory droplets containing bacteria, and viruses can spread infection only to susceptible individuals in proximity to the source (Gallis, 1976). Likewise, as mentioned above, organisms that thrive in building air and water-handling systems may be aerosolized and inspired by humans. It has been shown that many microorganisms are capable of producing volatile organic compounds (VOCs) (Kaminski et al., 1974). Low levels of these compounds have been associated with moldy or mildewy odors; higher concentrations conceivably can be irritants or systemic toxins. Microorganisms also produce a variety of higher-molecular-weight toxins (Burge, 1987). However, the postulated involvement of secondary metabolites produced by fungi as a cause of SBS (Jarvis, 2003) has not been confirmed by controlled studies (Fung and Hughson, 2003), leaving open the question of whether microbial products, as well as the organisms themselves, cause illness in the SBS context.

The size of particles containing bacterial or viral pathogens or their products determines the duration of time that pathogens are in the air. Their settling velocity is a function of particle size and determines the "hang time" in air that limits the probability of transfer between persons in the environment. Hatch and Gross (1964) showed that, in a confined space with a 10 ft ceiling, settling due to gravity would reduce the concentration of particles greater than 13 μm by 63% in less than 10 min. A comparable reduction due only to gravity would require several hours for particles of 2–3 μm (approximately the size of a single bacterium). The removal of particles from the air is also dependent on the extent of building ventilation and filtration. Therefore, the risk of direct respiratory

Table 35.1 Microorganisms Causing Respiratory Infection or Sensitization When Inhaled

Disease	Causative Organism	Primary Reservoir	Reference
Bacterial Disease			
Pneumonia	*Streptococcus pneumoniae*	Humans	Baquero et al. (2002)
Pneumonia (nosocomial)	*Klebsiella pneumoniae*	Humans	Podschun and Ullman (1998)
Pneumonia	*Haemophilius influenzae*	Humans	Watt et al. (2003)
Walking pneumonia	*Mycoplasma pneumoniae*	Humans	Loens et al. (2003)
Q fever	*Coxiella bunetii*	Animals (sheep)	Marrie (2003)
Ornithosis, psittacosis, parrot fever	*Chlamydia psittaci*	Birds (domestic and wild)	Eidson (2002)
Brucellosis[a]	*Brucella melitensis*	Animals	Kaufmann et al. (1990)
Legionaires' disease, pontiac fever	*Legionella pneumoniae* *Legionella ansia*	Water (indoor and outdoor)	Shelton et al. (2000); Breiman et al. (1990); Lee and West (1991)
Tuberculosis	*Mycobacterium tuberculosis*	Humans	Young and Duncan (1995)
Hypersensitivity pneumonitis	*Thermoactinomyces*	Heated water, soil, compost, moldy hay	Burge (1990)
Diphtheria	*Corynebacterium diphtheriae*	Humans	Hadfield et al. (2000)
Pertussis, whooping cough	*Bordetella pertussis*	Humans	Yeh (2003)
Inhalation anthrax	*Bacillus anthracis*	Animals	Mock and Fouet (2001)
Bubonic plague	*Yersinia pestis*	Animals	Rollins et al. (2003)
Pneumonic plague[a]			
Tularemia	*Francisella tularensis*	Animals	Feldman (2003)
Viral Disease			
Influenza	Influenza viruses	Humans, birds, swine	Webby and Webster (2003)
Croup, bronchiolitis	Parainfluenza viruses, respiratory syncytial viruses (RSV)	Humans	Wright et al. (2002)
Mumps[a]	Mumps (paramyxovirus)	Humans	Galazka et al. (1999)
Measles	Measles virus	Humans	Schneider-Schaulies and ter Meulen (1999)
Common cold	Rhinoviruses	Humans	Savolainen et al. (2003)
Chicken pox[a] (shingles)	Varicella-Zoster virus	Humans	Gershon (1995)
Smallpox[a]	Variola virus	Humans	Henderson et al. (1999)
Algal, Fungal, and Protozoan Disease			
Asthma, rhintis	*Alternaria, Cladosporium* *Penicillium,* various algae	Outdoor air, dead plant litter, indoor/outdoor damp organic material, water reservoirs	Burge (1990)
Hypersensitivity pneumonitis	Protozoa	Water reservoirs	Burge (1990)

Table 35.1 Microorganisms Causing Respiratory Infection or Sensitization When Inhaled (Continued)

Disease	Causative Organism	Primary Reservoir	Reference
Pulmonary aspergillosis	*Aspergillus*	Soil, compost	Herman (1980)
Coccidioidomycosis	*Coccidioides immitis*	Soil of arid regions	Deresinski (2001)
Histoplasmosis	*Histoplasma capsulatum*	Animals, soil, keratonaceous materials (feathers)	Joseph Wheat (2003)

[a] Diseases transmitted via respiratory tract, but signs of infection are seen elsewhere in the body.

exchange of infectious particles is greatest from those small particles dispersed from the respiratory tracts of infected individuals, and the hazard from the inhalation of particles resuspended from dust deposits on the floor is limited by their relatively large size.

Microorganisms are dispersed into the air as liquid droplets varying in size from greater than 100 μm to less than 10 μm. Salem and Aviado (1970) reported that of the respiratory activities of sneezing, coughing, and talking, sneezing produced the highest proportion of the small droplets. It has long been known that nearly all of the airborne small droplets containing bacteria originate from the front of the mouth; only a few originate from the nose (Duguid, 1945). The spread has been observed by high-speed stroboscopic light photography.

As far back as 1934, Wells demonstrated that large water droplets evaporate rapidly and the residual particles of solute, which he termed droplet nuclei, were only a few micrometers in size. The smaller droplets resulting from a sneeze remain suspended in air, evaporate quickly, and leave droplet nuclei a few micrometers in diameter, which may or may not contain microorganisms. He also showed that even in an atmosphere of 90% relative humidity, droplets with diameters in the order of 80 μm evaporate before settling to the floor from a height of 6 ft.

The droplet nuclei settle very slowly because of their small size and may remain suspended in air almost indefinitely, especially in a room in which the movements of air and people keep them suspended. This creates an environment with a high concentration of potentially infective particles. Larger particles are expelled up to a distance of 2 m and at a velocity of at least 142 ft/sec (Gallis, 1976; Jennison, 1942). These large particles, though quick to settle from air, can be resuspended by sweeping, dusting, movement such as walking, and wind.

35.1.3 Particle Size and Dose–Response

As discussed above, the particle size of bioaerosols determines the duration of its availability in the atmosphere, the distance it can travel, and the site of its ultimate deposition in the respiratory tract of the next host. The same principles govern how deeply particles of a given size are inhaled. Hatch (1961) showed that for droplet nuclei (2–3 μm), pulmonary deposition is higher than that for the upper respiratory tract, whereas for dust-borne bacteria and liquid droplets, which are larger in size, deposition is essentially limited to the nasopharyngeal area (Figure 35.1). Wells et al. (1948) demonstrated that, to establish disease, the infectious particles must be deposited as virulent organisms at the critical site and that only droplet nuclei can contribute significantly to the atmospheric spread of diseases initiated by deposition of infectious particles in the lungs. This was demonstrated in their quantitative study of tuberculosis infection in rabbits. Using suspensions of *Mycobacterium tuberculosis* as single organisms of 2–3 μm and 13 μm, they found that the number of tubercles developing in the rabbits' lungs approximated the number of inhaled organisms of 2–3 μm, whereas only 6% of the 13-μm particles reached the lungs to produce tubercles. These studies not only confirmed their

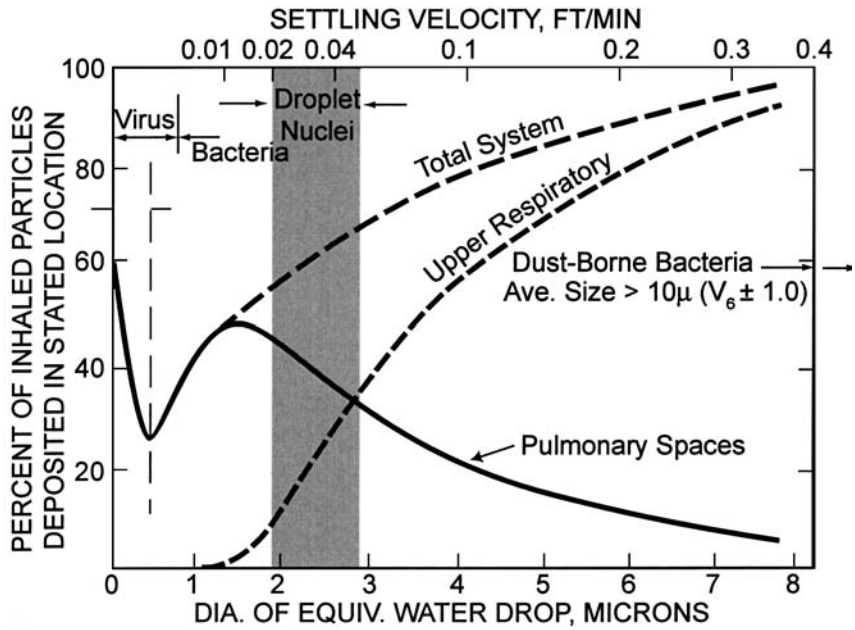


FIGURE 35.1 Total and regional deposition of inhaled particles in relation to aerodynamic particle size, showing the relative positions of single viral and bacterial particles, droplet nuclei, and dust-borne bacteria on the size–deposition curve (from Hatch [1961]).

prediction that larger particles would not reach the depths of the lungs but also demonstrated the selectivity of tissue susceptibility. Cells of *M. tuberculosis* implanted on the mucosa of the upper respiratory tract proved to be innocuous. Effective contact required implantation on a particular part of the respiratory tract, or a part from which susceptible tissue can be reached. This is in contrast to chemical aerosols where deposition, while also dependent on particle size, can cause damage and lethality at any region of the respiratory tract. (For this reason, Salem et al. [1992] recommended that particle sizes between 1 and 4 μm be used for toxicity testing in rodents.)

Early studies of other diseases acquired by inhalation produced similar results. Druett et al. (1953) found striking differences in the atmospheric concentration of anthrax spores of different sizes required to produce disease in 50% of the exposed animals. There was a 17-fold increase in the concentration of particles larger than 12 μm to produce disease than with anthrax spores in a particle. Goodlow and Leonard (1961) confirmed this finding with aerosol particles of *Francisella tularensis* of different sizes. To produce 50% mortality in guinea pigs and rhesus monkeys required 3 and 17 cells of 1-μm particle size, 6500 and 240 cells of 7-μm particle size, 20,000 and 540 cells of 12-μm particle size, and 170,000 and 3,000 cells of 22-μm particle size, respectively. Brucellosis, a zoonotic disease, can be transmitted from its animal reservoir to humans by the inhalation of infectious aerosols in addition to skin and conjunctival contact as well as ingestion. Aerosol transmission of brucellosis, which has a very low infectious dose, has been widely accepted as a potential aerosol hazard in laboratories and must be handled at Biosafety Level 3 (BSL3) containment (Olle-Goig and Canela-Soler, 1987; Staszkiewicz et al., 1991).

35.1.4 Inhalation of Bioaerosols

The traditional study of inhalation of bioaerosols in animal models has had two main goals: to be able to estimate infectivity of a given agent in humans, and to be able to define the mechanisms of the pathogenesis of the airborne disease. Animal studies have provided a scientifically sound

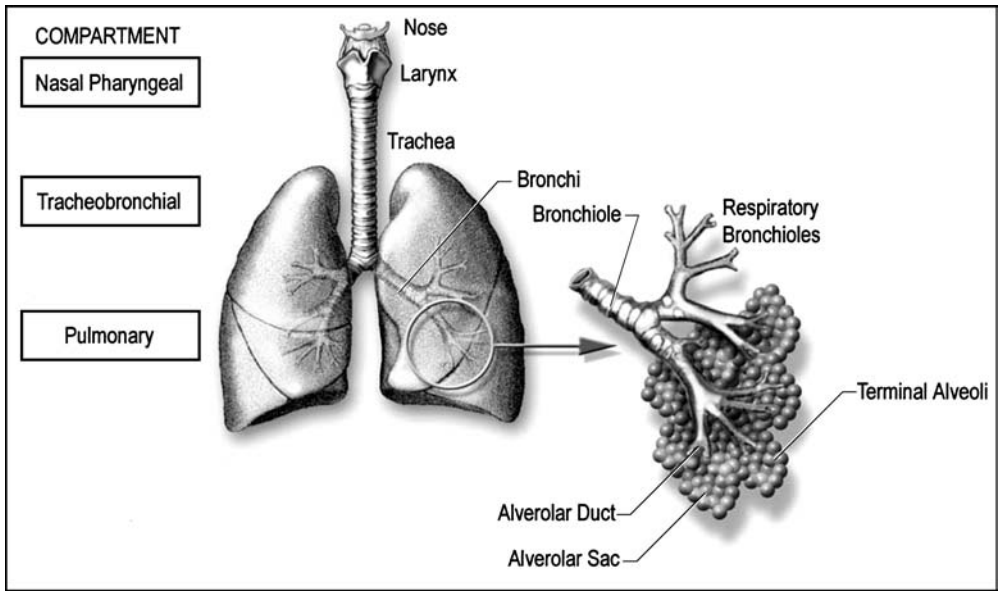


FIGURE 35.2 Major anatomical components of the respiratory system.

approach to providing cause-effect data under well-controlled and defined conditions under a legion of exposure conditions.

Both the site of deposition and the total dose of an airborne infectious agent delivered to the respiratory tract can be significantly affected by the geometry of the pulmonary anatomy; the route of breathing (nose versus oral-nasal); the depth and rate of airflow; and the physical properties that govern particle transport and deposition. Studies of these factors over the past 50 years have significantly improved our ability to (1) understand the pathogenesis of the infectious disease process; (2) extrapolate laboratory animal studies to humans; (3) predict and assess health risk associated with airborne pathogens; and (4) provide guidance in identifying a most effective mode of treatment for respiratory infections.

35.2 ANATOMICAL AND PHYSIOLOGICAL FACTORS IN RESPIRATORY DEPOSITION OF INFECTIOUS ORGANISMS

The anatomical structure and the physical dimensions of the respiratory system are important factors that govern the deposition and fate of inhaled organisms. The respiratory system of terrestrial mammals can be conveniently divided into three major components or regions (Figure 35.2). Each of these regions has anatomical features that influence the entry, transport, deposition, and clearance pattern of airborne microorganisms (reviewed in Gardner et al., 1988; Crystal et al., 1991; McClellan and Henderson, 1989; Miller and Menzel, 1989). This brief review will provide basic understanding of the anatomical and physiological factors associated with the inhalation of bioaerosols.

The uppermost (nasopharyngeal) region, as discussed here, consists of the area extending from the nares down to the epiglottis and larynx at the entrance to the trachea. The mouth is included in this region as an alternate entry route during mouth breathing. This region has a complicated morphology consisting of the turbinates, epiglottis, glottis, pharynx, and larynx. The complicated shape of the nasal passages is not solely related to the role of the nose as an olfactory organ, but also plays a major physiological function in the modification of inspired air prior to its entry into the lungs. Upon inspiration, the air enters the nose through the anterior nares passing into the airway passages where it is ultimately carried to the pharynx. These airway passages are lined with small hairs and

membranes that are populated with goblet and mucous-secreting cells. The high surface-to-volume ratio facilitates humidification and warming of the incoming air. In this region, filtration hairs and cilia, and impaction on (and adhesion to) the mucous membranes removes the largest inhaled particles, preventing them from being deposited deeper in the respiratory system.

The tracheobronchial (TB) region consists of the conducting airways beginning at the trachea and extending down to the terminal bronchioles. The TB region functions to deliver inspired air to the deeper portions of the lung. The dimensions and number of branching airways varies from species to species, making definitive extrapolation of animal studies to humans difficult. The trachea is an elastic tube supported by 16–20 cartilaginous rings that circle about three-fourths of its circumference and is the first and largest of a series of branching airways. Air enters the left and right lungs via two major bronchi that branch off the trachea. Each bronchus distributes air into separate lobes; in humans, the left lung consists of an upper and lower lobe, and the right lung has an upper, middle, and lower lobe. Humans differ from some laboratory species in this respect. Although rats also have five lobes, they have only a single lobe on the left and four on the right. The conducting airways in each lobe consist of up to 18–20 dichotomous branches from the bronchi to the terminal bronchiole. Beyond the terminal bronchi, the airways become very thin-walled and are referred to as respiratory bronchioles, which have numerous small air sacs (alveoli) protruding from its walls).

The lowest (pulmonary) region primarily functions in gas exchange. However, because it contacts the smallest inhaled particles, it also possesses features that function in pulmonary clearance and immunological defense. This region begins with the partially alveolated respiratory bronchioles. The epithelium of the respiratory bronchioles is nonciliated. Each respiratory bronchiole divides into alveolar ducts. These ducts are actually bronchioles where almost their entire wall has been completely alveolated. These ducts lead ultimately into the alveolar sacs. The total number of ducts and sacs has been estimated to be 7×10^6 and 8.4×10^6, respectively (Gardner et al., 1988). The alveoli, which are evaginations of the alveolar sacs, are thin walled and surrounded by a meshwork of blood capillaries. In the alveolus, the atmosphere and the blood are brought into an intimate contact, in which an exchange of O_2 and CO_2 can take place. The surface of the alveoli is primarily made up of two types of cells. Type 1 cells are very thin and cover the greatest surface area. Type 2 cells are larger, display numerous microvilli, and secrete a surface-lining surfactant fluid that reduces surface tension, lessening tendency of alveoli to collapse. The total respiratory surface of the lung has been estimated to be 50 m^2 during expiration and as much as 100 m^2 during the deepest inspiration.

35.3 DOSIMETRY FACTORS FOR INHALED MICROORGANISMS

As mentioned, aerosolization often greatly reduces the viability of microorganisms, and the actual number of infectious organisms reaching the respiratory tract may be greatly reduced. In considering the delivery of an agent in a bioaerosols to a test subject, it is important to distinguish between the concentration generated and the dose delivered. Posology is the study of *dose* and *dosage.* Although the term dose and dosage are often used interchangeably, dose correctly is the total amount of a test article administered. *Dose* refers to the total amount of the microorganism that is actually inhaled, delivered, and deposited to the tissue. A dose of 10 mg, for example, means that this is the amount of material administered whether it is to a mouse, rat, dog, or human, no matter by what route (oral, by inhalation, etc). *Dose* refers to the total amount of the microorganism that is actually inhaled, delivered, and deposited to the tissue. *Dosage,* on the other hand, is a relative amount; it is the amount of material administered relative to the body weight, body surface, and/or time (mg kg^{-1}, mg m^{-2}). Concentration or dosage in inhalation studies is the amount of material per unit volume, that is, the amount of a substance in the air or in the test medium. Dosage is often expressed as concentration or concentration and duration (i.e., LC_{50} as mg kg^{-1}, mg kg^{-3}, ppm, ppb, or LCt_{50} as mg-min m^{-3}) and the route of administration is usually specified.

Developing a quantitative understanding of the relationship among exposure concentration, dose delivered to the tissue, and a specific pulmonary response is a basic fundamental goal of inhalation

scientists. The term *deposition* refers specifically to the amount of inhaled, airborne agent that is deposited in the respiratory tract. The fate of the organism can be expressed in terms of *clearance,* which refers to the subsequent translocation of and removal of deposited agent from the respiratory tract, and *retention,* which refers to inhaled agent that remains in the respiratory tract and is not cleared.

The infective dose for microbial aerosols has been shown in animals to be greatly influenced by particle size, which in turn, controls the site of deposition. Particle size is also critical in situations in which one part of the respiratory system is more susceptible to the inhaled organism than another. Airborne microbes may comprise particles varying in size from a single virus (as small as 0.1 μm in diameter) to the largest fungal spores and pollens (50–100 μm).

A number of generic factors can significantly influence the deposition of particles. The arrangement of ventilation in the aerosol test chamber is important because the physics of airflow governs deposition of the agent in the chamber. The rate and depth at which the test subject breathes influences the volume of air and, hence, the mass of infectious agent entering the respiratory tract and the total surface area over which deposition can occur. The route of breathing (oral, nasal, or oronasal) influences filtering efficiency of inhaled materials and thus, impacts the dose delivered to the lower respiratory tract.

Although most adults are nasal breathers at rest, they may resort to chronic or periodic mouth breathing under certain conditions such as exercise, nasal obstruction, or in the presence of chemical irritants. As respiratory demands increase, the proportion of air entering via the mouth also increases. Such action can significantly alter the pattern of deposition and, thus, the response to the inhaled substance. For instance, nearly 100% of particles having an aerodynamic size of about 10 μm or larger are deposited in the nasopharyngeal region during nasal breathing. This compares with only about 65% deposition of such particles under conditions of oronasal breathing. There is also increased penetration of larger particles deeper into the respiratory tract with oronasal breathing. Although nasal breathing offers an effective means of protecting sensitive lower respiratory tract tissues from airborne particles, it should be remembered that rodents do not breathe through the mouth, a factor that must be taken into consideration when extrapolating such rodent data to humans. Also, a great difference exists in the complexity of the nasal passages, resulting in differences in nasal airflow patterns between humans and the laboratory animal that may account for species-specific lesion distribution following inhalation exposure to certain agents.

Chemical agents can alter physiological responses during exercise by causing pulmonary function changes (e.g., increased airway resistance through constriction) which tend to decrease the volume of air penetrating to the alveoli and can result in a shift to rapid, shallow breathing. Exercise has been shown to have a pronounced effect on pulmonary tissue uptake of gaseous pollutants but have little effect on tracheobronchial tissue. The deposition, clearance, and retention of inhaled particles have been extensively reviewed (National Research Council, 1991; Bates et al., 1989; Gardner et al., 1988).

For particles, the overriding factors influencing regional respiratory tract deposition are those based on the aerodynamic properties, which, in turn, depend on a variety of physical properties. Particles of the same physical size do not necessarily behave the same aerodynamically. Because a denser particle will tend to fall faster than a less dense particle of the same diameter, the size of airborne particles is expressed in terms of their "aerodynamic diameter" which is the diameter equivalent to that of a theoretical spherical particle with a density of 1 that has the same terminal settling velocity (i.e., behaves aerodynamically in the same way) as the particle in question. Using such an adjustment permits a more valid comparison among particles of different physical sizes. These differences between actual and aerodynamic sizes are important in predicting respiratory deposition of inhaled particles.

35.4 MECHANISMS OF AGENT DEPOSITION

To begin causing an infection, or injury due to toxic or immunological effects, an agent must be deposited in a significant dose onto susceptible tissue. Figure 35.3 illustrates the five mechanisms

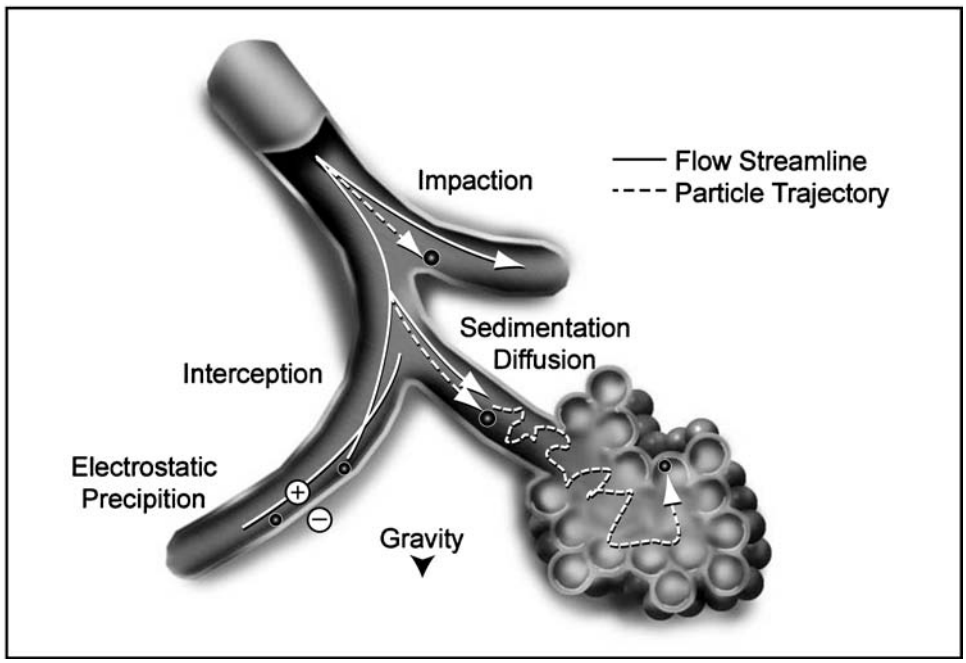


FIGURE 35.3 Mechanisms of particle deposition in the respiratory system.

by which particle deposition can occur: impaction, sedimentation, Brownian diffusion, interception, and electrostatic precipitation. Electrostatic attraction and interception will not be considered here; electrostatic deposition of particles to the walls of respiratory airways plays a minor, if any role and is not important for the inhalation deposition of most environmental contaminants, whereas interception is more important for fiber deposition.

Impaction is the main mechanism for the deposition of particles having an aerodynamic size of $\geqslant 2.0\ \mu m$. Larger particles are removed from the inhaled air by the mechanism of impaction at the various bifurcations. Impaction onto an airway surface occurs when a particle's momentum prevents it from changing course in an area where there is rapid change in the direction of the airflow. The largest size particles are deposited in the anterior regions of the nose and are then most effectively cleared by mechanical means, including sneezing, coughing, and nose blowing. Deposited microbes may also be cleared from the upper airways by mucociliary transport from the airways to the throat, where it may be swallowed or expectorated. The probability of impaction increases with increased air velocity, rate of breathing, particle size, and density.

Sedimentation is deposition due to gravity and is important for particles with an aerodynamic size of $\geqslant 0.5\ \mu m$ in medium and small airways, where the air velocity is relatively low. Such particles will fall out of the airstream at a constant rate when the total force due to air buoyancy and air resistance balances the gravitational forces on the airborne particle.

Brownian diffusion is a major mechanism for deposition in airways where the airflow is low, such as found in the alveoli and bronchiole. The smallest particles ($\geqslant 0.5\ \mu m$) may be deposited onto the airway walls because of their bombardment by surrounding air molecules. Figure 35.1 depicts the regional deposition of inhaled aerosols as a function of aerodynamic diameter. The degree of virulence of an organism may also be due to certain enzymes and other metabolic substances that are produced by the organisms. These biological products can play a significant role in determining the outcome of an infection.

35.4.1 Biological Factors Influencing Airborne Contagion

We discussed above the factors that influence the deposition and retention of inhaled biological materials or microorganisms for one main reason: the likelihood that exposure to a given pathogen will result in disease is directly correlated with the number of organisms that reach sites in the host where infection can occur. This number varies from organism to organism. The average number of individual infectious organisms required to cause disease in a given host is called the infective dose. The infective dose varies not only with the species and/or strain of the microorganism, but also with the host. Not all hosts are equally susceptible to each strain of a pathogen; for instance, (*L*ive *V*accine *S*train) causes a mild, if any, infection in humans and has been studied and used as a human vaccine against tularemia. However, *F. tularensis* strain LVS causes a severe infection in mice. A number of environmental factors also play a role in making the host susceptible to a pathogen. Stresses on the host such as fatigue, poor nutrition, age, other infections, and the interaction of the infectious agent with pollutants can modify and complicate the etiology and pathogenesis of airborne disease (Gardner, 1988; Coffin and Gardner, 1972; Graham et al., 1987; Parker et al., 1989). Prior exposure to the pathogen is often protective, as some pathogens do not vary significantly the molecular structures they display to the immune system. However, as in the common cold and influenza (as discussed later) this is not always the case.

Organisms that have evolved the capacity to cause disease are said to possess the property of *pathogenicity.* Pathogenicity genes are those that, when inactivated by mutation, prevent the organism from causing disease with any degree of severity. This idea is complementary to that of *virulence,* which describes the severity of the disease caused by a pathogen. In general, the number of organisms required to cause disease is usually proportional to their virulence. Bacteria such as *Francisella tularensis* and *Brucella abortus* are infectious at doses as low as 10s of organisms; the estimates for the infectious dose of *Bacillus anthracis* vary, but most fall in the range of thousands to tens of thousands of spores (http://www.usamriid.army.mil/education/bluebook.html). The mass of an infective dose of *B. anthracis* spores is far below the lethal dose of even the most potent toxin, botulinum toxin.

Once deposited in the respiratory tract, a pathogen must be able to grow and multiply to produce disease. This is accomplished by increasing in number in a local lesion or by spreading systemically throughout the host. The success of the infection depends on (1) the specific properties of the invading organism to produce disease and (2) the manner in which the infected host responses to the microbial invasion. The mechanisms of pathogenicity and virulence of infectious organisms

Table.35.2 Some Principal Bacterial Toxins

Species	Toxin	Associated Disease
Clostridium botulinum	Type-specific neurotoxins	Botulism (neurotoxin, paralytic)
Clostridium perfringens	α-Toxins and others	Gas gangrene
Clostridium tetani	Tetanospasmin; tetanolysin	Tetanus
Corynebacterium diphtheriae	Diptheria toxin	Diphtheria (modifies enzymes)
Staphylococcus aureus	α, β, γ-toxins; leukocidin Enterotoxin	Staphylococcal pyogenic infections Staphylococcal food poisoning
	Erythrogenic toxin	Staphylococcal scarlet fever
Streptococcus pyogenes	Steptolysins O and S Erythrogenic toxin	Streptococcal pyogenic infections
Shigella dysenteriae	Neurotoxin	Bacillary dysentery
Salmonella typhi	Endotoxins	Gastroenteritis, enteric fever
Vibrio cholerae	Endotoxins	Cholera

are nearly as varied as the agents themselves, and a complete review is beyond the scope of this chapter. However, it is possible conceptually to divide bacteria into those whose primary means of damaging the host is the production of toxins and those whose action is primarily intracellular. The latter include species that produce single polypeptide toxins such as *Staphylococcus aureus* (staphylococcal enterotoxins), large, multisubunit toxins such as *Clostridium botulinum* (botulinum toxin), or complex multisubunit toxins, such as *Bacillus anthracis* (the tripartite toxin consisting of lethal factor, edema factor, and the so-called protective antigen) (Mock and Fouet, 2001). Others are listed in Table 35.2. Vaccines that protect against the effects of these organisms are generally preparations of toxoids, inactive forms of the toxins that are still recognizable by the immune system. Intracellular bacterial pathogens include *Salmonella typhimurium, Listeria monocytogenes, Coxiella burnetii* (the causative agent of Q fever), and *Brucella abortus.* Some bacterial pathogens act in both manners simultaneously. Viruses cause damage directly to host cells by co-opting host cell metabolic functions, and redirecting them to the replication and spread of progeny viral particles.

35.5 METHODS OF STUDY

The classical criteria for determining whether a given microorganism is the causative agent for a particular disease were first set forth by Robert Koch and emerged from his seminal studies of anthrax and tuberculosis. His use of animals to passage microorganisms to obtain pure cultures led to his formulation of what became known as *Koch's Postulates.* Formalized in 1882, they state that to prove that an organism causes a disease, it is necessary to demonstrate:

- That the organism is present in every instance of a given disease;
- That, when removed from an infected host, the organism could be grown in pure culture;
- That the disease could be reproduced in healthy experimental animals by introducing the cultured microorganism several generations removed from the initial isolate; and
- That the organism could then be re-isolated from the experimental animal and freshly cultured.

The postulates are not directly applicable to all organisms that cause disease. Early on, before the nature of viruses was fully understood and before cell culture techniques were available, the application of the postulates to viruses was problematic. Currently, they cannot be applied ethically to diseases that, like HIV, infect only humans. (The third postulate, requiring the intentional infection of a suitable host, is not likely to pass scrutiny with Institutional Review Boards reviewing the use of humans in research.) Therefore, much work in HIV research has been devoted to the development of suitable animal models and determining the degree to which the infection of chimpanzees with Simian Immunodeficiency Virus mimics AIDS in humans. Although controlled human clinical studies offer the best opportunity to directly relate an infectious agent to human disease, ethical tests can have no long-term residual effects, restricting such studies to exposures to organisms with limited or no pathogenicity, or in extreme cases to organisms for which highly effective treatments are readily available.

The primary strength of well-designed animal studies is the degree to which variables can be controlled, in ways generally not available in the human clinical setting. The researcher has the choice of a wide range of concentrations, exposure regimens, biological agents, biological parameters, and test species. Such studies are most useful in studying the mechanisms of pathogenesis of disease. Because many physiological mechanisms are common to animals and humans, the worker studying infectious disease hypothesizes that if an agent is infective in several animal species, it is likely to cause similar effect in humans. However, as mentioned above,

care must be taken in attempting to quantitatively extrapolate the effective airborne infectious concentration in animals to the human and that certain defense mechanisms may differ in various species.

Apart from information obtained from the direct clinical/laboratory observation of human and animal disease, epidemiological studies (on various time–space scales) can determine the correlation between exposure and disease, but have great difficulty in proving causal relationships because of the many confounding variables. Such studies can show an association between exposure and effects, and identify qualitatively the potential for human risk.

35.6 EXPIRIMENTAL INFECTIONS OF THE LUNG

Laboratory-induced pulmonary infections have been useful in understanding the (1) pathogenicity of microorganisms, (2) environmental factors that alter susceptibility to respiratory disease, and (3) mechanisms of host resistance to infection. By using the appropriate animal model, one can measure subtle defects of the total pulmonary defense system by testing the lungs ability to efficiently defend itself against invading, potentially pathogenic organisms. The three experimental techniques that have been most widely used in the laboratory to expose a test animal to an airborne infectious organism include (1) aerosolization, (2) intratracheal instillation, and (3) intranasal inoculation.

Aerosolization of the microorganism most closely simulates an airborne exposure (whether naturally occurring or intentional). This method provides the most uniform exposure of the animal and is applicable to a wide variety of pathogenic organisms. To conduct such exposures requires expensive equipment and a team of scientists with expertise in aerosol sciences, engineering, and biology as well as specialized containment to ensure the health and safety of the investigators (Phalen, 1984; Gardner, 1988). Organisms may also be instilled intratracheally in an anesthetized animal. Although these studies are less costly, one cannot guarantee a uniform distribution throughout the lung as with inhalation exposure (Hatch et al., 1981; Phalen, 1984). Also, the need to anesthetize the test animal results in an unnatural condition of exposure and alters the host's physiology in ways that may affect early events in the infection process. Intranasal inoculation of microorganisms has been used for years. With this method, one must depend on the aspiration of the inoculum to spread the infectious organism from the upper respiratory tract to the conducting airways and deep lung. Both this technique and intratracheal instillation can result in considerably more animal-to-animal variation than with the aerosol challenge.

Ideally, whichever experimental method and host animal are chosen, the study design must (1) yield reproducible data; (2) permit correlation among species of test animals; (3) ensure the sensitivity of the test animal to the microorganism, and (4) facilitate the comparison of data with that from other laboratory studies. The test organism must be able to (1) multiply within the infected susceptible hose tissue, (2) be quantifiable at the administered dose, (3) trace and follow during the infectious and recovery phase; (4) not greatly influence mortality rate with small variations in dose or virulence, and (5) withstand the vigors of aerosolization if the route of administration is by inhalation. Such *in vivo* exposure studies can be used to provide vital information on survival statistics, growth, replication, and fate of the infectious organism in the host tissue and mechanistic studies using sensitive biochemical and immunological measurements.

Although it is unlikely that there will be acceptable substitutes to whole-animal studies in the near future, the advent of microarray technology is beginning to offer a higher throughput method of examining the effects of infection on host cell transcription (Kato-Maeda et al., 2001). The entire genetic complement of both hosts and pathogens is being studied at each stage of infection to determine the alterations in each organism stimulated by the presence of the other, to understand better both the molecular details of pathogenesis and resistance, and to provide novel markers for the detection and diagnosis of disease.

35.7 EMERGING ISSUES IN AIRBORNE DISEASE: ENGINEERED AND NATURALLY OCCURRING EMERGING INFECTIOUS DISEASES

35.7.1 Engineered Microorganisms

Few debates in modern biology stir so much argumentative ardor, both within and outside the scientific community, as the release to the environment of genetically engineered organisms, from salmon to cotton, from corn to cows. Particularly hotly debated is the release of engineered microorganisms (Wilson and Lindow, 1993). As mentioned in the introduction, ice-nucleating strains of *P. syringae* are capable of injuring plants by nucleating the formation of ice crystals on leaf surfaces, causing frost damage. The bacterium benefits presumably by consuming substrates leaked from damaged leaf tissue. It was found that strains of *P. syringae* engineered to remove the gene responsible for ice nucleation served as an effective competitive inhibitor for native ice-nucleating strains, providing a means for biological control of frost damage on crop plants (Wilson and Lindow, 1994). Field trials of this work were highly controversial, and protesters damaged experimental field plots.

Genetic engineering has also been reported to have been employed for intentionally nefarious purposes. Ken Alibek, a worker in the offensive biological warfare program of the former Soviet Union, described efforts there to modify pathogens to evade existing vaccines, resist antibiotic treatment (for bacterial pathogens), and combine the properties of different viral pathogens in a single agent (Alibek, 1999). The success of these efforts is partly a matter of record, and partly a matter of conjecture. The addition of a hemolysin gene from *Bacillus cereus* allowed a virulent strain of *B. anthracis* to evade vaccination with strains to which the hemolysin gene had not been added (Pomerantsev et al., 1997). Antibiotic-resistant strains of *Burkholderia mallei* (formerly *Pseudomonas mallei*) were isolated by classical selection methods, ostensibly to study the effect of resistance to additional drugs not used in the initial selection (Stepanshin et al., 1994; Manzeniuk, 1994). The success of the program in enhancing or modifying viruses remains a topic of discussion. However, a group in Australia studying mouse ectromelia virus reported that the addition of the gene encoding mouse IL4 greatly enhanced the virulence of the virus under study (Jackson et al., 2001), suggesting that modifications to enhance viral virulence are possible. However, critics of the work stated that the result was predictable (Müllbacher and Lobigs, 2001).

35.7.2 Influenza

A concern potentially greater than the intentional release of a pathogen is the emergence of novel, previously undescribed variants of known diseases, or entirely new diseases, that are easily transmitted through the air. The influenza pandemic of the early twentieth century killed over 700,000 in the United States and upwards of 40 million people worldwide (Reid and Taubenberger, 2003); common variants of influenza strain A cause over 30,000 deaths in the Unites States each year and sicken millions more. The strains that caused pandemics in 1968 and 1957 are thought to have arisen from genetic reassortment of the hemagglutinin gene between human and avian influenza strains. Recently, the highly virulent H5N1 and H7N7 strains of avian influenza, among others, bridged the avian-human barrier in Asia, a direct transfer not seen before 1997 (Webby and Webster, 2003). Influenza vaccine manufacturers were not able to respond quickly with effective vaccines, highlighting the need for additional resources in both strain monitoring and rapid vaccine development. The 1918 variant is also likely to have arisen through genetic rearrangement among strains, but the mechanisms are not yet clear.

35.7.3 Multidrug-Resistant Tuberculosis

Tuberculosis (TB) is another disease that is easily spread by airborne droplets, created by the coughing and sneezing of patients. No other disease has killed as many people; there were approximately

90 million fatalities worldwide due to TB in the twentieth century alone, by one estimate (Young and Duncan, 1995). Approximately one-third of the global population is believed to carry *M. tuberculosis* (Drobniewski et al., 2003), and over 8 million cases are diagnosed each year, resulting in about 2 million deaths. Initial successes in antibiotic therapy led to unrealistically optimistic claims that TB would be eradicated. Unfortunately, the last decade has seen a surge in the incidence of strains of *M. tuberculosis* that are resistant to two of the most widely used antitubucular drugs, isoniazid (an inhibitor of one-carbon metabolism) and rifampicin (an inhibitor of RNA polymerase). The cause of this emergence is in part rooted in poor public hygiene: the overcrowding of prisons and the inconsistent use of antibiotics. Additionally, coinfection with HIV and TB contributes to the elevated mortality due to TB. Advances in the fight against TB lie in several areas: new techniques, including polymerase chain reaction (PCR), for early diagnosis (especially the identification of multi drug-resistant strains), the identification of new drug targets in *M. tuberculosis* through the use of genomics and microarray technology and continuing efforts to improve public health infrastructure.

35.7.4 SARS

The appearance of severe acute respiratory syndrome (SARS) has been called "the first pandemic of the 21st century" (Peiris et al., 2003). SARS infected more than 8000 patients, resulting in over 700 deaths in 26 countries within months after its emergence in the Guangdong Province of mainland China. Caused by a newly discovered coronavirus (SARS-CoV), SARS is likely to have bridged the animal-to-human species barrier among people involved in the raising of small mammals for food, including civet cats. Epidemiological studies suggest that SARS-CoV is less transmissible than originally thought (each case giving rise to 2–4 additional cases); however, a few infected individuals have been responsible for the subsequent infection of larger groups of people, in so-called super-spreading events. The authors concluded that SARS-CoV is sufficiently transmissible to cause a very large epidemic if no preventive public health measures are taken, but not so contagious that good public hygiene could not control the spread of the disease. Empirical evidence supporting this conclusion may be the experience of Canada, in which rapid quarantine of cases and all of their contacts halted the spread of SARS-CoV in several weeks. These and other public health measures worldwide were based on an estimate of the maximal incubation period being 10 days.

35.7.5 Poxviruses

One of the great triumphs of modern medicine is the eradication of Variola virus (smallpox) by an aggressive, worldwide immunization campaign (Henderson et al., 1999). Smallpox was fatal in only 30% of cases, but severely disfigured survivors and was very readily transmissible by several means, including aerosols created by the coughing of patients. The eradication of smallpox was made easier because the disease was confined entirely to humans; there are no other known hosts for Variola virus in nature. However, other members of the poxvirus family can also infect humans, albeit with less virulence than Variola virus. In 2003 there were several reports of persons, including at least three children, in the midwestern United States who contracted monkeypox virus from prairie dogs that had been cohoused with exotic rodent pets imported from western Africa (Anderson et al., 2003). Allegations that the former Soviet Union grew large stocks of Variola virus (Alibek, 1999) revived the fear of the reemergence of smallpox as a biological weapon (Poupard et al., 1984) and caused the United States and other western governments to invest in a program to update and improve the vaccine for smallpox (Poland and Neff, 2003). While the route of infection was unclear, what is evident from consideration of influenza, SARS, and monkeypox is that the increased global transportation of people and trade in animals will continue to contribute to the spread of emerging diseases, especially those readily transmitted by the airborne route.

35.7.6 Biological Weapons and Bioterrorism

The choice of a particular agent for use in a biological attack depends on several factors. Military consideration of the use of biological weapons stressed organisms with the greatest virulence, lowest infective doses, ease of large-scale production, and the ability to be disseminated as an aerosol without significantly decreasing the viability of the organism (Regis, 1999). *Bacillus anthracis* possesses all of these traits, making it perhaps the most intensively studied organism for this purpose. While not reported to have been genetically modified, the strain of *Bacillus anthracis* used in the mail attacks in late 2001 was reported to have been carefully milled and prepared so that the size of spore-carrying particles was ideal for deep inhalation (Jernigan et al., 2001), suggesting that the perpetrator had at least a basic understanding of the aerosol science underlying the transmission of disease.

Similarly, powdered ricin toxin has been found in a recent biological "incident" in the offices of the U.S. Senate majority leader. Ricin toxin, derived from the seeds of castor bean plants (Figure 35.4), is a potent toxin that acts by catalytically disabling ribosomes, the cellular organelles that translate genetic information into active proteins (Lord et al., 2003). Theoretically a single ricin

FIGURE 35.4 Castor bean plants (*Ricinus communis* L.) grown in a research greenhouse by one of the authors (K.P.O'C).

molecule can kill one cell. The ubiquity and "dual-use" nature of castor products make restricting the availability of the plants problematic. Castor plants are found worldwide and can be purchased over the Internet, making the raw ingredient for this material perhaps one of the most widely available biological agents of concern. Castor plants are widely cultivated because their other important product, castor oil, has a multitude of uses as a lubricant, chemical feedstock, food and cosmetics additive, and precursor for the synthesis of plastics. Ricin toxin itself is under legitimate study as a component of "immunotoxins," medicines directed specifically at tumor cells that spare surrounding healthy tissue (Lord et al., 1994).

It is important to remember, however, that terrorists are capable of finding biological "weapons of opportunity" from among the more commonplace pathogens. Members of a fringe religious community in Oregon committed the largest known biological attack in the United States in 1984, spraying a clinical isolate of *Salmonella typhimurium* over salad bars in the town of Antelope. The timing of the attack was intended to sicken a number of people sufficiently to influence the outcome of a local election (Torok et al., 1997). It is believed that a member of the group obtained the *Salmonella* culture from a local clinical laboratory. Over 750 cases of gastroenteritis were associated with the attack.

35.8 SUMMARY

We have reviewed briefly the aerosol science and biological factors that contribute to the spread of biological agents (organisms and their products) as bioaerosols. A major challenge in the study of biological aerosols is the application of what is known to the sampling of air over large occupied areas, for the purpose of public health and biological defense. The low infective doses required by some agents to cause disease and the complex environmental background of both synthetic and natural materials in air present a significant challenge to workers attempting to protect cities from the intentional release of airborne pathogens. Aerosol physicists are faced with the problem of effective sampling, and microbiologists must contend with the challenge of devising ever more sensitive assays for the detection of pathogens and toxins that are accurate and nearly real-time in their ability to report the presence of danger. Modelers must devise better simulations of airflow in the complex landscape of cities, as well as improve the models by which the spread of diseases in increasingly mobile human and animal populations can be predicted. Perhaps most daunting of all, the public policies regarding the investment in this infrastructure, and what is done when an attack is detected, are rightly a subject of current, lively, and on-going debate.

REFERENCES

Al-Dagal, M. and Fung, D.Y., Aeromicrobiology-a review, *Crit. Rev. Food Sci. Nutr.*, 29, 333–340, 1990.

Alibek, K., *Biohazard*, Random House, New York, 1999.

Anderson, M.G., Frenkel, L.D., Homann, S., and Guffey, J., A case of severe monkeypox virus disease in an American child: emerging infections and changing professional values, *Pediatr. Infect. Dis. J.*, 22, 1093–1096, 2003.

Baquero, F., Baquero-Artigao, G., Canton, R., Garcia-Rey, C., Antibiotic consumption and resistance selection in Streptococcus pneumoniae, *J. Antimicrob. Chemother. Suppl.*, S2, 27–37, 2002.

Bates, D.V., Dungworth, D.L., Lee, P.N., McClellan, R.O., and Roe, F.J.C., *Assessment of Inhalation Hazards*, Springer-Verlag, New York, 1989.

Beggs, C.B., Noakes, C.J., Sleigh, P.A., Fletcher, L.A., and Siddiqi, K., The transmission of tuberculosis in confined spaces: an analytical review of alternative epidemiological models, *Int. J. Tuberc. Lung Dis.*, 7, 1015–1026, 2003.

Breiman, R.F., Cozen, W., Fields, B.S., Mastro, T.D., Carr, S.J., Spika, J.S., and Mascola, L., Role of air sampling in investigation of an outbreak of Legionnaires' disease associated with exposure to aerosols from an evaporative condenser, *J. Infect. Dis.*, 161, 1257–1261, 1990.

Burge, H.A., Toxigenic potential of indoor microbial aerosols, In *Short-Term Bioassays in the Analysis of Complex Environmental Mixtures*, Sandhu, S.S., Demarini, D.M., Mass, M.J., Moore, M.M., and Mumford, J.L., eds., Plenum, New York, 1987.

Burge, H., Bioaerosols: Prevalance and health effects in the indoor environment, *J.Allergy Clin. Immunol.*, 86, 687–701, 1990.

Burge, P.S., Sick building syndrome, *Occup. Environ. Med.*, 61, 185–190, 2004.

Coffin, D.L. and Gardner, D.E., Interaction of biological agents and chemical air pollutants, *Ann. Occup. Hyg.*, 15, 219–235, 1972.

Crystal, R.G., West, J.B., Barnes, P.J., Cherniack, N.S., and Weibel, E.R., Eds., *The Lung: Scientific Foundation*, Raven Press, New York, 1991.

Deresinski, S.C., Coccidioidomycosis: efficacy of new agents and future prospects, *Curr. Opin. Infect. Dis.*, 14, 693–696, 2001.

Drobniewski, F.A., Caws, M., Gibson, A., and Young, D., Modern laboratory diagnosis of tuberculosis, *Lancet Infect. Dis.*, 3, 141–147, 2003.

Druett, H.A., Henderson, D.W., Packman, L., and Peacock, S., Studies on respiratory infection. 1. The influence of particle size on respiratory infection with anthrax spores, *J. Hyg.*, 51, 359, 1953.

Duguid, J.P., The number and sites of origin of droplets expelled during expiratory activities, *Edinb. Med. J.*, 52, 385–401, 1945.

Edwards, J.H., Griffiths, A.J., and Mullins, J., Protozoa as sources of antigens in humidifier fever, *Nature*, 264, 438, 1976.

Eidson, M., Psittacosis/avian chlamydiosis, *J. Am. Vet. Med. Assoc.*, 221, 1710–1712, 2002.

Feldman, K.A., Tularemia, *J. Am. Vet. Med. Assoc.*, 222, 725–730, 2003.

Fung, F. and Hughson, W.G., Health effects of indoor fungal bioaerosol exposure, *Appl. Occup. Environ. Hyg.*, 18, 535–544, 2003.

Gallis, H.A., Microbial ecology and normal flora of the human body, in *Zinsser Microbiology*, Joklik, W.K. and Willet, H.P., eds., Appleton-Century-Crofts, New York, 1976, pp. 404–412.

Galazka, A.M., Robertson, S.E., and Kraigher, A., Mumps and mumps vaccine: a global review, *Bull. World Health Org.*, 77, 3–14, 1999.

Gardner, D.E., Crapo, J.D., and Massaro, E.J., Eds., *Toxicology of the Lung*, Target Organ Toxicology series, Raven Press, New York, 1988.

Gardner, D.E., The use of experimental infections to monitor improvements in pulmonary defense, *J. Appl. Toxiocol.*, 6, 385–388, 1988.

Gershon, A.A., Varicella-zoster virus: prospects for control, *Adv. Pediatr. Infect. Dis.*, 10, 93–124, 1995.

Goodlow, R.J. and Leonard, F.A., Viability and infectivity of microorganisms in experimental airborne infection, *Bacteriol. Rev.*, 25, 182, 1961.

Graham, J.A., Gardner, D.E., Blomer, E.J., House, D.E., Menache, M.G., and Miller, F.J. Influence of exposure patterns of nitrogen dioxide and modifications by ozone on susceptibility to bacterial disease in mice, *J. Toxicol. Environ. Health*, 21, 113–125, 1987.

Hadfield, T.L., McEvoy, P., Polotsky, Y., Tzinserling, V.A., and Yakovlev, A.A., The pathology of diphtheria, *J. Infect. Dis.*, Suppl. 1, S116–S120, 2000.

Hatch, G.E., Slade, R., Boykin, E., Miller, F.G., and Gardner, D.E., Correlation of effects of inhaled versus intratracheally injected metals on susceptibility to infections, *Am. Rev. Respir. Dis.*, 124, 167–173, 1981.

Hatch, T.F., Distribution and deposition of inhaled particles in respiratory tract, *Bacteriol. Rev.*, 25, 237–240, 1961.

Hatch, T.F. and Gross, P., *Pulmonary Deposition and Retention of Inhaled Aerosols*, Academic Press, New York, 1964, pp. 137–145.

Henderson, D.A., Inglesby, T.V., Bartlett, J.G., et al. Smallpox as a biological weapon: medical and public health management, JAMA, 281:2127–2137, 1999.

Hirano, S.S. and Upper, C.D., Bacteria in the leaf ecosystem with emphasis on Pseudomonas syringae-a pathogen, ice nucleus, and epiphyte, *Microbiol. Mol. Biol. Rev.*, 64, 624–653, 2000.

Jackson, R.J., Ramsay, A.J., Christensen, C.D., Beaton, S., Hall, D.F., and Ramshaw, I.A., Expression of mouse interleukin-4 by a recombinant ectromelia virus suppresses cytolytic lymphocyte responses and overcomes genetic resistance to mousepox, *J. Virol.*, 75, 1205–1210, 2001.

Jarvis, B.B., Stachybotrys chartarum: a fungus for our time, *Phytochemistry*, 64, 53–60, 2003.

Jennison, M.W., Atomizing of mouth and nose Secretions into the air as revealed by high-speed photography, in *Aerobiology*, Moulton, F.R., ed., American Association for the Advancement of the Sciences, Washington, DC, 1942, pp. 106–128.

Jernigan, D.B., Raghunathan, P.L., Bell, B.P., Brechner, R., Bresnitz, E.A., et al. Investigation of bioterrorism-related anthrax, United States, 2001: epidemiologic findings, *Emerg. Infect. Dis.*, 8, 1019–1028, 2001.

Joseph Wheat, L., Current diagnosis of histoplasmosis, *Trends Microbiol.*, 11, 488–494, 2003.

Kaminski, E., Stawicki, S., and Wasowicz, E., Volatile flavor compounds produced by molds of aspergillus, penicllium, and fungi Imperfecti, *Appl. Microbiol.*, 27, 1001, 1974.

Kaufmann, A.F., Fox, M.D., Boyce, J.M., Anderson, D.C., Potter, M.E., Martone, W.J., and Patton, C.M., Airborne spread of brucellosis, in *Airborne Contagion*, Kundsin, R.B., Ed., Annals of New York Academy of Science, New York, 1980. pp. 105–114.

Kato-Maeda, M., Gao, Q., and Small, P.M., Microarray analysis of pathogens and their interaction with hosts, *Cell Microbiol.*, 3, 713–719, 2001.

Ketchum, P.M., *Microbiology: Concepts and Applications*, John Wiley & Sons, New York, 1988.

Kundsin, R.B., Opening Remarks. In R.B. Kundsin, (ed.), Airborne contagion. Annals of New York Academy of Sciences, New York, 1980, pp.1–2.

Lange, D.J., Herpesviruses, in Zinsser Microbiology, Joklik, W.K. and Willett, H.P., Eds., Appleton-Century-Crofts, New York, 1976, pp.947–949.

Lee, J.V. and West, A.A., Survival and growth of Legionella species in the environment, *J. Appl. Bacteriol.* (Symp. Suppl.) 70, 121S–129S, 1991.

Loens, K., Ursi, D., Goossens, H., and Ieven, M., Molecular diagnosis of Mycoplasma pneumoniae respiratory tract infections, *J. Clin. Microbiol.*, 41, 4915–4923, 2003.

Lord, M.J., Jolliffe, N.A., Marsden, C.J., Pateman, C.S., Smith, D.C., Spooner, R.A., Watson, P.D., and Roberts, L.M., Ricin. Mechanisms of cytotoxicity, *Toxicol. Rev.*, 22, 53–64, 2003.

Lord, J.M., Roberts, L.M., and Robertus, J.D., Ricin: structure, mode of action, and some current applications, *FASEB J.*, 8, 201–208, 1994.

Mannis, M.J., Tamaru, R., Roth, A.M., Burns, M., and Thirkill, C., Acanthamoeba sclerokeratitis, *Arch. Ophthalmol.*, 104, 1313, 1986.

Manuel, J.S., Dedicated outdoor air systems: rx for sick buildings, *Environ. Health Perspect.*, 111, A712–A715, 2003.

Manzeniuk, I.N., Stepanshin, I.G., and Svetoch, E.A., Evaluation of the potential of chemotherapy of glanders caused by Pseudomonas mallei strains resistant to sulfanilamides and trimethoprim, *Antibiot Khimioter.*, 39, 30–34, 1994.

Marrie, T.J., *Coxiella burnetii* pneumonia, *Eur. Respir. J.*, 21, 713–719, 2003.

McClellan, R.O. and Henderson, F.F., Eds., Concepts in Inhalation Toxicology, Hemisphere Publishing Co., New York, 1989.

Miller, F.J. and Menzel, D.B., Eds., *Extrapolation Modeling of Inhaled Toxicants*, Hemisphere Publishing Co., New York, 1989.

Mock, M. and Fouet, A., Anthrax, *Annu. Rev. Microbiol.*, 55, 647–671, 2001.

Müllbacher, A. and Lobigs, M., Creation of killer poxvirus could have been predicted, *J. Virol.*, 75, 8353–8355, 2001.

National Research Council, *Human Exposure Assessment for Airborne Pollutants: Advances and Opportunities*, National Academy Press, Washington, DC, 1991.

Olle-Goig, J.E. and Canela-Soler, J., An outbreak of *Brucella melitensis* infection by airborne transmission among laboratory workers, *Am. J. Public Health*, 77, 335–338, 1987.

Parker, R.F., Davis, J.K., and Cassell, G.H., Short term exposure to nitrogen dioxide enhances susceptibility to respiratory mycoplasmosis and decreases in intrapulmonary killing of mycoplasma pulmonia, *Am. Rev. Respir. Dis.*, 140, 502–512, 1989.

Peiris, J.S.M., Yuen, K.Y., Osterhaus, A.D.M.E., and Stöhr, K., The Severe Acute Respiratory Syndrome, *JAMA*, 349, 2431–2441, 2003.

Phalen, R.F., *Inhalation Studies: Foundation and Techniques*, CRC Press, Boca Raton, FL, 1984.

Podschun, R. and Ullmann, U., *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors, *Clin. Microbiol. Rev.*, 11, 589–603, 1998.

Poland, G.A. and Neff, J.M., Smallpox vaccine: problems and prospects, *Immunol. Allergy Clin. North Am.*, 23, 731–743, 2003.

Pomerantsev, A.P., Staritsin, N.A., Mockov, Y.V., and Marinin, L.I., Expression of cereolysine AB genes in *Bacillus anthracis* vaccine strain ensures protection against experimental hemolytic anthrax infection, *Vaccine*, 15, 1846–1850, 1997.

Poupard, J.A., Miller, L.A., and Granshaw, L., The use of smallpox as a biological weapon in the French and Indian War of 1763, *ASM News*, 55, 122–124, 1989.

Regis, E., *The Biology of Doom: The History of America's Secret Germ Warfare Project*, Henry Holt and Company, New York, 1999.

Reid, A.H. and Taubenberger, J.K., The origin of the 1918 pandemic influenza virus: a continuing enigma, *J. Gen. Virol.*, 84, 2285–2292, 2003.

Rollins, S.E., Rollins, S.M., and Ryan, E.T., *Yersinia pestis* and the plague, *Am. J. Clin. Pathol.*, 119 (Suppl.) S78–S85, 2003.

Salem, H. and Aviado, D.M., Physiology of the cough reflex, in *International Encyclopedia of Pharmacology and Therapeutics*, Secion 27, Vol. 1. *Antitussive Agents*, Salem, H. and Aviado D.M., Eds., 1970, pp. 233–270.

Salem, H., Principles of inhalation toxicology, in *Inhalation Toxicology*, Salem, H., ed., Marcel Dekker, New York, 1987, pp. 1–3.

Salem, H., Kennedy, G.L., Morris, J.B., Roloff, M.V., Ulrich, C.E., Valentine, R., and Wolff, R.K., Recommendations for the conduct of acute inhalation limit tests., *Fundam. Appl. Toxiocol.*, 18, 321–327, 1992.

Savolainen, C., Blomqvist, S., and Hovi, T., Human rhinoviruses. *Paediatr. Respir. Rev.*, 4, 91–98, 2003.

Schneider-Schaulies, S. and ter Meulen, V., Pathogenic aspects of measles virus infections, *Arch. Virol. Suppl.*, 15, 139–158, 1999.

Shelton, B.G., Kerbel, W., Witherell, L., and Millar, J.D., Review of Legionnaires' disease, *AIHAJ*, 61, 738–742, 2000.

Spendlove, J.C. and Fannin, K.F., Source, significance, and control of indoor microbial aerosols: human health aspects, *Public Health Rep.*, 98, 229, 1983.

Spengler, J.D. and Sexton, K., Indoor air pollution: a public health perspective, *Science*, 221, 9–17, 1983.

Staszkiewicz, J., Lewis, C.M., Colville, J., Zervos, M., and Band, J., Outbreak of *Brucella melitensis* among microbiology laboratory workers in a community hospital. *J. Clin. Microbiol.*, 29, 287–290, 1991.

Stepanshin, I.G., Manzeniuk, I.N., Svetoch, E.A., and Volkovoi, K.I., In vitro development of fluoroquinolone resistance in the glanders pathogen, *Antibiot. Khimioter.*, 39, 30–33, 1994.

Torok, T.J., Tauxe, R.V., Wise, R.P., et al., A large community outbreak of salmonellosis caused by intentional contamination of restaurant salad bars, *JAMA*, 278, 389–395, 1997.

Watt, J.P., Levine, O.S., and Santosham, M., Global reduction of Hib disease: what are the next steps? Proceedings of the meeting Scottsdale, Arizona, September 22–25, 2002, *J. Pediatr.*, 143 (6 Suppl), S163–S187, 2003.

Webby, R.J. and Webster, R.G., Are we ready for pandemic influenza? *Science*, 302, 1519–1522, 2003.

Wells, W.F., On air-borne infection: II. Droplets and droplet nuclei, *Am. Hyg.*, 20, 611, 1934.

Wells, W.F., Air-borne contagion and air hygiene, Harvard University Press, Cambridge, MA, 1955.

Wells, W.F., Ratcliffe, H.L., and Crumb, C., On the mechanism of droplet-nucleus infection. II. Quantitative experimental air-borne tuberculosis in rabbits, *Am. J. Hyg.*, 47, 11, 1948.

Wilson, M. and Lindow, S.E., Release of recombinant microorganisms, *Annu. Rev. Microbiol.*, 47, 913–944, 1993.

Wilson, M. and Lindow, S.E., Ecological similarity and coexistence of epiphytic ice-nucleating (Ice+) *Pseudomonas syringae* strains and a non-ice-nucleating (Ice-) biological control agent, *Appl. Environ. Microbiol.*, 60, 3128–3137, 1994.

Wright, R.B., Pomerantz, W.J., and Luria, J.W., New approaches to respiratory infections in children. Bronchiolitis and croup, *Emerg. Med. Clin. North Am.*, 20, 93–114, 2002.

Yeh, S.H., Pertussis: persistent pathogen, imperfect vaccines, *Expert Rev. Vaccines*, 2, 113–127, 2003.

Young, D. and K. Duncan. 1995. Prospects for new interventions in the treatment and prevention of mycobacterial disease, *Annu. Rev. Microbiol.*, 49, 641–673.

36 Emerging Biothreats: Natural and Deliberate

Jessica M. Mann and Nancy D. Connell

CONTENTS

36.1 INTRODUCTION

Emerging and reemerging pathogens continue to maintain a constant presence in the world, presenting new challenges to health care workers and scientists. Some of these pathogens are also considered potential weapons of mass destruction ("select agents") or cause exotic diseases, placing them with classic agents of concern such as anthrax and smallpox. Pathogens have the ability to cause epidemics, and with global travel and intermixing populations, the possibility for large pandemics is an ever-present concern. Many select agents are prevalent in the world, still causing disease in many people every year. Some pathogens that have been relatively well controlled have developed resistance to antibiotics and other drugs used to treat disease. The deliberate release of an agent or emergence of a new strain or organism could lead to the rapid spread of a disease throughout the world before the health care system realized there was a problem: The recent severe acute respiratory syndrome (SARS) is an excellent example of the phenomenon. The possibility of this scenario has led the scientific, health care, and public health communities to look outside their realms of expertise and develop collaborations with each other. These groups now realize that rapid, coordinated recognition of an outbreak, whether natural or deliberate, is essential for its control.

In present-day society, a large proportion of the population is susceptible to new emerging diseases, such as West Nile Virus and SARS, as well as to the classic pathogens that cause smallpox, plague, polio, etc. Most people in developed countries have been vaccinated against those bacteria and viruses that ravaged populations centuries ago. Smallpox has since been eradicated and a large percentage of the world's population has not been vaccinated or exposed to the disease. This makes smallpox a scary and efficient weapon, because one case of smallpox disease might represent an epidemic in the unvaccinated and unprotected society. Other diseases of interest to those in the arena of biological defense include anthrax, plague, and tularemia. Also, emerging pathogens such as West Nile Virus and the virus that causes SARS have been the objects of significant attention in the scientific community because of their high prevalence and pathogenicity.

Bioterrorism is not a new phenomenon. Plague and smallpox have been used in war; *Salmonella typhimurium* was used in a domestic U.S. terrorist event by the Rajneeshee cult in 1984. Cult members in Oregon sprinkled bacteria on salad bars, causing hundreds of people to fall ill with food poisoning. We will explore ancient epidemics, both natural and deliberate, caused by agents that are today considered to be weapons of mass destruction.

The greatest pandemic in the world was caused by *Yersinia pestis*, the causative agent of plague, in the 1300s, and massive epidemics continued throughout the 1500s and 1600s. Also known as the Black Death, plague killed millions of people, approximately a third of the population of Europe and Asia. The disease generated social upheaval, leading to the destruction of the feudal system in England and the decreasing role of the Church in politics (Cartwright, 1972). Plague was also used during this time by the Tartars in the Siege of Kaffa. The Tartars threw the dead bodies of plague victims over the walls into Kaffa, to spread the disease, in an effort to take control of the trading post. The Tartars were carriers of the Black Death as they continued their travels. This was an early example of using a disease to fight an opponent, in other words, an early form of biological warfare (Cartwright, 1972).

Plague and smallpox caused such devastating effects due to the lack of protection within the population against infectious disease. Conditions were not sanitary, rodents ran rampant throughout villages and cities, carrying fleas and spreading the plague. Because of the high morbidity and mortality seen with these epidemics, it is possible that it was pneumonic plague instead of the more common bubonic form. Similarly, smallpox was prevalent in populations that had not been previously exposed to the disease. For example, when exploration of the Americas began, settlers brought smallpox with them, and the susceptible tribes experienced extreme mortality. As new generations were born, smallpox was again an issue because the small children were susceptible. There were instances of people self-vaccinating against smallpox, a method described as variolation. This method was useful at times, but many people still contracted the disease. In the twentieth century, a vaccine was produced for smallpox, antibiotics and vaccines were developed for plague, and technological advances for sanitation and rodent control were developed and helped to bring these pathogens under control. Smallpox has since been eradicated, because humans are its only host, but there are still instances of plague, mainly bubonic, in the world every year in endemic areas, such as the southwestern United States.

Today, potential agents of biowarfare are categorized by the Centers for Disease Control into three classes, A, B, and C. The regulations that created this categorization were developed in the wake of several domestic incidents with possession of biological agents for terrorist purposes. The regulations have become more stringent in the wake of the September 11th attacks and the October 2001 anthrax letters. Category A and B agents are called Select Agents and are strictly regulated by federal and state laws in the United States. Agents are placed in categories based on their availability, ease of dissemination and production, and morbidity and mortality from disease. Category A agents include some discussed in this chapter, such as anthrax, plague, tularemia, botulinum toxin, and smallpox. These agents are listed as "A" agents because they are easily produced, easily disseminated, can cause extreme public panic, and will require special efforts from the public health system to contain the outbreaks. Infections by these organisms tend to be acute; some are highly contagious and very rare. Category B agents include glanders, ricin toxin, and the tick-borne encephalitis viruses among many others. These organisms are not as easy to disseminate and/or the disease is not as acute, and morbidity and mortality figures are less than those of Category A agents. Category C includes agents that could be engineered for mass production, such as multidrup-resistant tuberculosis, Hantavirus, and tick-borne hemorrhagic fever viruses. These agents have the ability to cause a major public health impact and they are prevalent in society. Some agents listed as category C agents are those that are emerging or reemerging infections, such as tuberculosis.

Bacillus anthracis, the causative agent of anthrax, has played an important role throughout history in the fields of microbiology and immunology. Robert Koch used anthrax to develop his postulates in 1876, which established a microbial link to causation of disease for the first time. Louis Pasteur

developed the first live vaccine using this bacterium in 1881. Anthrax is found mainly in herbivores and is an accidental pathogen of humans, usually when a person handles contaminated hides or meat. This disease is called Woolsorter's disease but has almost been completely eliminated in developed nations by immunization (Broussard, 2001). In the second half of the nineteenth century, anthrax showed its ability to cause inhalational disease in humans when a group of Woolsorters fell ill because of the aerosolization of infectious spores in animal hides. This outbreak was the first reported incident of a respiratory infectious disease acquired in the workplace (Friedlander, 1997). The ability of *Bacillus anthracis* to sporulate and spread easily via aerosols thus causing great mortality led military leaders to recognize anthrax as a possible agent for biological warfare. This recognition and concern was affirmed when there was an accidental release of anthrax spores from a military research facility in Sverdlovsk, Russia in 1979. The release caused the largest inhalational anthrax epidemic in history, with a total of 68 confirmed deaths (Meselson et al., 1994). Casualties were seen in humans and in animals (Friedlander, 1997).

In the fall of 2001, bioterrorism presented itself in the United States in the form of letters containing white powder, which were sent through the U.S. mail to media companies and government buildings. These letters contained purified anthrax spores. There were cases of cutaneous and inhalational anthrax in several states, including Florida, New York, and New Jersey. Cross-contamination occurred in post offices via letters and equipment (Greene et al., 2002). Several post offices are still closed as of the summer of 2003. These attacks spawned new legislation regulating the import/transfer and possession of these agents more stringently than previous legislation.

36.2 ANTHRAX

Bacillus anthracis is a gram-positive, spore-forming, nonmotile bacillus (1–1.5 μm × 3–10 μm). The bacterium can grow readily on many different nutrient media and is nonhemolytic when grown on sheep blood agar plates, a characteristic used for identification and distinction from other species of *Bacillus.* In all growth conditions, *B. anthracis* forms a capsule, whereas sporulation occurs only in the environment or when the organism is experimentally starved for nutrients. The endospores are resistant to heat and dessicaiton and survive in the soil for decades. *B. anthracis* has two virulence plasmids; one codes for an antiphagocytic capsule and the other for a tripartite toxin. The capsule is composed of a poly-D-glutamic acid polymer which confers resistance to phagocytosis and which may also contribute to the resistance of anthrax bacteria to the lysis by serum cationic proteins (Friedlander, 1997).

The tripartite toxin is composed of three subunits, to form the A-B toxin binding, protective antigen (PA), lethal factor (LF), and edema factor (EF). Protective antigen is common to both toxins. If PA combines with LF it produces lethal toxin, which causes shock and death. If it combines with EF it will produce the edema toxin. PA binds to the surface of the target cell and delivers the enzymatic proteins to the cytosol. EF is an adenylate cyclase whose catalytic properties are dependent on the presence of eukaryotic cytoplasmic cofactor calmodulin and calcium. EF is responsible for affecting monocyte cytokine profiles, creating cAMP, which alters the water homeostasis and can also disrupt phagocytic antibacterial responses (Hanna, 1998). Lethal factor is a zinc metalloprotease, exhibits a cytotoxic effect on macrophages, and causes shock and death of cells. LF cleaves MEK1 which blocks activation of the mitogen-activated protein kinase kinase (MAPKK) pathways (Duesbery et al., 2001). The MAPKK isoforms MEK1 and 2 are the cytosolic targets for LF activity. These isoforms are dual specific kinases with roles in cell growth, proliferation, differentiation, and apoptosis and are also key mediators of signal transduction from the cell surface to the nucleus. The signaling pathways involving MAPKK1, 2, and 3 are crucial in the activation of macrophages and are involved in the production of cytokines (Pellizzari et al., 1999).

It has been shown that the deletion of the toxin plasmid (Friedlander, 1997), the capsule plasmid (Friedlander, 1997), or the gene coding protective antigen (Friedlander, 1997) can attenuate the organism. The gene for transcriptional activation, AtxA, which mediates the toxin and the capsule

genes, regulates virulence of the organism. This gene controls regulation in environments where the temperature is greater than 37°C, a carbon dioxide concentration of more than 5% and in the presence of serum (Broussard, 2001). Also, it has been shown that in mouse models lethal factor plays a more significant role in virulence than the edema toxin (Friedlander, 1997). The clinical manifestations of anthrax infection vary depending on the route of infection, but anthrax disease is not transmissible from person to person. Cutaneous anthrax, the most common form of anthrax disease, has an incubation period of 1–5 days. The first sign of disease is a small papule, which progresses into a vesicle after a few days. This vesicle will rupture and leave a necrotic ulcer, which then forms the characteristic black eschar. This eschar is present for approximately two to three weeks. This ulcer is relatively painless, and patients usually present with edema at the infection site, headache, fever, and malaise. Cutaneous anthrax is easily cured with antibiotic treatment.

Inhalational anthrax manifests with nonspecific symptoms 1–6 days postinfection. These symptoms include headache, fever, and malaise, and a nonproductive cough may be present. Symptoms persist for 2–3 days, followed by a short recovery period. Following this short recovery period, a sudden onset of respiratory stress will occur. A chest x-ray will show the characteristic widening of the mediastinum. Shock and death will follow within 24 h. Despite treatment, mortality is essentially 100%.

Gastrointestinal anthrax occurs after the ingestion of insufficiently cooked contaminated meat. This form of the disease begins with nausea, vomiting, fever, with severe abdominal pain following the first wave of symptoms. Mortality from this form can be as high as 50%. Cases of oropharyngeal anthrax have occurred also due to ingestion of contaminated meat. This manifests with a sore throat, or a local oral ulcer. Swelling of the neck can occur accompanied by a fever due to edema at the site. This form also has a mortality of approximately 50%.

Bacillus anthracis is listed as a Category A select agent. This bacterium is extremely easy and inexpensive to cultivate, because it grows readily on many different bacteriological media. *B. anthracis*, found ubiquitously in nature, is a zoonotic bacterium, thus eradication is not possible because the organism can be isolated from soil and still is a prevalent cause of disease in livestock. As previously mentioned, this bacterium forms protective spores, which allow it to persist in nonadvantageous environments. The spores are stable for decades in many different environments. The spores, when finely milled, are able to attach to the bronchi and begin growing and replicating. This powder is easily disseminated and is extremely effective at causing disease via the respiratory route. An additional characteristic that has led to the development of anthrax as a biological weapon is that the symptoms are nonspecific. Early diagnosis of anthrax is difficult, the presenting symptoms can be easily confused with the common cold or flu virus, but if it is not detected early, inhalational anthrax has an extremely high mortality rate.

36.3 PLAGUE

Yersinia pestis, the causative agent of plague, was responsible for three devastating pandemics and continues to be prevalent throughout the world. The first plague epidemic was in AD541, beginning in Egypt and sweeping across Europe, Africa, and Asia. Population losses were between 50 and 60% (Inglesby et al., 2000). The second pandemic lasted 130 years and is known as the Black Death. It began in 1346 and by its conclusion killed 20–30 million people; it had major political, cultural, and religious ramifications (Inglesby et al., 2000). The third pandemic, in China in 1855, spread to all inhabited continents and killed 12 million people in China and India alone (Inglesby et al., 2000). There have also been instances throughout history in which *Y. pestis* has been used as a biological weapon. During World War II (WWII), Unit 731, a branch of the Japanese Army dropped plague-infected fleas over areas of China, thus causing plague outbreaks (Inglesby et al., 2000). During the years after WWII, as a part of the United States Offensive Biological Weapons Program and the Soviet Union program, scientists developed techniques to aerosol the plague bacterium, thus eliminating the zoonotic spread of the disease. Also, in 1995 a microbiologist attempted to acquire plague illegally. This acquisition inspired new antiterrorism legislation in the United States.

Plague is a zoonotic infection, spread by rodents carrying fleas infected with the *Y. pestis* bacterium. *Y. pestis* is a nonmotile, gram-negative, non-spore-forming bacillus, with a bipolar staining morphology. The bacterium is a lactose nonfermenter, it is urease and indole negative, and it grows well at 28°C on MacConkey Agar or blood agar.

Yersinia bacteria persist in lymphatic tissue and Peyer's Patches. Bacteria colonize the liver and spleen and can cause death in three to four days. There are three manifestations of plague, bubonic, septicemic, and pneumonic. Bubonic plague is caused by a bite from an infected flea. A bubo will form at the site of infection approximately one day after the primary symptoms of fever, malaise, and chills appear. Persons infected with fleas may also develop the disease minus the bubos. This form is known as septicemic plague. There are instances where persons with bubonic plague develop septicemic plague. Septicemia plague can cause necrosis of blood vessels and intravascular coagulation, thus producing the characteristic gangrene on the nose, fingers, and toes. Secondary pneumonic plague can develop from the bubonic and septicemic forms in about 50% of cases. This form manifests when bacilli move to the lungs and patients experience symptoms of severe bronchitis (Inglesby et al., 2000). Primary pneumonic plague occurs due to the inhalation of bacilli and occurs rarely in the United States. This form of plague can be contracted after handling infected animals or after a deliberate release of aerosolized *Yersinia* bacteria. These bacteria are able to resist the host primary immune defenses because of a plethora of virulence factors.

Y. pestis possesses three virulence plasmids, pCD1 (also known as pYV [Cornelius, 2000]) and pMT1 and pPCP1 (Fields et al., 1999). This bacterium also possesses additional chromosomally encoded virulence factors; a possible adhesion protein and lipopolysaccharide (Benner et al., 1999). pMT1 encodes a murine toxin and a capsular fraction 1 protein. A plasminogen activator serine protease, Pla, is encoded by pPCP1. Pla is an outer membrane protease required for full virulence from peripheral infectious routes (Fields et al., 1999). The pCD1 is a 70–75 kb low-calcium response plasmid, which encodes the V antigen (LcrV) and the Yops proteins. Also encoded on this plasmid is a specialized apparatus for secretion and translocation of bacterial proteins into host cells. *Yersinia pestis* encodes a type III secretion system (Ysc) on pCD1, which translocates and secretes the Yops proteins and LcrV. The low-calcium response plasmid is thermally regulated by LcrF, a transcriptional activator, to maximally induce LcrV and the Yops proteins only when in contact with a eukaryotic cell or when the bacterium is in a calcium-deficient medium. LcrV and most of the Yops proteins have been shown to be essential for virulence (Fields et al., 1999). There are three categories of Yops proteins: those with direct antihost functions, translocation proteins, and regulatory proteins. Those with direct antihost function are mainly translocated into the cell and can cause cytotoxicity (YopE) and apoptosis induction (YopJ) and inhibit platelet aggregation *in vitro* by binding to thrombin (yopM) (Heuck, 1998). The regulatory proteins (YopN, LcrG, LcrV, and LcrQ) mediate cell contact dependent induction of Yop gene expression and secretion (Heuck, 1998).

Y. pestis is also included in the Category A listing of select agents. Unlike anthrax, a person with pneumonic plague can transmit the disease to other persons within close proximity. This transmissibility along with the ease of production and dissemination placed *Y. pestis* in the category favored by those looking to develop biological weapons. This bacterium is also a zoonotic pathogen, using fleas and rodents as its host carriers. Plague still occurs throughout the world, in the southwestern area of the United States, for example, mainly in the bubonic form. Pneumonic plague, the form that would most likely be seen in a biological attack, has a high mortality rate, and there is no vaccine available for preventative treatment and protection of the population. As with anthrax, this disease presents with commonly seen, nonspecific symptoms, thus clouding the ability to properly diagnose and treat patients.

36.4 TULAREMIA

Francisella tularensis, the causative agent of tularemia, is one of the most infectious of the biothreat agents. The disease, known also as rabbit fever, was first described in 1911 as a plaguelike disease in rodents and as a severe and fatal disease in humans. Large outbreaks occurred in Europe and the

USSR in the 1930s and 1940s due to a contaminated water supply (JAMA, 2001). There were also zoonotic cases in the United States at the same time (Dennis et al., 2001). This disease gained notoriety as a result of being a prevalent laboratory-acquired biohazard because of the extreme infectivity (LD_{50} = 10 CFU) (Dennis et al., 2001). Effective methods to disseminate the tularemia bacterium were developed by the United States in the 1950s and 1960s as a part of the proliferation and development of offense biological weapons. Unit 731, the Japanese unit known for its use of biological weapons in warfare, studied tularemia from 1932 to 1945 (Dennis et al., 2001).

Tularemia is caused by a small, nonmotile, aerobic, gram-negative, coccobacillus. Strains of *F. tularensis* are divided into two biovars, types A and B. The two types are differentiated based on virulence testing, biochemical reactions, and epidemiological factors. Type A strains, such as the SCHU strain, are highly virulent in humans and animals and are most commonly found in North America. Type B strains are relatively avirulent and are most common in Europe. A major difference between these types is that type A strains can make acid from glycerol, whereas type B strains lack this ability.

Little is known about the virulence of *F. tularensis*. The organism survives in macrophages and has the ability to combat host responses to survive and replicate. There have been studies focusing on the murine model of tularemia disease. This model demonstrates that neutrophils are critical in the first few days of infection (Stenmark et al., 1999). In the early stages of murine infection, interleukin 12 (IL-12) and tumor necrosis factor alpha (TNFα) are expressed by mononuclear phagocytes. These cytokines then stimulate the production of interferon gamma (IFNγ) by NK cells. If these cytokines are neutralized early on in the infection process, the disease symptoms are lethally exacerbated (Stenmark et al., 1999).

Tularemia has been studied for years by many governments and other entities to develop techniques for dissemination and production of biological weapons. Because the infectivity of this agent, tularemia is also listed as a Category A agent. Tularemia is not transmissible person to person and does not cause an acute infection. Tularemia infections are usually limiting with low mortality but people are inconvenienced and incapacitated. There is no licensed vaccine for tularemia, but most cases are controlled by antibiotic treatment. *F. tularensis* is more fastidious than *B. anthracis* or *Y. pestis*, but is still an effective incapacitating agent.

36.5 BOTULISM

Botulinum intoxication occurs via exposure to the toxin or by infection with the *Clostridium botulinum* bacterium, which produces the most poisonous neurotoxin known. The extreme lethality and potency of botulinum toxin have led to its development as a biothreat agent beginning over sixty years ago: a single gram of dispersed and inhaled toxin could kill more than one million people. There have been instances of the use of this toxin throughout history, such as the production of toxin by the Japanese extremist group, Aum Shinrikyo. The group spread botulinum toxin aerosols over Tokyo three times between 1990 and 1995 (Arnon et al., 2001). The attempts failed because of poor microbiological techniques, deficient aerosol-generating equipment, and internal sabotage (Arnon et al., 2001).

Botulinum intoxication is a naturally occurring disease, affecting mainly children and people who have consumed contaminated home-canned food items. There are three forms of natural botulism: food borne, intestinal, and wound. Inhalational botulism is a man-made form of botulism and is caused by the dispersion of botulinum toxin aerosols. All forms of botulism exhibit virtually the same neurological symptoms, with abdominal cramps and vomiting preceding these symptoms in gastrointestinal intoxication instances (Arnon et al., 2001). These symptoms include double vision, and acute, febrile, symmetric descending paralysis. The trademark symptom of botulism is the presence of multiple cranial nerve palsies. The rapidity and onset of these symptoms directly relates to the amount of toxin that has absorbed into the bloodstream. Intoxication does not cross the blood–brain barrier, so patients with botulism are still lucid, even though speech may be slurred and they may appear lethargic (Arnon et al., 2001).

There are eight immunologically distinct botulinum neurotoxins (denoted A, B, C1, C2, D, E, F, and G). Types A, B, E, and F are responsible for human poisonings, with type A being the form most commonly responsible for food-borne outbreaks. The types of toxin are defined by the absence of cross-neutralization and serve as epidemiological markers (Arnon et al., 2001). The type A neurotoxin is a complex of hemagglutinin and neurotoxin. The hemagglutinin protects the neurotoxin from the stomach acids and lowered pHs to maintain stability and cause botulism disease (Boyd, 1995). The toxin is released in the intestine, absorbed, and then transported to the lymph via the bloodstream. Clinical symptoms will appear 24–72 h postabsorption. Botulinum toxin acts on the the peripheral nervous system, the cranial nerves, in particular, not the central nervouse system (CNS) (Boyd, 1995). The toxin is composed of a 100-kDa "heavy chain," simple dichain polypeptide joined to a 50-kDa "light chain" by a single disulfide bond (Arnon et al., 2001). The light chain is a zinc-containing endopeptidase that blocks acetylcholine-containing vesicles from fusing with the terminal membrane of motor neurons. This inhibition of transmission results in flaccid paralysis (Arnon et al., 2001). Fatality rates are high, approximately 75% for type A intoxication (Boyd, 1995) with the only treatment being a trivalent antitoxin containing neutralizing antibodies against types a, b, and e (Arnon et al., 2001).

There have already been attempts to use botulinum toxin as a biological weapon: Aum Shinrikyo tried on several occasions between 1990 and 1995 to release botulinum toxin in Tokyo (Arnon et al., 2001). The toxin's extreme toxicity and low infectious dose have led to its development by a number of countries, before and after the Biological Weapons Convention of 1972, in the effort to stockpile an effective bioweapon (Arnon et al., 2001).

36.6 SMALLPOX

Smallpox disease is caused by the Variola major virus. This is the only virus in history to be eradicated throughout the entire world. Humans are the only host of smallpox, and eradication occurred in 1977 with the last case of smallpox disease in Ethiopia (Henderson et al., 1999). Once the disease was eradicated, vaccination programs slowed and eventually ceased in 1980 after recommendation by the World Health Organization (WHO) (Henderson et al., 1999). With eradication came the agreement that there would be only two stocks of Variola major virus in the world, at the centers for disease control in Atlanta, GA, and in the Institute of Virus Preparations in Moscow, Russia (Henderson et al., 1999). There is a concern that some smallpox stocks may have strayed from Russia as a repercussion of the smallpox proliferation studies and the breakup of the Soviet Union (Henderson et al., 1999). This concern contributes to the fear that this virus could be used as a bioweapon. Variola major virus is an attractive bioweapon because of its ease of person-to-person transmission, high mortality rate, and the lack of an effective antiviral therapy (Bronze et al., 2002). The discovery of one case of smallpox is treated will be as an international health emergency (Henderson et al., 1999).

Smallpox is transmitted from person to person by infectious aerosols and by direct physical contact, primarily to close contacts and family members. The virus has the ability to survive for extended periods within the scabs, and contaminated bed sheets remain infectious. Approximately 10–15 days postexposure, an infected person will begin to experience the early symptoms of smallpox disease. These symptoms include headache, fever, and malaise. Three to four days after initial symptoms a red rash will appear over the entire body, with small blisters/vesicles. The vesicles mature into fluid-filled lesions, also called pustules, which then dry out and form scabs in the skin. These scabs will fall off and leave sunken marks in the skin. Unlike other diseases, every smallpox lesion is at the same stage at the same time. The lesions are most prominent in the face and extremities (Henderson et al., 1999). Mortality from smallpox is between 25 and 30% (Henderson et al., 1999). In addition there are alternate forms of smallpox, flat black pox and hemorrhagic black pox. In flat black pox (also called the malignant form) the pox are black, smooth, and velvety to the touch. Pus is not formed and skin can fall away from the body in sheets (Henderson et al., 1999). The hemorrhagic form of smallpox is usually always fatal. This form

occurs when blood seeps from the infected person's orifices, and internal membranes that line the organs can and will break down.

Smallpox is a double-stranded DNA (dsDNA) virus in the genus Orthopoxvirus (family Poxviridae). It is a brick-shaped virus, with a diameter of approximately 200 nm, which has the capability of replicating in the cytoplasm of cells. Unlike the other DNA viruses, it is not dependent on replication within the nucleus. To be infectious, the virus must be completely assembled. The DNA alone is not infectious. Cell-mediated and humoral responses by the host immune system are important to combat this disease. The complement system is also an important player in the innate immune response (Bernet et al., 2003). In an effort to combat the complement system, poxviruses have complement regulatory proteins (CRPs). CRPs differ with response to ligand specificity and the mechanism of convertase inactivation. (Rosengard et al., 2002). Also, the efficacy of the viral CRPs can determine whether a cell will be destroyed or whether it will be maintained as a site for viral replication (Rosengard et al., 2002). These proteins are encoded mainly in the terminal repeat sections of the Variola major chromosome. Other genes, important in modulation and virulence, are also encoded in these regions (Rosengard et al., 2002).

There are also enzymes called SPICE, smallpox inhibitor of complement enzymes, an encoded CRP, which is composed of four short consensus repeats. SPICE is a more potent inhibitor of the complement system and effectively prevents stability of the human complement proteins. This in turn allows the smallpox virus to overcome the classical and the alternative complement pathways (Rosengard et al., 2002).

There is a vaccine available against smallpox, made with the vaccinia virus. This vaccine can be administered up to four days postexposure and can prevent/lessen disease symptoms. Immunity will last up to ten years, with boosters available to provide longer lasting immunity. There are stockpiles of vaccines with a few million doses at the CDC. The federal government has recently enacted a new vaccination policy to ensure health care workers, first responders, and laboratory workers are immunized in the event of a smallpox incident (Traynor, 2003). Also, production of the vaccine has resumed to maintain enough vaccines for the U.S. population in the event of a large-scale release of smallpox or an incident of smallpox disease and the need for prophylactic public vaccinations (Traynor, 2002).

The likelihood that smallpox will appear in the world is slim, since it has been eradicated and research on the virus has largely ceased, or has been limited to two research sites, one in the United States and the second in Russia. Thus the appearance of smallpox might indicate deliberate infection from the use of the smallpox virus as a weapon. This virus is a most feared weapon because it has been eradicated and the majority of the world population is susceptible to infection. Mortality is approximately 30% in those who experience smallpox infection, and the disease is highly contagious. Smallpox virus is very stable in powdered purified form and is easily disseminated. Until the rash appears an infected person exhibits nonspecific symptoms of disease and is already contagious. This virus is the ideal weapon in that it is easily spread and transmissible, and large segments of the population are susceptible. The downside of this weapon is the environment one needs to produce the virus safely.

36.7 WEST NILE ENCEPHALITIS

West Nile Virus is an emerging pathogen that first appeared in North America in 1999. The appearance of an "old world" flavivirus in the "new world" is contributed to the increasing global commerce and travel (Petersen, 2001). West Nile virus (WN virus) was first isolated in 1937 in Uganda and has caused disease in Africa, southern Europe, central and southern Asia, and Oceania (Xiao et al., 2001). Outbreaks have been sporadic since the discovery of the virus in 1937, with only two being considered notable, Israel (1951–54 and 1971) and South Africa (1974) (Petersen, 2001). However, West Nile virus is considered an emerging disease because of several epidemiological factors that have developed over time. The virus has increasingly caused outbreaks of disease in

humans and in horses, with an apparent increase in the severity of the human cases and a high avian death rate accompanying the human outbreaks (Petersen, 2001). A new variant of the virus has emerged and now the WN viruses can be divided into two lineages. Class WN1 is associated with clinical human encephalitis and has been isolated from many countries worldwide. The WN2 viruses are clustered in Africa and are not associated with clinical human encephalitis (Petersen, 2001). Despite the different classifications, the two viruses are antigenically similar and also show similarity to other flaviviruses. This similarity accounts for the cross-reactions in serological testing in diagnostic laboratories and thus requires diagnostic tests to be more specialized to differentiate between the different classes of viruses.

WN virus is a zoonotic disease, spread by the bite of an infected mosquito. Many infected individuals are nonsymptomatic or their symptoms are not recognized. Some patients may experience a denguelike illness, and some may have frank meningitis and encephalitis. Unlike other closely related flaviviruses, WN virus targets the Purkinje cells of the cerebellum (Xiao et al., 2001). WN virus is a 30- to 35-nm icosehedral core virus composed of multiple copies of its 12-kDa capsid protein. The viral capsid contains a single-stranded positive strand RNA, 11,000 nt in length (DeGroot et al., 2001). An envelope surrounds the capsule, is derived of host-cell components, but has been modified by the insertion of two integral membrane glycoproteins, E (53 kDa) and prM (18–20 kDa) (Petersen, 2001). The fully infectious virion particle measures 45 nm to 50 nm in diameter (Petersen, 2001). The E-glycoprotein is the viral hemagglutinin and also mediates cell binding. This protein elicits the most virus-neutralizing antibodies (Petersen, 2001). The prM protein is cleaved to M protein late in virus maturation by a cellular protease and is then incorporated into the virion (Petersen, 2001). There is also a capsid protein and five nonstructural proteins, which includes an RNA-directed polymerase (DeGroot et al., 2001).

The pathogenesis of this virus is still largely unknown. The model for pathogensis is borrowed from dengue and JE virus, both flaviviruses and similar to WN virus. This model suggests that the Langerhans cells in the epidermis play an important role in the up-regulation of immunological proteins that respond to viral infection and are responsible for antigen processing and presentation to T cells (DeGroot et al., 2001). Also, the mobilization of dendritic cells and their presentation of antigen to T cells in lymphoid follicles may play an important role in the host response to WN virus infection (DeGroot et al., 2001). The incubation period of WN disease is 5–15 days, with detectable antibodies present at 3–7 days postinfection. However, to confirm WN virus infection, antibody testing must be performed in the acute and convalescent stages (DeGroot et al., 2001).

West Nile Virus is an emerging pathogen that most states in the United States are now battling. The disease goes largely unnoticed in the human population because many people do not exhibit symptoms, but many deaths occur in the avian population from the bites of mosquitoes. The spread of West Nile Virus illustrates the course followed by many emerging pathogens, and the pathogens' ability to spread to many geographic areas.

36.8 SARS

A novel Coronavirus, designated SARS-CoV, causes severe acute respiratory syndrome (SARS) (Rota et al., 2003). Coronaviruses are common cold viruses in humans, responsible for 15–30% of all colds in the United States each year, and also can cause infection of the gastrointestinal tract (Holmes, 2003b). In general, coronaviruses are species specific and are categorized into three classes based on host range, antigenic relationships, and genomic organization (Rota et al., 2003). Coronaviruses in groups one and two cause disease in mammals, whereas the group three viruses are avian specific. Coronaviruses are large, enveloped, positive stranded RNA viruses, with a genome of 29,727 nucleotides, for SARS-CoV. These viruses are the largest RNA viruses known, with very narrow host ranges and are very fastidious in cell culture (Rota et al., 2003). The genomic organization of SARS-CoV is characteristic of the coronavirus genome arrangement, 5′-replicase (rep), spike (S), envelope (E), membrane (M), and nucleocapsid (N)-3′ (Rota et al., 2003).

SARS was first identified in the People's Republic of China in 2002. Only after cases of severe respiratory disease sprouted in Hong Kong, Vietnam, and Canada did the World Health Organization recognize SARS as an emerging virus and issued a global alert for severe acute respiratory syndrome, thus giving a name to the coronavirus. Shortly thereafter, a coronavirus was isolated, in early 2003, which was shown to be the causative agent of SARS (Holmes, 2003a). SARS-CoV has been isolated from fecal material as well as respiratory specimens, which supports the hypothesis that this virus is spread through the oral and fecal routes, as are many other mammalian coronaviruses (Holmes, 2003a). Infection with SARS presents with a fever, nonproductive cough, and shortness of breath two to seven days postinfection. Mortality occurs in 3–10% of cases (Rota et al., 2003). There are no vaccines against SARS, although antiviral therapies against SARS are a topic of intense investigation. A promising approach is the development of protease inhibitors that will prevent the cleavage of the viral S glycoprotein or the processing of the RNA polymerase (Holmes, 2003a). As of June 11, 2003, there were 8430 cases of SARS worldwide, with 789 deaths (ProMed Digest 238; see footnote).

SARS provided a striking illustration of the ease with which an infectious disease can spread throughout the world, because most countries have experienced at least one case of SARS. The WHO implemented travel advisories in an attempt to control the spread of the disease across borders of towns, states, and countries. The SARS outbreak also serves as a model for transmission of an infectious biological weapon and highlights the strengths and weaknesses of the global health network.

36.9 CONCLUSION

The rapid development of antibiotics and vaccines during the post-WWII years led to a heady confidence of the successful eradication of infectious disease. In 1967, the U.S. Surgeon General William H. Stewart declared that infectious diseases were a thing of the past and that the attention of biomedical researches and public health officials should be focused on chronic diseases such as heart disease and cancer (Stewart, 1967). But in subsequent years, it has become increasingly clear that infectious disease is perhaps the most serious of all the contemporary challenges to global health.[1] The WHO estimates that 1500 people die of an infectious disease every hour; measles remains the leading killer of children; one person dies of tuberculosis every 15 sec. Added to the list of existing diseases are emerging infections; superimposed on these natural occurrences is the now real threat of intentional release of infectious organisms. The challenge ahead is to develop novel, coordinated, global approaches to prevention, detection, tracking, and containment of outbreaks of communicable diseases, whether unintentional or intentional.

REFERENCES

Arnon, S.S., Inglesby, T.V., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Fine, A.D., Hauer, J., Layton, M., Lillibridge, S., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T.M., Russell, P.K., Swerdlow, D.L., and Tonat, K., Working Group on Civilian Biodefense, Botulinum toxin as a biological weapon: medical and public health management, *JAMA*, 285, 1059–1070, 2001.

Benner, G.E., Andrews, G.P., Byrne, W.R., Strachan, S.D., Sample, A.K., Heath, D.G., and Friedlander, A.M., Immune response to Yersinia outer proteins and other Yersinia pestis antigens after experimental plague infection in mice, *Infect Immun.*, 67, 1922–1928, 1999.

Bernet, J., Mullick, J., Singh, A.K., and Sahu, A., Viral mimicry of the complement system, *J. Biosci.*, 28, 249–264, 2003.

[1] Access to ProMed digest is through http://www.promedmail.org. ProMed is a global electronic reporting system for outbreaks of emerging infectious diseases and toxins. This list serve is open to all sources.

Boyd, R.F., *Basic Medical Microbiology*, Lippincott Williams and Wilkins, Baltimore, 1995.

Bronze, M.S., H.M., Machado, L.J., Voskuhl, G.W., and Greenfield, R.A., Viral agents as biological weapons and agents of bioterrorism, *Am. J. Med. Sci.*, 323, 316–325, 2002.

Broussard, L.A., Biological agents: weapons of warfare and bioterrorism, *Mol. Diag.*, 6, 323–333, 2001.

Cartwright, F.F., *Disease and History*, Barnes and Noble Books, New York, 1972.

Cornelius, G.R., Molecular and cell biology aspects of plague, *Proc. Natl. Acad. Sci. USA*, 97, 8778–8783, 2000.

DeGroot, A., et al., Rapid determination of HLA-B*07 Ligands from the West Nile Virus NY99 Genome, *Emerg. Infect. Dis.*, 7, 706, 2001.

Dennis, D.T., I.T., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Fine, A.D., Friedlander, A.M., Hauer, J., Layton, M., Lillibridge, S.R., McDade, J.E., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T.M., Russell, P.K., and Tonat, K., Working Group on Civilian Biodefense, Tularemia as a biological weapon: medical and public health management, *JAMA*, 285, 2763–2773, 2001.

Duesbery, N.S., R.J., Webb, C.P., Koochekpour, S., Koo, H.M., Leppla, S.H., and Vande Woude, G.F., Suppression of ras-mediated transformation and inhibition of tumor growth and angiogenesis by anthrax lethal factor, a proteolytic inhibitor of multiple MEK pathways, *Proc. Natl. Acad. Sci. USA*, 98, 4089–4094, 2001.

Fields, K.A., N.M., Cowan, C., and Straley, S.C., Virulence role of Vantigen of Yersinia pestis at the bacteria surface, *Infection and Immunity*, 67, 5395–408, 1999.

Friedlander, A.M., Anthrax, in *Textbook of Military Medicine*, Vol. Part I: *Warfare, Weaponry and the Casualty*, Brigadier General Russ Zajtchuk, M.C., U.S. Army, Ed., Office of the Surgeon General of the Department of the Army of the United States of America, 1997, pp. 468–478.

Greene, C.M., Reefhuis, J., Tan, C., Fiore, A.E., Goldstein, S., Beach, M.J., Redd, S.C., Valiante, D., Burr, G., Buehler, J., Pinner, R.W., Bresnitz, E. and Bell, B.P., Epidemiologic investigations of bioterrorism-related anthrax, *Emerg. Infect. Dis.*, 8, 1048–1055, 2002.

Hanna, P., Anthrax pathogenesis and host response, *Curr. Top. Micobiol. Immunol.*, 225, 13–35, 1998.

Henderson, D.A., I.T., Bartlett, J.G., Ascher, M.S., Eitzen, E., Jahrling, P.B., Hauer, J., Layton, M., McDade, J., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T., Russell, P.K., and Tonat, K., Smallpox as a biological weapon: medical and public health management, *JAMA*, 281, 2127–2137, 1999.

Heuck, C., Type III secretion systems in bacterial pathogenesis of animals and plants, *Microbiol. Mol. Biol. Rev.*, 379–433, 1998.

Holmes, K.V., SARS-associated coronavirus, *N. Engl. J. Med.*, 348, 2003a.

Holmes, K.V., SARS coronavirus: a new challenge for prevention and therapy, *J. Clin. Investig.*, 111, 2003b.

Inglesby, T.V., D., D.T., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Fine, A.D., Friedlander, A.M., Hauer, J., Koerner, J.F., Layton, M., McDade, J., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T.M., Russell, P.K., Schoch-Spana, M., and Tonat, K., Plague as a biological weapon: medical and public health management, *JAMA*, 283, 2281–2290, 2000.

Meselson, M., G.J., Hugh-Jones, M., Langmuir, A., Popova, I,. Shelokov, A., and Yampolskaya, O., The Sverdlovsk anthrax outbreak of 1979, *Science*, 266, 1202–1208, 1994.

Pellizzari, R., G.-R.C., Vitale, G., Mock, M., and Montecucco, C., Anthrax lethal factor cleaves MKK3 in macrophages and inhibits the LPS/IFNgamma-induced release of NO and TNFalpha, *FEBS Lett.*, 462, 199–204, 1999.

Petersen, L.R.A.R., J.T., West Nile Virus: a re-emerging global pathogen, *Emerg. Infect. Dis.*, 7, 611, 2001.

Rosengard, A., Liu, Y., Nie, Z., and Jimenez, R., Variola virus immune evasion design: expression of a highly efficient inhibitor of human complement, *Proc. Natl. Acad. Sci. USA*, 99, 8803–8813, 2002.

Rota, P.A., O.M., Monroe, S.S., Nix, W.A., Campagnoli, R., Icenogle, J.P., Penaranda, S., Bankamp, B., Maher, K., Chen, M., Tong, S., Tamin, A., Lowe, L., Frace, M., DeRisi, J.L., Chen, Q., Wang, D., Erdman, D.D., Peret, T.C.T., Burns, C., Ksiazek, T.G., Rollin, P.E., Sanchez, A., Liffick, S., Holloway, B., Limor, J., McCaustland, K., Olsen-Rasmussen, M., Fouchier, R., Gunther, S., Osterhaus, A.D.M.E., Drosten, C., Pallansch, M.A., Anderson, L.J., and Bellini, W.J., Characterization of a novel coronavirus associated with Severe Acute Respiratory Syndrome, *Science*, 300, 1394–1399, 2003.

Stenmark, S., S.D., Bucht, A., and Sjostedt, A., Rapid local expression of interleukin-12, tumor necrosis factor alpha and gamma interferon after cutaneous Francisella tularensis infection in tularemia-immune mice, *Infect. Immun.*, 67, 1789–1797, 1999.

Stewart, W.H., A Mandate for State Action, presented at the Association of State and Territorial Health Officers, Washington, DC, December 4, 1967.

Traynor, K., Status of nation's smallpox vaccine reserve improves, *Am. J. Health Syst. Pharm.*, 59, 822, 827, 2002.

Traynor, K., Limited smallpox vaccinations to resume in United States, *Am. J. Health Syst. Pharm.*, 60, 219–220, 222, 2003.

Xiao, S.-Y., et al., West Nile Virus infection in the golden hamster (Mesocricetus anatus): a model for West Nile encephalitis, *Emerg. Infect. Dis.*, 7, 714, 2001.

37 Toxicology Associated with Respiratory Exposures to Fungi (Molds)

Marsha D.W. Ward, Yong Joo Chung, and MaryJane K. Selgrade

CONTENTS

37.1 INTRODUCTION

Fungi are a group of eukaryotic unicellular or multicellular microorganisms that lack chlorophyll. "Mold" is the common term for multicellular fungi that grow as microscopic filaments (hyphae). There are more than 200 different types of fungi to which people are routinely exposed (National Academy, 2002). Fungi are ubiquitous in both indoor and outdoor environments and human exposure is therefore inevitable and for the most part innocuous. However, the growth of molds in homes, schools, offices, and other public buildings has recently been implicated in a number of cases as the cause of a wide variety of human ailments and disabilities, and "toxic molds" have received a great deal of public and media attention over the past few years. This in turn has triggered increased scientific interest in the potential health effects of inhalation exposure to molds, particularly in indoor environments.

Respiratory exposures to fungi have been associated with an array of effects including irritation, which may originate from nonspecific inflammatory reactions or direct stimulation of nerve endings, specific allergic (hypersensitivity) responses, asthma, pulmonary hemosiderosis, and infection. Additionally, fungi produce and release metabolites, some of which are toxic to vertebrates. These mycotoxins may be cytotoxic, carcinogenic, estrogenic, vasoactive or neurotoxic (National Academy, 2000). However, ingestion has been the route of exposure for most mycotoxin studies. Specific human toxicity due to inhaled mycotoxins has yet to be definitively established (Fung and Hughson, 2003; Hardin et al., 2003). Because the leading pathogenic fungi for persons with nonimpaired immune

function do not normally grow in indoor environments, we will not consider infectivity in this chapter but will focus instead on immune-mediated injury as well as the potential for disease related to mycotoxin production.

Hypersensitivity pneumonitis and asthma are both immune-mediated diseases that have been associated with mold exposure. Asthma is a particular concern because the incidence of this disease in industrialized nations has increased dramatically over the past 25–30 years (Stafford et al., 2003; Centers for Disease Control, 2002), particularly among children. The indoor environment is thought to have some role in this increased incidence because children now spend 90–95% of their time indoors (Spengler and Sexton, 1983; Platts-Mills, 1995) and steps to conserve energy have greatly reduced dilution of indoor air by outdoor air. Immune-mediated effects associated with exposure to fungi can be divided into nonspecific inflammatory responses, specific hypersensitivity responses, and adjuvant effects. Because the indoor environment often includes a mix of several species of fungi and these fungi are complex mixtures of proteins and other components, any or all of these responses could occur as a result of fungal exposure.

Nonspecific inflammatory responses are characterized by the infiltration of polymorphonuclear leukocytes, production of inflammatory cytokines, edema, and some cell damage. These nonspecific responses do not result in the induction of immunological memory. Therefore on subsequent exposure local inflammation will again result, but there is no enhancement of the magnitude of the response and no change in the dose required to induce the response. Nonspecific inflammation may contribute to the exacerbation of asthma symptoms associated with moldy environments and could also be involved in the pulmonary hemosiderosis observed in some infants exposed to moldy environments. Inflammation also creates a milieu that is particularly conducive to the induction of allergic responses to specific proteins and may therefore mediate the adjuvant effect described below.

In contrast to nonspecific responses, hypersensitivity responses are characterized by an induction or sensitization period during which exposures may not cause any overt response, although the immune system is being primed. Once immunological memory is established, reexposure results in an exaggerated response and a lower dose of allergen is typically needed to elicit a response. Gell and Coombs (Janeway et al., 2001) originally described four hypersensitivity reactions based on immune mechanisms, three of these are important in mold hypersensitivities. Type I hypersensitivity (also called atopy) is mediated by antigen-specific cytophilic antibody (usually IgE) that binds to mast cells and basophils. When antigen cross-links these cell-bound antibodies, mediators are released causing immediate reactions, including the bronchoconstriction experienced by allergic asthmatics immediately after exposure to allergen. Type III reactions are the result of antigen-antibody (IgG) complexes that accumulate in tissues or the circulation, activate macrophages and the complement system, and trigger the influx of granulocytes and lymphocytes (inflammation). Pathologically this response may look very much like a type IV response although the underlying mechanisms are different. In the lung, type III responses are involved in hypersensitivity pneumonitis and may contribute to the late-phase asthmatic response, which occurs 6–8 h after allergen exposure and is characterized by mucus hypersecretion, bronchoconstriction, airway hyperresponsiveness to a variety of nonspecific stimuli, and airway inflammation characterized by eosinophils. Type IV or delayed-type hypersensitivity (DTH) is a cell-mediated rather than antibody-mediated process. Activated T cells release cytokines that cause accumulation and activation of macrophages, which in turn cause local damage. Type IV responses also are involved in hypersensitivity pneumonitis. Note that none of these responses are mutually exclusive.

Fungi may also cause adverse health effects through adjuvancy. An adjuvant is a compound administered in conjunction with an antigen that nonspecifically enhances the immune response to that antigen. Fungal cell walls are composed of acetylglucosamine polymer (chitin) fibrils embedded in a matrix of glucose polymers (1,3)β-D-glucans. These glucans are potent adjuvants and may facilitate the induction of both immediate and delayed type responses to fungal proteins (Wan et al., 1999).

37.2 EXPOSURE

Methods applicable to the assessment of mold exposures have recently been reviewed (Dillon et al., 1999; Burge, 2002; Levetin and Horner, 2002). Use of questionnaire or inspection survey information for visible signs or the characteristic smell of molds or dampness is the simplest and therefore most frequently used method. Unfortunately this provides little information on the degree of exposure and involves a certain amount of reporting bias. Settlement plates, dust samples (e.g., from vacuum cleaner bags), and air samples, have also been used as well as personal monitors (Toivola et al., 2002). Traditional methods for quantitative assessment of these samples have involved culturing and counting colonies or microscopic counts of fungal propagules (spores). The former technique fails to detect nonviable spores or fungal components where importantly allergens could be associated. Because there are few distinctive features among different species of indoor mold contaminants, the latter approach provides little opportunity for characterizing the specific types of molds present. More sophisticated approaches to quantitate exposure include surrogate measures (markers) of fungal mass and growth including ergosterol (the primary cell membrane sterol of most fungi) and (1,3)β-D-glucans. Another approach is polymerase chain reaction, a highly specific molecular method (Gaskell et al., 1997; Haugland et al., 1999) that is quantitative and provides information on specific species of fungi present in an environmental sample. Furthermore, high-performance liquid chromatography (HPLC) and other chromatographic methods have been developed to assess specific mycotoxins (Frisvad and Gravesen, 1994). Finally, where specific fungal allergens or other components have been identified, antibodies to these components in human sera can be monitored as biomarkers of exposure (von Emon et al., 2003).

Exposure assessment efforts have focused largely on fungal spores, most of which are about 10 μm in diameter. However, exposure to vegetative components of molds is also possible, particularly if they grow in and are dispersed by humidifying systems or heating and air-conditioning systems. A large degree of variability, both qualitative and quantitative, has been observed when repeated-exposure assessments have been performed in the same environment. This variability both within and between homes may in part explain why many sampling studies, unlike studies that rely on reported mold exposure, have been unable to show an association between mold exposure and symptoms (Douwes and Pearce, 2003). It is also possible that very-low-level exposures could be amplified by colonization of (or at least partial germination in) nasal passageways. For example, allergic fungal sinusitis has been described for *Aspergillus* (Leonard et al., 2001). Improvements in exposure assessment procedures and protocols may be the key to demonstrating more robust cause/effect relationships between mold exposure and health effects.

Fungal growth occurs only in the presence of moisture. The amount of water needed depends on availability of food material, temperature, and the fungal species. The most commonly identified fungi (both indoors and outdoors in all parts of the world) are species of *Penicillium, Cladosporium,* and *Aspergillus* (Kolstad et al., 2002). *Curvularia, Alternaria,* and *Fusarium* are also commonly encountered genera (Hargreaves et al., 2003). *Trichoderma* and *Stachybotrys,* which require high moisture levels for growth, are infrequent. However, *Stachybotrys* has been identified more frequently in buildings with mold problems (Kolstad et al., 2002).

In a few cases, animal studies have been used to study effects of respiratory exposures to several types of molds including *Aspergillus versicolor* (Korpi et al, 2003a), *Pencillium chrysogenum* (Schwab et al., 2003), *Metarhizium anisopliae* (Ward et al., 2000a, 2000b), and *Stachybotry chartarum* (Viana et al., 2002; Korpi, 2002). Approaches to respiratory exposures have included aerosol exposure using an atomizer and collection of aerosols through a side steam outlet to an optical particle counter for mass concentration determination, as well as simpler intranasal, intratracheal, or involuntary aspiration administration. In most cases, mice were exposed to a crude extract of fungal spores plus vegetative material or spores alone. However, in several cases (1,3)β-D-glucan was delivered as an aerosol to mice or guinea pigs (Fogelmark et al., 2001; Korpi et al., 2003b; Wan et al., 1999). There have been relatively few animal studies and approaches to exposure have varied considerably. Because the specifics of human exposure are poorly understood, it is difficult to mimic the human situation exactly.

37.3 IMMUNE-MEDIATED EFFECTS

37.3.1 Allergy/Asthma

Asthma is a complex chronic respiratory disorder characterized by pulmonary inflammation, mucus secretion, and intermittent airway constriction and airway hyperresponsiveness. These physiological responses result in symptoms of chest tightness, wheeze, and shortness of breath. Although asthma is most prevalent in young children, approximately half of these individuals stop wheezing by adolescence. The most severely asthmatic children usually have a clear allergic component, become symptomatic early in life, and continue through adolescence (Holt et al., 1999). The percentage of adult asthmatics that are allergic asthmatics is somewhat controversial with estimates ranging from 50% (Pearce et al., 1999) to >90% (Holt et al., 1999). It has been established that allergic asthma is a T-cell-mediated immune response driven by allergenic peptide presentation to memory Th2 cells (Holt et al., 1999) and is further characterized by a biphasic response composed of acute (IgE-mediated) and late-phase reactions, thought to be mediated by the presence of Th2 cells, mast cells, and eosinophils within the walls of the lower respiratory tract (Kobayashi et al., 2000).

The concept of molds as causative agents for allergy/asthma is not new. In fact many fungal genera have been associated with allergic lung disease, but only a few fungi are well studied and even fewer fungal allergens well characterized. The complexity and variability of fungal propagules in environmental samples make identification and characterization of fungal allergens a challenge. Because the question of what makes a protein an allergen has not been answered for allergy research in general, no structural motifs are available to easily pick mold allergens out of the mix. More traditional approaches to allergen identification, which rely on serum antibody responses to fungal components or positive skin testing, are hampered by the lack of standardized mold extracts for human testing.

A number of studies have shown an association between positive skin tests to molds, basidiospores (Lehrer et al., 1994; Chavasco et al., 1997), *Alternaria* and *Penicillium* (Eggleston et al., 1998), and asthma. Jacob et al. (2002) found *Cladosporium* and *Apergillus* spore counts were associated with increased risk of allergic sensitization. A prospective study of infants at high risk for asthma development found that these infants had significant risk for wheeze and persistent cough when exposed to high levels of *Penicillium* (Gent et al., 2002). Barnes et al. (2001), measuring the antigen concentration in the house dust of allergy clinic pediatric patients, found measurable amounts of the fungi *Alternaria* and *Cladosporium* among other allergens. However, major *Alternaria* (Alt a 1, Alt a 70kD) and house dust mite (Der p 1) allergens were significantly higher in the homes of asthmatics. Cooley et al. (1998) found a strong association between the presence of *Penicillium* species (especially *P. chrysogenum*) and *Stachybotrys* species and sick building syndrome. However, such species specific associations generally are not made or are complicated by positive results to multiple molds and/or other agents. An example of this complexity is Santilli and Rockwell's (2003) study of two Connecticut elementary schools with personnel reporting symptoms associated with poor indoor air quality. In both schools high levels of fungal contamination were found. Individuals that permitted skin testing, exhibited positive reactions to multiple molds. Table 37.1 is a summary of known allergens from inhaled fungi (from www.allergome.org, an allergenic molecules database).

An apparently unique hypersensitivity disorder resulting from the frank colonization of the lungs by *Aspergillus fumigatus* is called allergic bronchopulmonary aspergillosis (ABPA). ABPA is most common in patients with asthma and cystic fibrosis. Diagnostic criteria include *A. fumigatus*-specific responses such as immediate cutaneous reactivity, precipitating IgG antibodies, and elevated IgE, as well as peripheral eosinophilia coincident with chest radiographic infiltrates and the presence of *A. fumigatus* in sputum (Leonard et al., 2001).

Clearly, associations between mold exposures and allergic lung disease (as well as other adverse health effects) identified in epidemiological and case studies can be better elucidated in animal studies. A study of *P. chrysogenum*-exposed mice demonstrated that six weekly intranasal instillations of

TABLE 37.1 Summary of Inhaled Fungi Allergens from Allergome Database (www.allergome.org)

Fungi	No. identified	Examples of identified allergens	Biological function
Alternaria alternata, *Alternaria tenuis*	14	Alt a 3 Alt a 10	Heat shock proteins Aldehyde, dehydrogenase
Aspergillus fumigatus	20	Asp f 2 Asp f 13	Fibrinogen-binding protein Alkaline serine protease
Aspergillus flavus	3	Asp fl 1 Asp fl 18	Alkaline serine protease Vacuolar serine protease
Aspergillus niger	5	Asp n 18 Asp n Phytase	Vacuolar serine protease phosphatase
Aspergillus oryzae	3	Asp o 13 Asp o Lactase	Alkaline serine protease galactosidase
Candida albicans	5	Cand a CyP Cand a Enolase	Cyclophilin Enolase
Cladosporium herbarum	13	Cla h 3 Cla h 8	Aldehyde dehydrogenase Cold shock protein
Coprius comatus	7	Cop c 1	Leucine zipper protein
Epicoccum nigrum Epicoccum purpurascens	1	Epi p 1	Serine protease
Fusarium culmorum	3	Fus c 1	No function determined
Fusarium solani	2	Fus s 45kD	No function determined
Penicillium brevicompactum	1	Pen b 13	Alkaline serine protease
Penicillium citrinum	7	Pen c 1 Pen c 3	Alkaline serine protease Peroxisomal membrane protein
Penicillium chrysogenum	3	Pen ch 13 Pen ch 20	Alkaline serine protease *N*-acetyl glucosaminidase
Psilocybe cubensis	2	Psi c 2	Rotamase
Rhodotorula mucilaginosa	1	Rho m 1	Glycolytic enzyme
Saccharomyces cerevisiae Baker's Yeast	3	Sac c CyP Sac c MnSOD	Cyclophilin/rotamase Mn superoxide desmutase
Stachybotrys chartarum	1	Sta c Cellulase	Glycosyl hydrolase
Thermomyces lanuginosus	1	The l Lipase	Lipase
Trichophyton rubrum	2	Tri r 2, Tri r 4	Serine protease
Trichophyton tonsurans	2	Tri t 1 Tri t 4	No function determined Serine protease

viable (averaging 25% viability) *P. chrysogenum* conidia induced allergic asthmalike responses, including elevated total and specific IgE and IgG1 and eosinophilia compared with controls. However, instillation of nonviable conidia resulted in a significant increase in total serum IgG2a, suggesting a Th1-mediated immune response (Cooley et al., 2000). Subsequent studies found that mice did not develop allergic responses with intranasal low-level *P. chrysogenum* conidia

(10^2 viable conidia) exposures over 11 weeks (Schwab et al., 2003). Additionally, Schwab et al. (2003) found that intraperitoneal sensitization with an aqueous protease extract from viable *P. chrysogenum* spores resulted in significant increases in serum IgE and IgG1 when the mice were intranasally challenged with the protease extract or viable spores but not nonviable spores. These studies clearly suggest that viable *P. chrysogenum* spores can play a role in allergy development. Additionally, they suggest that the level of exposure is a critical factor in allergic sensitization.

We have evaluated the allergic potential of molds that are generally underrepresented in traditional sampling methods (*Stachybotrys chartarum;* Viana et al., 2002) or that may be used for indoor pest control (*Metarhizium anisopliae;* Ward et al., 1998, 2002a, 2002b). Involuntary aspiration by BALB/c mice to both *S. chartarum* and *M. anisopliae* extracts resulted in responses that are consistent with human allergic airway disease. Our studies demonstrated that these fungal extracts induced antigen nonspecific lung injury through increased permeability (bronchoalveolar lavage fluid [BALF] total protein) and cell damage (BALF lactase dehydrogenase [LDH] activity), as well as neutrophil influx. These nonspecific responses appeared shortly after exposure and waned by 3 days postexposure. Additionally, repeated exposure to these extracts induced responses indicative of an antigen-specific immune response. The responses included elevated BALF interleukin (IL)-5, serum IgE, and both immediate airway reactivity (specific fungal exposure) and hyperresponsiveness to nonspecific challenge (methacholine challenge). Figures 37.1 and 37.2 show representative data from *Metarhizium anisopliae* (M.D.W. Ward, unpublished data) and *Stachybotrys chartarum* (Viana et al., 2002) studies illustrating characteristic features of allergic asthma in this BALB/c mouse model. These studies suggest potential risks as a result of mold exposure. Currently studies are underway to identify the specific fungal allergens for these two mold extracts. Human study protocols to assess sera for antibodies to these allergens are currently under development. Such studies are needed before actual risks to humans can be adequately assessed.

37.3.2 Nonspecific Inflammation/Adjuvancy

Indoor air is a complex mixture that includes material from both bacterial (endotoxin, LPS) and fungal sources (glucans) that cause nonspecific inflammation and may act as adjuvants. Of particular interest for this chapter is (1,3)β-D-glucan, commonly isolated from fungal cell walls. Studies of human (1,3)β-D-glucan inhalation are somewhat limited. However, Rylander's (1999) review of field studies relating (1,3)β-D-glucan as a marker of biomass to increased extent of symptoms and inflammation suggested that (1,3)β-D-glucan may be a useful measure of fungal contamination/exposure in assessing risk of adverse health effects.

Several studies indicate that (1,3)β-D-glucan induces nonspecific inflammatory responses. The lung lavage fluid of mice intratracheally instilled with a (1,3)β-D-glucan rich *Pneumocystis carinii* cell wall fraction demonstrated a neutrophilic infiltrate and an increase tumor necrosis factor alpha (TNFα) levels (Vassallo et al., 2000). Additionally, primary mouse alveolar macrophages stimulated with this cell wall fraction *in vitro* produced proinflammatory responses such as the induction of TNFα and the potent neutrophil chemoattractant, macrophage-inflammatory protein-2 (MIP-2) (Vassallo et al., 2000). Fogelmark et al. (2001) found daily inhalation exposures of guinea pigs to (1,3)β-D-glucan for five weeks resulted in increased number of eosinophils in lung lavage, lung interstitium, and airway epithelium as well as an interstitial increase in lymphocytes. Endotoxin did not cause these effects and in fact modulated the effects of (1,3)β-D-glucan.

Other studies have shown that (1,3)β-D-glucan can enhance allergic responses to known antigens. Studies in mice have demonstrated that mice exposed to aerosolized (1,3)β-D-glucan in an ovalbumin (OVA) allergy model had higher levels of OVA-specific IgE and IgG1 then mice exposed to OVA alone (Ormstad et al., 2000; Wan et al., 1999). Additionally, there was no increase in IgG2a. Furthermore, Wan et al. (1999) found that (1,3)β-D-glucan increased eosinophils in lung lavage, as well as, increased IL-10 mRNA and decreased IL-12 mRNA in mouse lung cells. Collectively, these studies suggest that (1,3)β-D-glucan may act as an adjuvant during the initiation phase of allergic responses.

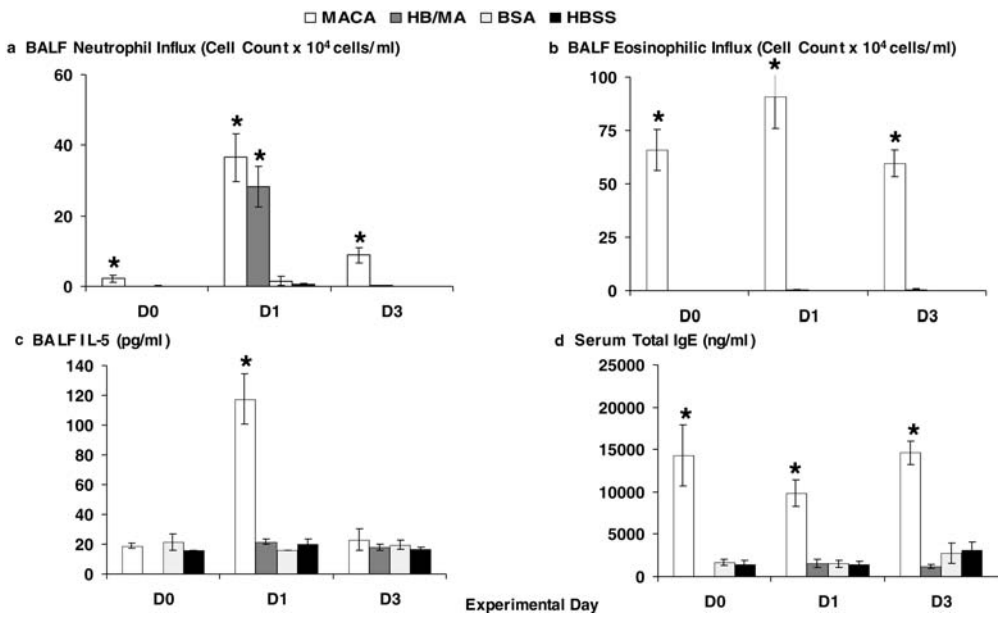


FIGURE 37.1 Mice were exposed 4 times to *Metarhizium anisopliae* extract (MACA), Hank's balanced salt solution (HBSS) (vehicle control), bovine serum albumin (BSA) (negative control), or one time to fungal extract (HB/MA) (nonspecific inflammatory control). Before (D0) aand at 1 day (D1) and 3 days (D3) following the fourth exposure, the mice were assessed for end points characteristic of allergic lung disease. (a) BALF neurotphil influx. (b) Eosionphil influx. (c) BALF IL-5. (d) Serum total IgE. *Significant difference from vehicle and negative controls ($p < 0.05$). Error bars represent standard error of the mean; $n = 5–6$ (M.D.W. Ward, unpublished data).

Fungal cell wall components may not be the only source of fungal-induced respiratory irritation. Acute exposures to *S. chartarum* (Korpi et al., 2002) and *Aspergillus versicolor* (Korpi et al., 2003a), fungi frequently found in water-damaged buildings, provoked a dose-dependent upper respiratory tract irritation in mouse airways. The investigators (Korpi et al., 2003b) found that pure β-glucan exposure resulted in lower sensory irritation responses than did *A. versicolor* extract. They concluded that β-glucan was unlikely to be the major source of the irritation but could not rule out ergosterol because its biological effects are unclear. In their *S. chartarum* study, they found that the extract caused sensory irritation in both immunized and nonimmunized mice. Additionally, repeated exposures resulted in a significantly increased IgE. Mice intratracheally instilled one time with spores of *Streptomyces californicus* isolated from the air of a water-damaged building had elevated proinflammatory cytokines (TNFα and IL-6) in their BALF (Jussila et al., 2001). Additionally, there was a dose-dependent increase in BALF total protein, albumin, hemoglobin, and LDH markers indicative of cytotoxicity. Jussila et al. (2001) concluded that *S. californicus* is a species capable of inducing adverse health effects.

37.3.3 Hypersensitivity Pneumonitis (HP)

Hypersensitivity pneumonitis (extrinsic allergic alveolitis) (reviewed in Patel et al., 2001), is an allergic lung syndrome considered to be features of both type III (elevated levels of IgG to the antigen) and type IV ($CD8^+$ lymphocytes) hypersensitivity responses. HP is caused by the inhalation of a variety of organic dusts. These dusts contain antigenic substances including fungal/bacterial components, animal proteins, and some chemicals. Although HP is relatively rare (only 1% of exposed individuals develop disease), it is a chronic disease that can progress to disabling or even fatal lung

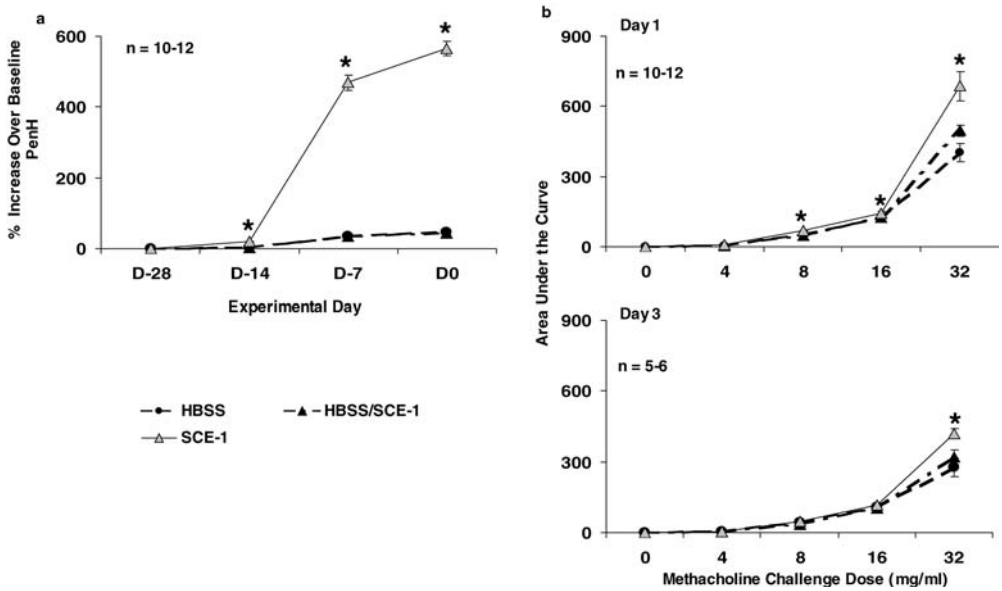


FIGURE 37.2 Respiratory immediate and hyperreactivity responses were assessed using enhanced pause (PenH) in a BUXCO whole-body plethysmograph. (a) Immediate respiratory physiological responses to aspirated *Stachybotrys chartarum* (SCE-1) in mice exposed to four doses of SCE-1, or Hank's balanced salt solution (HBSS) (vehicle control), or three doses of HBSS plus one dose of SCE-1 (HBSS/SCE-1) (nonspecific inflammatory control). (b) Respiratory hyperreactivity responses to doubling doses of methacholine at day 1 and day 3 after final exposure as previously described. *Significant difference from controls ($p < 0.05$). Error bars represent standard error of the mean. (Adapted from Viana et al., 2002.)

disease. HP, while somewhat difficult to characterize, is associated with the predominance of mononuclear inflammation of the lung interstitium, terminal bronchioles and alveoli. This inflammation is often associated with the most significant clinical feature, granuloma formation, possibly progressing to lung fibrosis. Historically, HP has predominantly resulted from occupational exposures and therefore has a variety of names based on occupation or antigen association. Table 37.2 provides a list of these along with the strain and species of organisms identified as the causative agent. There are a number of reports of HP in mushroom workers attributed to thermophilic actinomycetes (a spore-forming bacterium) in the organic dusts from compost. However, case studies have also identified the product mushroom as the source of disease (Matsui et al., 1992; Nakazawa and Tochigi, 1989; Tanaka et al., 2000). Additionally, home exposures to molds have been associated with HP (Yoshida et al., 1989; Apostolakos et al., 2001). A nonallergic, noninfectious, respiratory illness caused by inhalation of organic dust is organic dust toxic syndrome (ODTS). ODTS is usually an acute self-limited illness resulting from heavy airborne organic dust exposure. ODTS is superficially similar to HP. However, in ODTS chest roentgenograms are normal, serology is negative to a HP allergy panel, and lung lavage fluid has an increased number of neutrophils rather than a mononuclear cell influx. Additionally, repeated exposures do not lead to chronic lung disease as they generally do in the development of HP. In one study of 44 mushroom farm workers with chronic cough, 2 were diagnosed with HP, 6 with ODTS, 18 with postnasal drip syndrome, 3 with eosinophilic bronchitis, and 15 with cough variant asthma (Tanaka et al., 2002). A study of saw mill populations found a *Rhizopus micropsporus* ssp. *rhizopoliformis* to be the most prevalent mold associated with symptoms ranging from mucous membrane irritation to allergic alveolitis and including ODTS (Eduard et al., 1993). Two studies of Swedish farmers with allergic alveolitis and ODTS compared types of exposures to microorganisms (spore counts) or moldy agricultural products (hay, grain, etc.)

TABLE 37.2 Example of Occupational Fungi Exposure-Induced Hypersensitivity Pneumonitis

HP disease	Source	Associated Agent
Farmer's Lung	Moldy hay/grain	*Thermoactinomyces vulgaris*
Bagassosis	Moldy sugarcane	*Thermoactinomyces sacchari*
Maple bark stripper's disease	Mold bark	*Cryptostroma corticale*
Tobacco-worker's lung	Tobacco mold	*Aspergillus* species
Cheese-washer's lung	Cheese mold	*Penicillium casei*
Woodworker's lung	Moldy wood dust Wood pulp	*P. chrysogenum* *Alternaria* species
Summer-type HP	Mold in Japanese homes	*Trichosporon cutaneum*
Sauna-taker's lung	Contaminated water	*Aureobasidium* species
Sax lung	Saxophone mouthpiece	Candida albicans
Paprika-splitter's lung	Paprika	Mucor stolonifer

(Malmberg et al., 1988, 1993). Both studies found that the major difference in exposures for the two respiratory conditions was that allergic alveolitis appeared to require repeated exposures, whereas ODTS was associated with occasional heavy exposure to mold dust. These studies point out the various health outcomes from constitutively similar but quantitatively different in both dose and pattern of exposures.

37.4 MYCOTOXINS

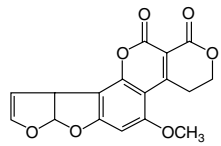
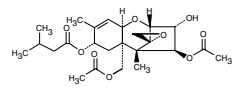
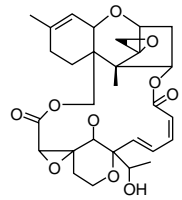
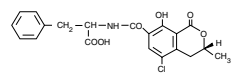
Mycotoxins, low-molecular-weight natural products, are secondary metabolites produced by filamentous fungi. They may be found in all components of fungi, for example, mycelia, spores, and matrix of molds (Palmgren and Lee, 1986; Wicklow and Shotwell, 1983). Because these components, especially spores, are readily airborne and respirable sizes, ranging from 7 to less than 1 μm in diameter (Hendry and Cole, 1993), inhalation is a likely route of exposure to mycotoxins. The diseases produced by exposure to mycotoxins are collectively called mycotoxicoses. This section describes mycotoxicoses in humans and animals due to inhaled mycotoxins, such as aflatoxins, trichothecenes, and ochratoxins. Table 37.3 lists the mycotoxins discussed in this chapter along with their toxic effects and structures.

37.4.1 Aflatoxins

Aflatoxins are found in a variety of crops used for human and animal consumption. The main sources for these toxins are *Aspergillus flavus* and *A. parasiticus,* producing B aflatoxins and B and G aflatoxins, respectively. The M aflatoxins are oxidative metabolites of B aflatoxins found in animal and human excretions including milk. Among these aflatoxins, aflatoxin B1 (AFB1) is the most potent natural carcinogen known (Squire, 1981) and the major aflatoxin produced by the fungi mentioned above. Aflatoxins are potent liver toxins and are carcinogenic by ingestion exposure.

AFB1 has been detected in respirable dust (Burg et al., 1981) and inhalation exposure has been associated with human and animal health effects. Some epidemiological studies showed an association of AFB1-containing grain dust with lung cancer incidence in certain occupational settings (Hayes et al., 1984; Olsen et al., 1988). Hayes et al. (1984) reported increased mortality for both total-cancer and respiratory cancer in aflatoxin-exposed individuals. Olsen et al. (1988) observed elevated risks for liver and biliary tract cancers among animal feed workers seemingly involving a

TABLE 37.3 List of Mycotoxins Mentioned in This Text and Their Adverse Effects in Animal Studies

Toxins	Source	Structure	Mode of action	Toxic effect
Aflatoxin B1	*Aspergillus* species		Formation of DNA adduct	Carcinogenic Mutagenic Hepatotoxic Teratogenic Immunosuppressive
T-2 toxin	*Fusarium* species		Inhibition of DNA and protein synthesis	Immunomodulating Cytotoxic
Macrocyclic trichothecenes	*Stachybotrys, Myrothecium,* and *Trichothecium* species	 Satratoxin G	Inhibition of DNA and protein synthesis	Immunomodulating Cytotoxic
Ochratoxin A	*Penicillium* and *Aspergillus* species		Formation of DNA adduct Protein synthesis inhibition Break of DNA single strand	Nephrotoxic Hepatotoxic Teratogenic Carcinogenic Immunosuppressive Oxidative stress

10-year latency period. Their daily "intake" of AFB1 by pulmonary exposure was estimated to be approximately 170 ng. Autrup et al. (1991) found aflatoxin bound to serum albumin, as an index of exposure to AFB1, in 7 of 45 workers exposed to AFB1 contaminated feed. Furthermore, 3 of the 7 serum-bound, aflatoxin-positive workers were known to have high exposures.

Jakab et al. (1994) demonstrated immune suppression in laboratory rats and mice exposed to AFB1 through either aerosol inhalation or intratracheal instillation, including suppression of alveolar macrophage phagocytosis. Additionally, intratracheal instillation also suppressed the primary splenic antibody response, suggesting that respiratory tract exposure to AFB1 can damage the adaptive as well as innate immune responses. Inhaled aflatoxin may also be carcinogenic (Zarba et al., 1992). In their nose-only aerosol inhalation experiments, rats were exposed for up to 120 min. The amount of aflatoxin-N7-guanine adducts in the liver was time-dependently increased, indicating that aerosol inhalation is an effective AFB1 exposure route resulting in genotoxic damage in the rat liver.

37.4.2 Trichothecenes

Trichothecenes are a family of sesquiterpenoid mycotoxins (compounds formed from three isoprene units), which include some of the most potent protein synthesis inhibitors known (Ueno, 1983). These mycotoxins have immunomodulatory effects in rats and mice (Bondy and Pestka, 2000). More than 180 trichothecenes have been identified and are categorized into four types based on functional groups attached to the trichothecene ring (Grove, 1993, 1996). Type A trichothecenes, the second most toxic category of the trichothecenes, include T-2 toxin, whereas type D trichothecenes include macrocyclic trichothecenes, such as satratoxins, verrucarin, and roridin. T-2 toxin and the macrocyclic trichothecenes are of concern regarding their inhalation toxicity.

T-2 toxin is found in certain agricultural commodities (e.g., wheat, corn, oat, rice, and rye) that become contaminated with *Fusarium sporotrichioides* and *Fusarium poae.* There are few data on inhalation exposure of humans to T-2. However, human illness associated with oral exposure to T-2 toxin occurred in Russia in the 1930s. This toxin was thought to be the main cause of alimentary toxic aleukia (Joffe, 1978). People under near famine were forced to consume overwintered grain that was contaminated with *F. poae* and *F. sporotrichioides.* The *F. sporotrichioides* species isolated from the epidemic produced 4.1 g of T-2 toxin/kg of infected millet (Joffe, 1986). In addition, T-2 toxin and other trichothecenes have been detected in the dust from office ventilation system (Smoragiewicz et al., 1993).

Studies have been conducted on the acute toxicity of T-2 toxin because of its potential use in chemical warfare. In animal experiments assessing the LD_{50}, inhaled T-2 toxin was at least 10 times more toxic than systemic administration and at least 20 times more toxic than dermal exposure (Creasia et al., 1987). In another acute inhalation toxicity study assessing LD_{50}, inhaled T-2 toxin was approximately 20 times more toxic in rats and at least twice as toxic in guinea pigs than intraperitoneally administered T-2 toxin (Creasia et al., 1990). However, these results were not consistently demonstrated in swine (Pang et al., 1987).

Macrocyclic trichothecenes are 10 to 100 times more toxic than other types of trichothecenes based on mouse LD_{50} studies, HeLa cell cytotoxicity tests, and rabbit reticulocyte protein synthesis inhibition assays (Ueno, 1983). *Stachybotrys chartarum* is best known as a producer of macrocyclic trichothecenes: mainly satratoxin H, G, F, roridins E and L-2 (Jarvis et al., 1998; Nielsen et al., 1998; Vesper et al., 2000). Satratoxins have been detected in quantities ranging from 2 to 15 ng/cm^2 in building materials heavily contaminated by *S. chartarum* (Nielsen et al., 1998).

Human exposure, presumably by inhalation, to *S. chartarum* spores has been associated with outbreaks of *Stachybotrys*-associated human disease (Dearborn, 1997; Etzel et al., 1998; Johanning et al., 1996). Symptoms reported by exposed individuals include recurrent cough, irritation of the eyes and skin, mucous membrane disorders, headache, and fatigue, which are also characteristics of indoor air illnesses. Notably, pulmonary hemorrhage and hemosiderosis in infants have been associated with toxigenic *S. chartarum*-contaminated homes (Etzel et al., 1998). Spores of this fungus were isolated from the lungs of a child with pulmonary hemorrhage (Elidemir et al., 1999). In addition, macrocyclic trichochecenes and other metabolites have been detected in dust and air samples from homes contaminated with *Stachybotrys* (Nielsen et *al*., 2002; Vesper et al., 2000; Yike et al., 1999). However, the Centers for Disease Control (CDC) reviewed literature for the role of *Stachybotrys* mycotoxins in building-related disease and announced that there is inadequate evidence to support a causal relationship between symptoms or illness among building occupants and exposure to mycotoxins (Page and Trout, 2001). With this controversy, a number of review articles about *Stachybotrys* and their mycotoxins in human health have been published. Evidence is consistent with the possibility that *Stachybotrys* spores and their toxins may adversely affect human health; however, the evidence is not conclusive (Chapman et al., 2003; Etzel, 2003; Hodgson and Dearborn, 2002; Kuhn and Ghannoum, 2003; Miller et al., 2003; Revankar, 2003; Terr, 2001).

In addition, macrocyclic trichothecenes have been etiologically associated with stachybotryotoxicosis in horse and sheep in Central Europe (Bata et al., 1985; Harrach et al., 1981, 1983; Hintikka, 1978). Clinical findings of this disease include leukopenia, thrombocytopenia, hemorrhage, arrhythmias, and death (Forgacs, 1972). Intranasal (Nikulin et al., 1997) and intratracheal (Rao et al., 2000b) exposures to *Stachybotrys* spores can cause inflammation in mouse lungs. When the spores are introduced to the lung, the number of alveolar macrophages, which can not only phagocytose the spores but also secrete various immune mediators, are increased (Rao et al., 2000b). Relatedly, Rao et al. (2000a) demonstrated that removal of toxins from *Stachybotrys* spores reduces inflammatory effects in the lungs of mice. Yike et al. (2002) studied stachybotrystoxicosis in infant rats after intratracheal instillation. Death due to hemorrhagic lungs, increased respiratory resistance, inflammation in lungs, and increased macrophages, lymphocytes, and neutrophils in BAL fluid were reported. Extraction of metabolites from spores showed a minimal

effect on the parameters measured in BAL fluid. These studies imply that toxins from the spores may enhance the hemorrhagic and inflammatory response in this organ. Alveolar type II cells are sensitive to exposure to *S. chartarum* spores and to macrocyclic trichothecene, isosatratoxin-F in a juvenile mouse model (Mason et al., 2001) and consequently pulmonary surfactant is accumulated in the alveolar space after exposure (Rand et al., 2002). This increased pulmonary surfactant may be a host defense mechanisms or the result of cellular trauma. Recently, localization of satratoxin G (SG), macrocyclic trichothecene, after intratracheal exposure was evaluated in mouse lungs (Gregory et al., 2004). Alveolar macrophages and alveolar type II cells showed SG labeling, especially in nuclear heterochromatin, RER and/or lysosomes. These observations indicate that the alveolar macrophages may play an important role in the sequestration and immobilization of the toxin.

37.4.3 Ochratoxins

Ochratoxins are commonly found in cereal products, especially in barley-containing food products that are contaminated with *Penicillium* (mainly *P. verrucosum*) and *Aspergillus* (mainly *A. alutaceus*) species of storage fungi. Airborne dust and fungal conidia can also be sources of Ochratoxin A (Richard et al., 1999; Skaug et al., 2001). Ochratoxin A is a nephrotoxin to all animal species studied to date and is most likely toxic to humans (Marquardt and Frohlich, 1992). In addition, ochratoxin A is also hepatotoxic, immunosuppressive, teratogenic, and carcinogenic in animal studies (Kuiper-Goodman and Scott, 1989) and is considered a possible human carcinogen (category 2B) (Beardall and Miller, 1994).

A case of acute renal failure most likely due to inhalation of ochratoxin was reported in a woman spending 8 h in a moldy granary (Di Paolo et al., 1993). She developed transient respiratory distress and within 24 h had developed tubulonecrosis which required over 3 weeks to achieve resolution. Exposure to toxins was not demonstrated in this study, but a strain of *A. ochraceus* producing ochratoxin was isolated from the wheat. In another study accumulated dust samples in heating duct contained 300–1500 ppb of ochratoxin A in a household where signs resembling ochratoxin poisoning in animals occurred (Richard et al., 1999). The human residents complained of increased thirst, edema, skin rash, and general lethargy. Ochratoxin A was intratracheal administered in rat and toxicokinetics of ochratoxin A was studied. The lung efficiently absorbed the ochratoxin; the biological half-life of ochratoxin A was 127 h (Breitholtz-Emanuelsson et al., 1995).

37.5 CONCLUSIONS

In conclusion, respiratory exposures to fungi and fungal extracts have been associated with a variety of health effects. Animal studies suggest that both immune-mediated and direct toxic effects may result from such exposures. However, to date the database for both human and animal studies is rather limited. It is clear that there are still a number of questions, both qualitative and quantitative, that need to be answered before the risks associated with indoor exposure to fungi can be adequately assessed. Improved exposure assessment capabilities and current public interest make this an area that requires and deserves further investigation.

ACKNOWLEDGMENTS

Disclaimer: This chapter has been reviewed by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflects the views and policies of the agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. This work was partially supported by Cooperative Training Agreement CT829471.

REFERENCES

Apostolakos, M.J., Tossmoore, H., and Beckett, W.S., Hypersensitivity pneumonitis from ordinary residential exposures, *Environ. Health Perspect.* 109, 979–981, 2001.

Autrup, J.L., Schmidt, J., Seremet, T., and Autrup, H., Determination of exposure to aflatoxins among Danish workers in animal-feed production through the analysis of aflatoxin B1 adducts to serum albumin, *Scand. J. Work Environ. Health*, 17, 436–440, 1991.

Barnes, C., Tuck, J., Simon, S., Pacheco, F., Hu, F., and Portnoy, J., Allergenic materials in the house dust of allergy clinic patients, *Ann. Allergy Asthma Immunol.*, 86, 517–523, 2001.

Bata, A., Harrach, B., Ujszaszi, K., Kis-Tamas, A., and Lasztity, R., Macrocyclic trichothecene toxins produced by Stachybotrys atra strains isolated in Middle Europe, *Appl. Environ. Microbiol.*, 49, 678–681, 1985.

Beardall, J.M. and Miller, J.D., Disease in humans with mycotoxins as possible causes. In Mycotoxins in grains, in *Compounds Other than Aflatoxin*, Miller, J.D. and Trenholm, H.L., Eds., Eagan Press, St. Paul, MN, 1994, pp. 487–539.

Bondy, G.S. and Pestka, J.J., Immunomodulation by fungal toxins, *J. Toxicol. Environ. Health B Crit. Rev.*, 3, 109–143, 2000.

Breitholtz-Emanuelsson, A., Fuchs, R., and Hult, K., Toxicokinetics of ochratoxin A in rat following intratracheal administration, *Nat. Toxins*, 3, 101–103, 1995.

Burg, W.A., Shotwell, O.L., and Saltzman, B.E., Measurements of airborne aflatoxins during the handling of contaminated corn, *Am. Ind. Hyg. Assoc. J.*, 42, 1–11, 1981.

Burge, H.A., An update on pollen and fungal spore aerobiology, *Curr. Rev. Allergy Clin. Immunol.*,110, 544–552, 2002.

CDC (Centers for Disease Control and Prevention), Self-reported asthma prevalence among adults—United States, 2000, *MMWR Morb. Mortal. Wkly. Rep.*, 50, 682–686, 2001.

CDC (Centers for Disease Control and Prevention), Surveillance for asthma—United States, 1980–1999, *MMWR Morb. Mortal. Wkly. Rep.*, 51, 1–13, 2002.

Chapman, J.A., Terr, A.I., Jacobs, R.L., Charlesworth, E.N., and Bardana, E.J., Jr., Toxic mold: phantom risk *vs.* science, *Ann. Allergy Asthma Immunol.*, 91, 222–232, 2003.

Chavascp, J.K., Gambale, W., de Siqueria, A.M., Fiorini, J.E., Croce Portocarrero, M., Mendes, L.G., Jr., and Nascimento, L.C., Evaluation of the allergenicity of spore and mycelia extracts of *Pisolithus tinctorius, Rev. Inst. Med. Trop. S. Paulo*, 39, 245–252, 1997.

Cooley, J.D., Wong, W.C., Jumper, C.A., Hutson, J.C., Williams, H.J., Schwab, C.J., and Straus, D.C., An animal model for allergic penicilliosis induced by the intranasal instillation of viable *Penicillium chrysogenum* conidia, *Thorax*, 55, 489–496, 2000.

Cooley, J.D., Wong, W.C., Jumper, C.A., and Straus, D.C., Correlation between the prevalence of certain fungi and sick building syndrome, *Occup. Environ. Med.*, 55, 579–584, 1998.

Creasia, D.A., Thurman, J.D., Jones, L.J., III, Nealley, M.L., York, C.G., Wannemacher, R.W., Jr., and Bunner, D.L., Acute inhalation toxicity of T-2 mycotoxin in mice, *Fundam. Appl. Toxicol.*, 8, 230–235, 1987.

Creasia, D.A., Thurman, J.D., Wannemacher, R.W., Jr., and Bunner, D.L., Acute inhalation toxicity of T-2 mycotoxin in the rat and guinea pig, *Fundam. Appl. Toxicol.*, 14, 54–59, 1990.

Dearborn, D.G., Pulmonary hemorrhage in infants and children, *Curr. Opin. Pediatr.*, 9, 219–224, 1997.

Dillon, H.K., Miller, J.D., Sorenson, W.G., Douwes, J., and Jacobs, R.R., Review of methods applicable to the assessment of mold to children, *Environ. Health Perspect.*, 109, 473–480, 1999.

Di Paolo, N., Guarnieri, A., Loi, F., Sacchi, G., Mangiarotti, A.M., and Di Paolo, M., Acute renal failure from inhalation of mycotoxins, *Nephron*, 6, 621–625, 1993.

Douwes, J. and Pearce, N., Invited commentary: Is indoor mold exposure a risk factor for asthma, *Am. J. Epidemiol.*, 158, 203–206, 2003.

Eduard, W., Sandven, P., and Levy, F., Serum IgG antibodies to spores in two Norwegian sawmill populations: relationship to respiratory and other work-related symptoms, *Am. J. Ind. Med.*, 24, 207–222, 1993.

Eggleston, P.A., Rosenstreich, D., Lynn, H., Gergen, P., Baker, D., Kattan, M., Mortimer, K.M., Mitchell, H., Ownby, D., Slavin, R., and Malveaux, F., Relationship of indoor allergen exposure to skin test sensitivity in inner city children with asthma, *J. Allergy Clin. Immunol.*, 4, 563–570, 1998.

Elidemir, O., Colasurdo, G.N., Rossmann, S.N., and Fan, L.L., Isolation of Stachybotrys from the lung of a child with pulmonary hemosiderosis, *Pediatrics*, 10, 964–966, 1999.

Etzel, R.A., Stachybotrys, *Curr. Opin. Pediatr.*, 15, 103–106, 2003.

Etzel, R.A., Montana, E., Sorenson, W.G., Kullman, G.J., Allan, T.M., Dearborn, D.G., Olson, D.R., Jarvis, B.B., and Miller, J.D., Acute pulmonary hemorrhage in infants associated with exposure to Stachybotrys atra and other fungi, *Arch. Pediatr. Adolesc. Med.*, 152, 757–762, 1998.

Fogelmark, B., Thorn, J., and Rylander, R., Inhalation of (1,3)-beta-D-glucan causes airway eosinophilia, *Mediat. Inflamm.*, 10, 13–19, 2001.

Forgacs, J., Stachybotryotoxicosis, in *Microbial Toxins*, Kadis, S., Ciegler, A., and Ajl, S.J., Eds., Academic Press, Inc., New York, 1972, pp. 95–128.

Frisvad, J.C. and Gravesen, S., *Penicillium* and *Aspergillus* from Danish homes and working places with indoor air problems: identification and mycotoxin determination, in *Health Implications of Fungi in Indoor Environments*, Samson, RA, Flannigan, B., Flannigan, M.E., Verhoeff, A.P., Adan, O.C.G., and Hoekstra, E.S., Eds., Elsevier, Amsterdam, 1994, pp. 281–290.

Fung, F. and Hughson, W.H., Health effects of indoor fungal bioaerosol exposure, *Appl. Occup. Environ. Hyg.*, 18, 535–544, 2003.

Gaskell, G.J., Carter, D.A., Britton, W.J., Tovey, E.R., Benyon, F.H., and Lovborg, U., Analysis of the internal transcribed spacer regions of ribosomal DNA in common airborne allergenic fungi, *Electrophoresis*, 18, 1567–1569, 1997.

Gent, J.F., Ren, P., Belanger, K., Triche, E., Bracken, M.B., Holford, T.R., and Leaderer B.P., Levels of household mold associated with respiratory symptoms in the first year of life in a cohort at risk for asthma, *Environ. Health Perspect.*, 110, A781–A786, 2002.

Gregory, L., Pestka, J.J., Dearborn, D.G., and Rand, T.G., Localization of Satratoxin-G in Stachybotrys chartarum spores and spore-impacted mouse lung using immunocytochemistry, *Toxicol. Pathol.*, 32, 26–34, 2004.

Grove, J.F., Macrocyclic trichothecenes, *Nat. Prod. Rep.*, 10, 429–448, 1993.

Grove, J.F., Non-macrocyclic trichothecenes. Part 2, *Prog. Chem. Org. Nat. Prod.*, 69, 1–70, 1996.

Hardin, B.B., Kelman, B.J., and Saxon, A., Adverse human health effects associated with molds in the indoor environment, *J. Occup. Environ. Med.*, 45, 470–478, 2003.

Hargreaves, M., Parappukkaran, S., Morawska, L., Hitchins, J., He, C., and Gilbert, D., A pilot investigation into associations between indoor airborne fungal and non-biological particle concentrations in residential houses in Brisbane, Austrialia, *The Science of The Total Environment*, 312, 89–101, 2003.

Harrach, B., Bata, A., Bajmocy, E., and Benko, M., Isolation of satratoxins from the bedding straw of a sheep flock with fatal stachybotryotoxicosis, *Appl. Environ. Microbiol.*, 45, 1419–1422, 1983.

Harrach, B., Mirocha, C.J., Pathre, S.V., and Palyusik, M., Macrocyclic trichothecene toxins produced by a strain of Stachybotrys atra from Hungary, *Appl. Environ. Microbiol.*, 41, 1428–1432, 1981.

Haugland, R.A., Vesper, S.J., and Wymer, L.J., Quantitative measurement of Stachybotrys chartarum conidia using real time detection of PCR products with the TaqMan(TM) fluorogenic probe system, *Mol. Cell. Probes*, 13, 329–340, 1999.

Hayes, R.B., van Nieuwenhuize, J.P., Raatgever, J.W., and ten Kate, F.J., Aflatoxin exposures in the industrial setting: an epidemiological study of mortality, *Food Chem. Toxicol.*, 22, 39–43, 1984.

Hendry, K.M. and Cole, E.C., A review of mycotoxins in indoor air, *J. Toxicol. Environ. Health*, 38, 183–198, 1993.

Hintikka, E.L., Mycotoxins, mycotoxicoses, in *Mycotoxigenic Fungi*, Wyllie, T.D. and Morehouse, L.G., Eds., Marcer Dekker, New York, 1978, pp. 87–89.

Hodgson, M. and Dearborn, D.G., Human pulmonary disease and exposure to Stachybotrys chartarum and other toxigenic fungi, *J. Occup. Environ. Med.*, 44, 705–707, 2002.

Holt, P.G., Macaubas, C., Stumbles, P.A., and Sly, P.D., The role of allergy in the development of asthma, *Nature*, 402, B12–B17, 1999.

Jacob, B., Ritz, B, Gehring, U., Koch, A., Bischof, W., Wichmann, H.E., and Heinrich, J., Indoor exposure to molds and allergic sensitization, *Environ. Health Perspect.*, 110, 647–653, 2002.

Jakab, G.J., Hmieleski, R.R., Zarba, A., Hemenway, D.R., and Groopman, J.D., Respiratory aflatoxicosis: suppression of pulmonary and systemic host defenses in rats and mice, *Toxicol. Appl. Pharmacol.*, 125, 198–205, 1994.

Janeway, C.A., Travers, P., Walport, M.M., and Shlomchik, M.J., *Immunobiology: The Immune System in Health and Disease*, Garland Publishing, New York, 2001, pp 471–500.

Jarvis, B.B., Sorenson, W.G., Hintikka, E.L., Nikulin, M., Zhou, Y., Jiang, J., Wang, S., Hinkley, S., Etzel, R.A., and Dearborn, D., Study of toxin production by isolates of Stachybotrys chartarum and Memnoniella

echinata isolated during a study of pulmonary hemosiderosis in infants, *Appl. Environ. Microbiol.*, 64, 3620–3625, 1998.

Joffe, A.Z., *Fusarium poae* and *F. sporotrichoides* as principle causal agent of alimentary toxic aleukia, in *Mycotoxic Fungi, Mycotoxins, Mycotoxicosis*, Willie. T. and Morehouse, L., Eds., Marcel Dekker, New York, 1978, pp. 27–41.

Joffe, A.Z., Effects of fusariotoxins in humans, in *Fusarium Species: Their Biology and Toxicology*, Joffe, A.Z., Ed., Wiley, New York, 1986, pp. 441–444.

Johanning, E., Biagini, R., Hull, D., Morey, P., Jarvis, B., and Landsbergis, P., Health and immunology study following exposure to toxigenic fungi (Stachybotrys chartarum) in a water-damaged office environment, *Int. Arch. Occup. Environ. Health*, 68, 207–218, 1996.

Jussila, J., Komulainen, H., Huttunen, K., Roponen, M., Halinen, A., Hyvarinen, A., Kosma, V-M., Pelkonen, J., and Hirvonen, M-R., Inflammatory responses in mice after intratracheal instillation of spores of *Streptomyces californicus* isolated from indoor air of a moldy building, *Toxicol. Appl. Pharm.*, 171, 61–69, 2001.

Kobayashi, T., Miura, T., Haba, T., Sato, M., Serizawa, I., Nagai, H., and Ishizaka, K., An essential role of mast cells in the development of airway hyperresponsiveness in a murine asthma model, *J. Immunol.*, 164, 3855–3861, 2000.

Kolstad, H.A., Brauer, C., Iversen, M., Sigsgaard, T., and Mikkelsen, S., Do indoor molds in nonindustrial environments threaten workers health? A review of the epidemiologic evidence, *Epidemiol. Rev.*, 24, 203–217, 2002.

Korpi, A., Kasanen, J.P., Raunio, P., Kosma, W.M., Virtanen, T., and Pasanen, A.L., Effects of aerosols from nontoxic Stachybotrys chartarum on murine airways, *Inhalation Toxicol.*, 14, 521–540, 2002.

Korpi, A., Kasanen, J.-P., Raunio, P., and Pasanen A.-L., Acute effects of *Aspergillus versicolor* aerosols on murine airways, *Indoor Air*, 13, 260–266, 2003a.

Korpi, A., Kasanen, J.P., Kosma, W.M., Rylander, R., and Pasanen, A.L., Slight respiratory irritation but not inflammation in mice exposed to (1, 3)-beta-D-glucan aerosols, *Mediat. Inflamm.*, 12, 139–146, 2003b.

Kuhn, D.M. and Ghannoum, M.A., Indoor mold, toxigenic fungi, and Stachybotrys chartarum: infectious disease perspective, *Clin. Microbiol. Rev.*, 16,144–172, 2003.

Kuiper-Goodman, T. and Scott, P.M., Risk assessment of the mycotoxin ochratoxin A, *Biomed. Environ. Sci.*, 2, 179–248, 1989.

Lehrer, S.B., Hughes, J.M., Altman, L.C., Bousquet, J., Davies, R.J., Gell, L., Li, J., Lopez, M., Malling, H.J., Mathison, D.A., Sastre, J., Schultze-Werninghaus, G., and Schwartz, H.J., Prevalence of basidiomycete allergy in the USA and Europe and its relationship to allergic respiratory symptoms, *Allergy*, 49, 460–465, 1994.

Levetin, E. and Horner, W.W., Fungal aerobiology: exposure and measurement, *Chem. Immunol.*, 81, 10–27, 2002.

Leonard, C.T., Berry, G.J., and Ruoss, S.J., Nasal-pulmonary relations in allergic fungal sinusitis and allergic bronchopulmonary aspergillosis, *Clin. Rev. Allergy Immunol.*, 21, 5–15, 2001.

Malmberg, P., Rask-Andersen, A., Hoglund, S., Kolmodin-Hedman, B., and Read-Guernsey, J., Incidence of organic dust toxic syndrome and allergic alveolitis in Swedish farmers, *Int. Arch. Allergy Appl. Immunol.*, 87, 47–54, 1988.

Malmberg, P., Rask-Andersen, A., and Rosenhall, L., Exposure to microorganisms associated with allergic alveolitis and febrile reactions to mold dust in farmers, *Chest*, 103, 1202–1209, 1993.

Marquardt, R.R. and Frohlich, A.A., A review of recent advances in understanding ochratoxicosis, *J. Anim. Sci.*, 70, 3968–3988, 1992.

Mason, C.D., Rand, T.G., Oulton, M., MacDonald, J., and Anthes, M., Effects of Stachybotrys chartarum on surfactant convertase activity in juvenile mice, *Toxicol. Appl. Pharmacol.*, 172, 21–28, 2001.

Matsui, S., Nakazawa, T., Umegae, Y., and Mori, M., Hypersensitivity pneumonitis induced by shiitake mushroom spores, *Intern. Med*, 31, 1204–1206, 1992.

Miller, J.D., Rand, T.G., and Jarvis, B.B., Stachybotrys chartarum: cause of human disease or media darling? *Med. Mycol.*, 41, 271–291, 2003.

Nakazawa, T. and Tochigi, T., Hypersensitivity pneumonitis due mushroom (*Pholiota nameko*) spores, *Chest*, 95, 1149–1151, 1989.

National Academy of Sciences, *Clearing the Air: Asthma and Indoor Air Exposures*, National Academy Press, Washington, DC, 2000, pp. 158–175.

Nielsen, K.F., Hansen, M., Larsen, T.O., and Thrane, U., Production of trichothecene mycotoxins on water-damaged gypsum boards in Danish buildings, *Int. Biodeterioration Biodegradation*, 42, 1–7, 1998.

Nielsen, K.F., Huttunen, K., Hyvarinen, A., Andersen, B., Jarvis, B.B., and Hirvonen, M.R., Metabolite profiles of Stachybotrys isolates from water-damaged buildings and their induction of inflammatory mediators and cytotoxicity in macrophages, *Mycopathologia*, 154, 201–205, 2002.

Nikulin, M., Reijula, K., Jarvis, B.B., Veijalainen, P., and Hintikka, E.L., Effects of intranasal exposure to spores of Stachybotrys atra in mice, *Fundam. Appl. Toxicol.*, 35, 182–188, 1997.

Olsen, J.H., Dragsted, L., and Autrup, H., Cancer risk and occupational exposure to aflatoxins in Denmark, *Br. J. Cancer*, 58, 392–396, 1988.

Ormstad, H., Groeng, E-C., Lovik, M., and Hetland, G., The fungal cell wall compenent b-1,3-glucan has an adjuvant effect on the allergic response to ovalbumin in mice, *J. Tox. Environ. Health*, Part A, 61, 55–67, 2000.

Page, E.H. and Trout, D.B., The role of Stachybotrys mycotoxins in building-related illness, *Aihaj*, 62, 644–648, 2001.

Palmgren, M.S. and Lee, L.S., Separation of mycotoxin-containing sources in grain dust and determination of their mycotoxin potential, *Environ. Health Perspect.*, 66, 105–108, 1986.

Pang, V.F., Lambert, R.J., Felsburg, P.J., Beasley, V.R., Buck, W.B., and Haschek, W.M., Experimental T-2 toxicosis in swine following inhalation exposure: effects on pulmonary and systemic immunity, and morphologic changes, *Toxicol. Pathol.*, 15, 308–319, 1987.

Patel, A.M., Ryu, J.H., and Reed, C.E., Hypersensitivity pneumonitis: current concepts and future questions, *J. Allergy Clin. Immunol.*, 108, 661–670, 2001.

Pearce, N., Pekkanen, J., and Beasley, R., How much asthma is really attributable to atopy? *Thorax,* 54, 268–272, 1999.

Platts-Mills, T.A.E., Is there a dose-response relationship between exposure to indoor allergens and symptoms of asthma? J. *Allergy Clin. Immunol.*, 96, 435–440, 1995.

Rand, T.G., Mahoney, M., White, K., and Oulton, M., Microanatomical changes in alveolar type II cells in juvenile mice intratracheally exposed to Stachybotrys chartarum spores and toxin, *Toxicol. Sci.*, 65, 239–245, 2002.

Rao, C.Y., Brain, J.D., and Burge, H.A., Reduction of pulmonary toxicity of Stachybotrys chartarum spores by methanol extraction of mycotoxins, *Appl. Environ. Microbiol.*, 66, 2817–2821, 2000a.

Rao, C.Y., Burge, H.A., and Brain, J.D., The time course of responses to intratracheally instilled toxic Stachybotrys chartarum spores in rats, *Mycopathologia*, 149, 27–34, 2000b.

Revankar, S.G., Clinical implications of mycotoxins and Stachybotrys, *Am. J. Med. Sci.*, 325, 262–274, 2003.

Richard, J.L., Plattner, R.D., May, J., and Liska, S.L., The occurrence of ochratoxin A in dust collected from a problem household, *Mycopathologia*, 146, 99–103, 1999.

Rylander, R., Airway responsiveness and chest symptoms after inhalation of endotoxin or (1→3)-β-D glucan, *Indoor Built Environ.*, 5, :106–111, 1996.

Santilli, J. and Rockwell, W., Fungal contamination of elementary schools: a new environmental hazard, *Ann. Allergy Asthma Immunol.*, 90, 203–208, 2003.

Schwab, C.J., Cooley, J.D., Brasel, T., Jumper, C.A., Graham, S.C., and Straus, D.C., Characterization of exposure to low levels of viable Penicillium chrysogenum conidia and allergic sensitization induced by a protease allergen extract from viable P. chrysogenum conidia in mice, *Int. Arch. Allergy Immunol.*, 130, 200–208, 2003.

Skaug, M.A., Eduard, W., and Stormer, F.C., Ochratoxin A in airborne dust and fungal conidia. *Mycopathologia*, 151, 93–98, 2001.

Smoragiewicz, W., Cossette, B., Boutard, A., and Krzystyniak, K., Trichothecene mycotoxins in the dust of ventilation systems in office buildings, *Int. Arch. Occup. Environ. Health*, 65, 113–117,1993.

Spengler, J.D. and Sexton, K., Indoor air pollution: a public health perspective, *Science*, 221, 9–17, 1983.

Squire, R.A., Ranking animal carcinogens: a proposed regulatory approach, *Science*, 214, 877–880, 1981.

Stafford, R.S., Ma, J., Finkelstein, S.N., Haver, K., and Cockburn, I., National trends in asthma visits and asthma parmacotherapy, 1978–2002, *J. Allergy Clin. Immunol.*, 111, 729–735, 2003.

Tanaka, H., Sugawara, H., Saikai, T., Tsunematsu, K., Takahashi, H., and Abe, S., Mushroom worker's lung caused by spores of *Hypsizigus marmoreus* (Bunashimeji): elevated serum surfactant protein D levels, *Chest*, 118, 1506–1509, 2000.

Tanaka, H., Saikai, T., Sugawara, H., Takeya, I., Tsunematsu, K., Matsuura, A., and Abe, S., Workplace-related chronic cough on a mushroom farm., *Chest*, 122, 1080–1085, 2002.

Terr, A.I., Stachybotrys: relevance to human disease, *Ann. Allergy. Asthma. Immunol.*, 87, 57–63, 2001.

Toivola, M., Alm, S., Reponen, T., Kolari, S., and Nevalainen, A., Personal exposures and microenvironmental concentrations of particles and bioaerosols, *J. Environ. Monit.*, 4, 166–174, 2002.

Ueno, Y., General toxicology, In: *Trichothecenes: Chemical, Biological and Toxicological Aspects*. Ueno, Y., Ed., pp. 135–162. Elsevier, New York, 1983.

Van Emon, J.M., Reed, A.W., Yike, I., and Vesper, S.J., Measurement of stachylysin™ in serum to quantify human exposures to the indoor mold Stachybotrys chartarum, *J. Occup. Environ. Med.*, 45, 582–591, 2003.

Vassallo, R., Standing, J.E., and Limper, A.H., Isolated *Pneumocystis carinii* cell wall glucan provokes lower respiratory tract inflammatory responses, *J. Immunol.*, 164, 3755–3763, 2000.

Vesper, S., Dearborn, D.G., Yike, I., Allan, T., Sobolewski, J., Hinkley, S.F., Jarvis, B.B., and Haugland, R.A., Evaluation of Stachybotrys chartarum in the house of an infant with pulmonary hemorrhage: quantitative assessment before, during, and after remediation, *J. Urban Health*, 77, 68–85, 2000.

Viana, M.E., Coates, N.H., Gavett, S.H., Selgrade, M.J.K., Vesper, S.J., and Ward, M.D.W., An extract of *Stachybotrys chartarum* causes allergic responses in a BALB/c mouse model, *Toxicol. Sci.*, 70, 98–109, 2002.

Wan, G.H., Li, C.S., Guo, S.P., Rylander, R., and Lin, R.H., An airborne mold-derived product, ∃-1,3-D-glucan, potentiates airway allergic responses, *Eur. J. Immunol.*, 29, 2491–2497, 1999.

Wicklow, D.T. and Shotwell, O.L., Intrafungal distribution of aflatoxins among conidia and sclerotia of Aspergillus flavus and Aspergillus parasiticus, *Can. J. Microbiol.*, 29, 1–5, 1983.

Ward, M.D.W., Madison, S.L., Andrews, D.L., Sailstad, D.M., Gavett, S.H., and Selgrade, M.J.K., Comparison of respiratory responses to Metarhizium anisopliae extract following local and systemic sensitization, *Toxicology*, 147, 133–145, 2000a.

Ward, M.D.W., Madison, S.L., Sailstad, D.M., Gavett, S.H., and Selgrade, M.J.K., Allergen-triggered airway hyperresponsiveness and lung pathology in mice sensitized with the biopesticide, *Metarhizium anisopliae*, by BALB/c mice, *Toxicology*, 143, 141–154, 2000b.

Ward, M.D.W., Sailstad, D.M., and Selgrade, M.J.K., The bio-pesticide, *Metarhizium anisopliae*, elicits allergic response in BALB/c mice, *Toxicol. Sci.*, 45, 195–203, 1998.

Yike, I., Allan, T., Sorenson, W.G., and Dearborn, D.G., Highly sensitive protein translation assay for trichothecene toxicity in airborne particulates: comparison with cytotoxicity assays, *Appl. Environ. Microbiol.*, 65, 88–94, 1999.

Yike, I., Miller, M.J., Sorenson, W.G., Walenga, R., Tomashefski, J.F., Jr., and Dearborn, D.G., Infant animal model of pulmonary mycotoxicosis induced by Stachybotrys chartarum, *Mycopathologia*, 154, 139–152, 2002.

Yoshida, K., Ando, M., Sakata, T., and Araki, S., Prevention of summer-type hypersensitivity pneumonitis: effect of elimination of *Trichosporon cutaneum* from the patients' homes, *Arch. Environ. Health*, 44, 317–322, 1989.

Zarba, A., Hmieleski, R., Hemenway, D.R., Jakab, G.J., and Groopman, J.D., Aflatoxin B1—DNA adduct formation in rat liver following exposure by aerosol inhalation, *Carcinogenesis*, 13, 1031–1033, 1992.

38 Inhalation Toxicity of Botulinum Neurotoxin

Michael Adler

CONTENTS

38.1 INTRODUCTION

Clostridium botulinum is a spore-forming, anaerobic gram-positive bacillus found in soils, aquatic sediments, and decaying animal carcasses (Sathyamoorthy and DasGupta, 1985; Hatheway, 1989). Toxigenic strains of *C. botulinum* elaborate seven antigenically distinct protein neurotoxins (types A, B, C1, D, E, F, and G). These botulinum neurotoxins (BoNTs) act primarily on the neuromuscular junction, causing paralysis of skeletal muscle by impairing the release of the neurotransmitter, acetylcholine (ACh). The clinical symptoms of botulism reflect toxin-induced blockade of ACh release from neuromuscular and neuroeffector junctions (Ambache, 1951; Simpson, 1981).

The basic syndrome of BoNT intoxication is similar for all naturally occurring forms and does not vary appreciably among serotypes (Simpson, 1981; Habermann and Dreyer, 1986; Hatheway, 1989; Brin, 1997). The earliest symptoms generally include visual disturbances, followed by dysphasia, dysphonia, dysarthria, reflecting an especially high susceptibility of cranial efferent terminals to BoNT action (Sellin, 1981; Habermann and Dreyer, 1986; Brin, 1997). A descending generalized skeletal muscle weakness may then develop, progressing from the upper to lower extremities. Involvement of the diaphragm and intercostal muscles can lead to ventilatory failure and death unless appropriate supportive care is provided (Shapiro et al., 1989; Robinson and Nahata, 2003). Among the distinguishing characteristics of botulism are the symmetrical nature of the muscle weakness, the descending progression of symptoms, and the absence of central nervous system or sensory involvement (Simpson, 1981; Brin, 1997; Shapiro et al., 1989; Hatheway, 1989; Simpson, 2004).

Symptoms are usually observed 12–36 h after exposure, although onset times as short as 4 h or as long as 8 days have been reported (Shapiro et al., 1989). The preponderance of symptoms, including the potentially lethal respiratory collapse, stems from inhibition of neuromuscular transmission. Gastrointestinal disturbances that sometimes accompany foodborne botulism (constipation, nausea, diarrhea) may be of parasympathetic origin (Ambache, 1951); the latter may also be a consequence of ingestion of other bacteria or bacterial toxins in the contaminated food product (Lund, 1990; Bonventre, 1979).

The BoNTs are secreted in an anaerobic environment as relatively inactive 150-kDa single-chain protoxins (range, 140–167 kDa), surrounded by complex of several associated proteins. The protoxin is subsequently cleaved to form the active disulfide-linked dichain neurotoxin consisting of a ~100-kDA heavy chain (HC) and ~50-kDa light chain (LC) (DasGupta and Sugiyama, 1972). The BoNTs have three functional domains. The carboxy-terminal end of the HC interacts with gangliosides and putative high-affinity protein receptors of motor neurons (Dong et al., 2003). The amino-terminal region of the HC promotes translocation of the LC into the cytosol (Koriazova and Montal, 2003). The LC is a zinc-containing endoprotease that cleaves serotype- and substrate-specific sites in the SNARE (soluble *N*-ethylmaleamide sensitive factor attachment protein receptors) complex, which leads to a cessation of evoked transmitter release (Brin, 1997; Simpson, 2004).

Substantial work in the past decade has led to the identification of the targets for the protease activity of the BoNT LC. Serotypes A and E cleave the active zone protein SNAP-25 near its carboxy terminus: type A cleaves the final 9 amino acids and type E cleaves the last 26 residues (Schiavo et al., 1993; Binz et al., 1993). Serotypes B, D, F, and G cleave various sites on synaptobrevin/VAMP, a small transmembrane protein located on the surface of small synaptic vesicles (Schiavo et al., 1992; Yamasaki et al., 1994). Serotype C1 cleaves the active zone protein syntaxin as well as SNAP-25 (Blasi et al., 1993; Williamson and Neale, 1998).

38.2 MANIFESTATIONS OF BOTULISM

38.2.1 Foodborne Botulism

Worldwide, BoNT intoxication is most commonly associated with food poisoning, although in the United States, infant botulism (see section 38.2.2) is currently more prevalent. Foodborne botulism results from ingestion of preformed toxin in foods that are contaminated with toxin spores. For BoNT production to occur, the spores must germinate under anaerobic conditions (Hutchinson, 1992). Other permissive factors include a low-acid environment (pH $>$ 4.6), water activity $>$0.94, temperature between 4 and 40°C and lack of adequate preservatives (Baird-Parker and Freame, 1967; Shapiro et al., 1998). Currently, the primary vehicle for foodborne botulism is improperly prepared home-preserved food products, often involving vegetables with a low-acid content. Other sources are restaurants that use unsafe food preparation procedures and contaminated commercially canned food products; the latter has become rare since the introduction of modern canning methods.

Although implicated in only a small fraction of all foodborne illnesses ($<$0.1%), the clinical syndrome of botulism is so severe that each outbreak is viewed as a potential health crisis and extensive surveillance and control measures have been mandated by the Centers for Disease Control and Prevention (Shapiro et al., 1998). Prompt recognition of an outbreak and early treatment with serotype-specific botulinum antitoxin is essential to limit the number of casualties and the severity of the disease.

In the United States, approximately half of all cases of foodborne botulism (average, 24 per year) is caused by serotype A and the remainder is contributed equally by serotypes B and E. Although the number of outbreaks of foodborne botulism have remained relatively constant, the case-to-fatality ratio has improved markedly; during the first half of the twentieth century, foodborne botulism was associated with a 60% mortality, whereas for the past decade, mortality has decreased to less than 10% of symptomatic individuals (Shapiro et al., 1998).

38.2.2 Infant Botulism

Currently the most prevalent form of BoNT intoxication in the United States is infant botulism (Arnon, 1998), which is a consequence of ingestion or inhalation of clostridial spores of serotypes A or B, which colonize the large intestines, germinate, and elaborate toxin into the bloodstream. Young infants, especially between 2 and 4 months of age, are especially susceptible to infant botulism. Although infant botulism may be acquired by inhalation of spores, this differs from "inhalation botulism" discussed in section 38.4 because, in the latter, preformed aerosolized toxin rather than spores are inhaled. Spores do not generally pose a threat in older infants or in most adults (Arnon, 1995, 1998; Cox and Hinkle, 2002; Simpson, 2004).

The characteristic symptoms of infant botulism are poor sucking, constipation, generalized weakness, and respiratory insufficiency that can progress to respiratory failure (Cox and Hinkle, 2002). The risk factors are not completely understood, but the incidence falls sharply after 28 weeks of age, which is likely to be related to development of a more diversified intestinal flora. The latter has been shown to suppress germination and growth of *Clostridium botulinum* spores in mice (Sugiyama and Mills, 1978). Honey has been the one product most often implicated in infant botulism and it is therefore recommended that honey not be given to infants in the susceptible age group (Spika et al.,1989; Arnon, 1998).

Although infant botulism was not recognized until a large outbreak occurred in California in 1976, it currently accounts for approximately 70% of all cases in the United States (Arnon, 1998; Shapiro et al., 1998). Because infant botulism results from a continual elaboration of BoNT, it is more effectively treated by antitoxin than is foodborne botulism. A human botulinum immune globulin preparation (BabyBIG), containing antibodies to BoNT serotypes A and B, has recently been approved by the U.S. Food and Drug Administration for use in infant botulism. In addition, the symptoms in infant botulism tend to develop more gradually than in classical foodborne intoxication, which generally has a more acute presentation.

Under rare conditions, adults may manifest a syndrome similar to infant botulism. Such cases generally occur in hospitalized patients treated with a long course of multiple antibiotics that eliminate the normally suppressive intestinal flora. Other predisposing factors include inflammatory bowel disease and surgical alterations of the bowel (Arnon, 1995).

38.2.3 Wound Botulism

The rarest form of naturally occurring BoNT intoxication is wound botulism in which *C. botulinum* organisms colonize a wound and elaborate toxin; wound botulism currently accounts for less than 25% of all BoNT outbreaks (Sandrock and Murin, 2001). The majority of cases are caused by serotype A and the remainder by serotype B (Shapiro et al., 1998). The neurological symptoms of wound botulism differ little from that of foodborne botulism except for the general absence of gastrointestinal symptoms. Historically, this form of botulism was so uncommon that it was not even recognized until the last half of the twentieth century. From its discovery in 1943 until 1985, only 33 incidents of wound botulism were documented (Merson and Dowell, 1973; Shapiro et al., 1998). An examination of these cases indicated that wounds susceptible to *Clostridium botulinum* are generally described as penetrating, with avascular areas, but need not appear obviously infected or necrotic. Additional risk factors include compound fractures and extensive crush injuries (Merson and Dowell, 1973; Shapiro et al., 1998). Contamination of wounds with *Clostridium botulinum* spores leads to germination and colonization at the site of injury; systemic botulism occurs from toxin transmitted via the bloodstream to distant targets (Merson and Dowell, 1973).

From 1980 to the present time, wound botulism has been observed predominantly in illicit drug users, following repeated subcutaneous administration of narcotics, or in individuals with nasal or sinus lesions from chronic cocaine abuse (Shapiro et al., 1998; Sandrock and Murin, 2001; Chang and Ganguly, 2003). Recent increases in subcutaneous and intranasal routes for drug abuse have led to a greater incidence of wound botulism. During the past decade alone, wound botulism in the population

above has exceeded the total reported in the preceding 40 years by a factor of almost 3 (Shapiro et al., 1998; Sandrock and Murin, 2001). Early antitoxin infusion (<12 h after presentation) increases that rate of recovery and reduces the need for artificial ventilation, whereas use of antibiotics and debridement of the wound does not appear to alter the time course of the disease (Sandrock and Murin, 2001).

For reasons that are not completely understood, wounds are much more likely to be contaminated by *Clostridium tetani* than with *Clostridium botulinum.* Although an aggressive vaccine program has nearly eliminated tetanus in developed nations, the absence of universal tetanus vaccination in many "third world" countries results in over 300,000 cases of tetanus annually; a large number of these occur in neonates, often by infection of the umbilical stump (Snydman, 1989).

38.3 BIOWARFARE AND BIOTERRORIST THREAT

During and shortly after World War II, BoNT was developed as a biological weapon because of its ability to cause extensive morbidity and mortality (Middlebrook and Franz, 1997). BoNT reverted once again to this role with the Iraqi stockpiling BoNT prior to the Persian Gulf War of 1991, and with the rise of terrorist and "religious" groups such as the Japanese Aum Shinrikyo cult, who have not only produced but actually deployed biological and chemical agents (Arnon et al., 2001). As a potential biological weapon, BoNT would be used as an aerosol or as a food contaminant. The former produces a form of the disease designated as inhalation botulism that is not found in nature (Robinson and Nahata, 2003); the latter would produce foodborne botulism, indistinguishable from the natural disease with the exception of an unusually large clustering of cases.

The critical issues regarding inhalation botulism from the point of view of treatment options are potency, rate of onset, and the presence of unique symptoms.

38.4 INHALATION BOTULISM

38.4.1 Unique Characteristics

Relative to the extensive database on naturally occurring botulism, there are comparatively few reports on inhalation botulism. With the exception of the single human outbreak described below, there are no documented cases of human exposure by the inhalation route; each case of inhalation botulism must therefore be viewed with suspicion and considered as a hostile act (Robinson and Nahata, 2003).

Among the earliest studies to examine different routes of BoNT administration was that reported by Cardella (1964). This author compared intraperitoneal (i.p.), respiratory and oral BoNT challenges in guinea pigs. Inhalation was achieved by exposing animals to static aerosols of toxin, and over 60% of the particles were smaller than 5 μm in diameter. The results are provided in Table 38.1.

Inhalation exposure was found to be intermediate in lethality between i.p. and oral administration. This may be surprising at first because many toxins are more lethal by inhalation than by injection. Thus T-2 mycotoxin, tetrodotoxin, and saxitoxin are all more toxic by inhalation than by parenteral administration (Creasia et al., 1990). However, it must be borne in mind that BoNT is a large protein,

Table 38.1 Lethality of BoNT Serotypes A-E by Various Routes of Administration in the Guinea Pig[a]

Serotype	i. p.	Aerosol	Oral
A	5.2	141	717
B	4.2	350	306
C1	1.6	87	177
D	4.1	186	436
E	34.3	778	—

[a] The doses are normalized to mouse i.p. LD_{50} units.

and aerosolized toxin must gain entry by first binding to receptors in airway epithelial cells and then undergo active transport. Because the BoNT receptors in the airways must be finite and saturable, only a fraction of the inhaled dose may be presumed to gain entry into the general circulation.

In an elegant study, Park and Simpson (2003) demonstrated that BoNT/A or the pure HC can bind to airway epithelial cells and undergo energy-dependent transcytosis, with net transport being in the apical-to-basolateral direction. This was the first direct demonstration of BoNT entry relevant to inhalation exposure.

38.4.2 Human Case Report of Inhalation Botulism

To date, only one human outbreak of inhalation botulism has ever been reported (Holzer, 1962). Three laboratory investigators in Germany exposed rabbits and guinea pigs to an aerosol of pure BoNT/A that was stabilized by addition of colloids. They performed autopsies on the animals the following day wearing protective gloves but no mask or respirator. Exposure to BoNT/A apparently occurred when the skin of the animals was removed, and the toxin that was deposited on the fur from the aerosol exposure became airborne and was inhaled. All three investigators became symptomatic, and in each case, after a 3-day latent period. The most prominent symptoms were dysphagia, dysphonia, dizziness, headaches, and blurred vision. All three individuals were hospitalized for approximately 1 week, and showed a positive serum test for the presence of BoNT by the mouse bioassay. Two of the patients received infusions of equine antitoxin, but it is unclear whether this led to a significant improvement in their recovery. The nature of the symptoms and the relatively rapid recovery described in this case report, are consistent with systemic botulism involving a relatively low dose of toxin.

38.4.3 Absence of Specific Pulmonary Symptoms from Inhalation of BoNT

Although BoNT gained entry via the lungs in the above-mentioned human intoxications, no alteration of pulmonary function was reported in any of the three laboratory investigators. Since inhalation of BoNT would be expected to generate high initial concentrations of toxin in the lungs, it may be thought that severe pulmonary dysfunction would result from inhalation of aerosolized BoNT. However, inhibition of ACh release by BoNT causes relaxation of bronchiolar smooth muscle, which would improve rather than depress respiration (Abad Santos et al., 2003).

In subsequent animal experiments, it was demonstrated that no specific pulmonary alterations result from inhalation of BoNT, even at lethal doses (Franz et al., 1993; Gelzleichter et al., 1999). This is consistent with findings that (with the possible exception of serotype C1), exposure to BoNT does not result in morphological alterations, even at the ultrastructural level (Duchen, 1971). The absence of cytotoxicity allows BoNT to be used as a therapeutic agent for the treatment of dystonias, movement disorders, and muscle spasms in humans (Brin, 1997).

In contrast to BoNT, inhalation of many biowarfare toxins leads to potentially fatal necrotic lesions, especially of the airways. Thus, Poli et al. (1996) demonstrated that inhalation of ricin in mice led to diffuse airway epithelial necrosis, characterized by severe interstitial edema and inflammation, and that all mice died 48–96 h after an aerosol challenge with ricin. Inhalation of anthrax spores has been reported to produce splenomegaly, enlargement of lymph nodes and hemorrhages in various organs, particularly the lungs, mediastinum, meninges, and gastrointestinal tract (Vasconcelos et al., 2003). Similarly, inhalation of aerosolized staphylococcal enterotoxin B resulted in pulmonary lesions that included severe, diffuse interstitial and alveolar pulmonary edema, leukocytic infiltrates, perivascular edema, and alveolar fibrin deposition (LeClair et al., 1996).

38.4.4 Treatment of Inhalation Botulism

A critical question for botulinum therapeutics is whether the conventional vaccine and antitoxin currently used for foodborne and wound botulism would be effective in treating patients following

an inhalation exposure of BoNT. Because inhalation botulism does not occur in nature, and the only experience in humans comes from the laboratory workers described earlier, it is necessary to determine whether the currently used vaccine and antitoxin are effective following inhalation challenge of BoNT.

38.4.4.1 Efficacy of the Vaccine (Toxoid) in Inhalation Botulism

BoNT serotypes A–E are converted into toxoid for immunization by incubation for several weeks in 0.6% formalin at 35 to 37°C under mildly acidic conditions (pH 5.5–6.5) (Cardella, 1964). This vaccine has been available for immunization of high-risk individuals, such as laboratory personnel who handle BoNT, for over 50 years. There are extensive data on the efficacy of the pentavalent vaccine in oral and i.p. challenge by BoNT but there is comparatively little awareness of its efficacy in inhalation botulism (Cardella, 1964; Shapiro et al., 1998). In experiments where guinea pigs were immunized with the pentavalent toxoid and challenged by i.p., oral, or inhalation BoNT, the vaccine was found to be equally protective against an inhalation challenge as against challenge by the other routes of administration. Guinea pigs that were immunized with 1 ml of pentavalent toxoid were able to survive an aerosol challenge of ~2000 LD_{50} of BoNT 50 days later (Cardella, 1964).

Although the vaccine is effective against an aerosol challenge of BoNT, it is unlikely that the population vulnerable to an aerosol attack will have been vaccinated. Two compelling factors argue against mass vaccination for botulism. First, the incidence of botulism from natural sources is exceedingly rare. During the past three decades, the total number of cases of botulism in the United States has averaged approximately 100 per year from all causes (Shapiro et al., 1998). This would hardly justify prophylaxis of large segments of the population. Second, BoNT serotypes A and B are currently the treatments of choice for movement disorders, and immunized individuals would be precluded from this treatment if they developed disorders of muscle tone (Brin, 1997).

38.4.4.2 Efficacy of the Antitoxin in Inhalation Botulism

Because a postexposure therapeutic for botulinum intoxication is not currently available (Adler et al., 2001), the most effective treatment for BoNT intoxication is infusion of antitoxin as soon as possible after exposure is established. Both human- and equine-derived antitoxin have been tested for protection against an inhalation challenge of BoNT, and both were found to be highly effective. In a pivotal study, Rhesus monkeys were injected with 8–16 IU/kg of human hyperimmune globulin or 14–143 IU of equine $F(ab')_2$ antitoxin. These yielded plasma titers of <0.02 to 0.6 IU for BoNT serotype A. The animals were challenged head-only with an ~6 LD_{50} dose of aerosolized liquid BoNT/A 48 h later (Franz et al., 1993). All animals not only survived this challenge but were also free of any signs of BoNT intoxication. Animals even survived challenge by BoNT/A 6 weeks after a single administration of 16 IU/kg of human hyperimmune globulin. Control animals, on the other hand, died 2–4 days after BoNT/A challenge and exhibited clinical signs of intoxication 12–18 hr before death. These signs were similar to those observed with other forms of botulism in non-human primates and consisted of, in order of onset, muscle weakness, intermittent ptosis, poor head control, dysphagia, and finally lateral recumbency.

Additional experiments were carried out in which the high dose of equine $F(ab')_2$ antitoxin (143 IU/kg) was given 46 h after an aerosol exposure of BoNT/A. In general, animals that were highly symptomatic at the time of antitoxin administration died during the next two days, whereas those with minimal or no signs at the time of antitoxin infusion recovered over the next 10 days and survived. From these data, the window for antitoxin administration in inhalation botulism is similar to that for other forms of botulism and is highly dependent on the severity of symptoms before therapy is initiated. This is reasonable since antitoxin acts by neutralizing BoNT that is free in circulation or bound to nerve terminals but not yet internalized (Shapiro et al., 1998).

A similar study was performed on passively immunized guinea pigs with comparable results (Gelzleichter et al., 1999). Animals were injected i.p. with human botulinum immune globulin

obtained from volunteers vaccinated with botulinum toxoid to generate a serum titer of 0.25 IU/ml; 0.25 IU is considered to be protective by the U.S. Centers for Disease Control and Prevention. Guinea pigs were challenged 24 h later with aerosolized BoNT serotypes A, B, C1, D, or E at doses corresponded to 25 $\times$ LCt_{50}. Ninety percent of guinea pigs survived the aerosol challenge by serotypes A, B, and E, and all animals survived challenge by serotypes C and D. It was also found that surviving guinea pigs were asymptomatic, thus passive immunization also prevented all BoNT-related signs. Since the antibodies were derived from human volunteers, these data further suggest that the volunteer would have survived inhalation challenge by BoNT.

38.5 CONCLUSIONS

BoNT is the most toxic substance known to mankind and has the potential for both alleviating a variety of muscle disorders (Brin, 1997) and for causing massive loss of life if deployed by terrorists or hostile nations (Arnon et al., 2001). BoNT also has its traditional role as the causative agent in foodborne, infant, and wound botulism. Deployment of BoNT by terrorists is expected to involve food contamination or aerosol exposure. Battlefield use of BoNT would most likely involve aerosol exposure from exploding munitions or aerial spraying. Although inhalation BoNT has not been well studied, there is sufficient information to indicate that the symptoms would be similar to foodborne botulism with the possible exception of diarrhea and nausea, and that the vaccine and antitoxin would still be effective. Moreover, the latency would also be similar to that observed for foodborne botulism, and the potency would be intermediate between parenteral and oral exposure to toxin. Because vaccination is not considered practical for the general population, and the antitoxin has a limited window, it is essential to develop therapeutic agents that will inhibit the catalytic activity of the LC and restore function in patients exhibiting moderate to severe symptoms (Adler et al., 2001). Much progress has already been made in this direction and it is reasonable to expect serotype selective inhibitors of the LC to be developed by the next decade.

ACKNOWLEDGMENTS

The authors thank Drs. Robert E. Sheridan, Sharad S. Deshpande, James P. Apland, James E. Keller, Heather A. Manley, Angela Purcell, George Oyler, Frank Lebeda, and Charles Millard for many fruitful and enlightening discussions.

REFERENCES

Adler, M., Oyler, G., Keller, J.E., and Lebeda, F.J., Pharmacological countermeasures for botulinum intoxication, in *Chemical Warfare Agents: Toxicity at Low Levels*, Somani, S.M. and Romano, J.A., Jr., Eds., CRC Press, Boca Raton,FL, 2001.

Abad Santos, F., Novalbos, J., Gallego Sandin, S., and Galvez Mugica, M.A., Regulation of bronchial tone in chronic obstructive pulmonary disease (COPD): role of muscarinic receptors, *An. Med. Int*., 20, 201–205, 2003.

Ambache, N., A further survey of the action of *Clostridium botulinum* toxin upon different types of autonomic nerve fibre, *J. Physiol. (Lond.)*, 113, 1–17, 1951.

Arnon, S.S., Botulism as an intestinal toxaemia, in *Infections of the Gastrointestinal Tract*, Blaser, M.J., Smith, P.D., Ravdin, J.I., and Guerrant, R.L., Eds., Raven Press, New York, 1995.

Arnon, S.S., Infant botulism, in *Textbook of Pediatric Infectious Diseases of the Gastrointestinal Tract*, Feigen, R.D. and Cherry, J.D., Eds., W.B. Saunders, Philadelphia, 1998.

Arnon, S.S., Schechter, R., Inglesby, T.V., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Fine, A.D., Hauer, J., et al., Botulinum toxin as a biological weapon: medical and public health management, *JAMA*, 285, 1059–1069, 2001.

Baird-Parker, A.C. and Freame, B., Combined effect of water activity, pH and temperature on the growth of Clostridium botulinum from spore and vegetative cell inocula, *J. Appl. Bacteriol*., 30, 420–429, 1967.

Binz, T., Blasi, J., Yamasaki, S., Baumeister, A., Link, E., Sudhof, T.C., John, R., and Niemann, H., Proteolysis of SNAP-25 by types E and A botulinal neurotoxins, *J. Biol. Chem.*, 269, 1617–1620, 1993.

Blasi, J., Chapman, E.R., Link, E., Binz, T., Niemann, H., and Jahn, R., Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin, *EMBO J.,* 12, 4821–4828, 1993.

Bonventre, P.F., Absorption of botulinal toxin from the gastrointestinal tract, *Rev. Infect. Dis.*, 1, 663–667, 1979.

Brin, M.F., Botulinum toxin: chemistry, pharmacology, toxicity and immunology, *Muscle Nerve*, 6, S146–S168, 1997.

Cardella, M.A., Botulinum toxoids, in *Botulism: Proceeding of a Symposium*, Lewis, K.H. and Cassel, K., Jr., Eds., Public Health Service Publication, Washington, DC, 1964.

Chang, G.Y. and Ganguly, G., Early antitoxin treatment in wound botulism results in better outcome, *Eur. Neurol*., 49, 151–153, 2003.

Cox, N. and Hinkle, R., Infant botulism, *Am. Fam. Phys*., 65, 1388–1392, 2002.

Creasia, D.A., Thurman, J.D., Wannemacher, R.W., Jr., and Bunner, D.L., Acute inhalation toxicity of T-2 mycotoxin in the rat and guinea pig, *Fundam. Appl. Toxicol*., 14, 54–59, 1990.

DasGupta, B.R. and Sugiyama, H., A common subunit structure in *Clostridium botulinum* types A, B and E toxins, *Biochem. Biophys. Res. Commun*., 48, 108–112, 1972.

Dong, M., Richards, D.A., Goodnough, M.C., Tepp, W.H., Johnson, E.A., and Chapman, E.R., Synaptotagmins I and II mediate entry of botulinum neurotoxin B into cells, *J. Cell Biol*., 162, 1293–1303, 2003.

Duchen, L.W., An electron microscopic study of the changes induced by botulism toxin in the motor end-plates of slow and fast skeletal muscle fibers of the mouse, *J. Neurol. Sci.,* 14, 47–60, 1971.

Franz, D.R., Pitt, L.M., Clayton, M.A., Hanes, M.A., and Rose, K.J., Efficacy of prophylactic and therapeutic administration of antitoxin for inhalation botulism, in *Botulinum and Tetanus Neurotoxins*, DasGupta, B.R., Ed., Plenum Press, New York, 1993.

Gelzleichter, T.R., Myers, M.A., Menton, R.G., Niemuth, N.A., Matthews, M.C., and Langford, M.J., Protection against botulinum toxins provided by passive immunization with botulinum human immune globulin: evaluation using an inhalation model, *J. Appl. Toxicol*., 19, 535–538, 1999.

Habermann, E. and Dreyer, F., Clostridial neurotoxins: handling and action at the cellular and molecular level, *Curr. Top. Microbiol. Immunol*., 129, 94–179, 1986.

Hatheway, C.L., Bacterial sources of clostridial neurotoxins, in *Botulinum Neurotoxin and Tetanus Toxin*, Simpson, L.L., Ed., Academic Press, Inc., San Diego, 1989.

Holzer, V.E., Botulismus durch inhalation, *Med. Klin*., 57, 1735–1738, 1962.

Hutchinson, D.N., Foodborne botulism. New techniques for preserving foods bring the need for greater awareness of the risks, *Br. Med. J.,* 305, 264–265, 1992.

Koriazova, L.K. and Montal, M., Translocation of botulinum neurotoxin light chain protease through the heavy chain channel, *Nat. Struct. Bi*ol., 10, 13–18, 2003.

LeClaire, R.D., Hunt, R.E., Bavari, S., Estep, J.E., Nelson, G.O., and Wilhelmsen, C.L., Potentiation of inhaled staphyloccocal enterotoxin B-induced toxicity by lipopolysaccharide in mice, *Toxicol. Pathol*., 24, 619–626, 1996.

Lund, B.M., Foodborne disease due to bacillus and clostridium species, *Lancet*, 336, 982–986, 1990.

Merson, M.H. and Dowell, V.R., Jr., Epidemiologic, clinical and laboratory aspects of wound botulism, *N. Engl. J. Med*., 289, 1105–1110, 1973.

Middlebrook, J.L. and Franz, D., Botulinum toxins, in *Medical Aspects of Chemical and Biological Warfare. Textbook of Military Medicine*, Sidell, F.R., Takafuji, E.T., and Franz, D.R., Eds., Office of the Surgeon General, Washington, DC, 1997.

Park, J.-B. and Simpson, L.L., Inhalational poisoning by botulinum toxin and inhalation vaccination with Its heavy-chain component, *Infect. Immun*., 71, 1147–1154, 2003.

Poli, M.A., Rivera, V.R., Pitt, M.L., and Vogel, P., Aerosolized specific antibody protects mice from lung injury associated with aerosolized ricin exposure, *Toxicon*, 34, 1037–1044, 1996.

Robinson, R.F. and Nahata, M.C., Management of botulism, *Ann. Pharmacother*., 37, 127–131, 2003.

Sandrock, C.E. and Murin, S., Clinical predictors of respiratory failure and long-term outcome in black tar heroin-associated wound botulism, *Chest*, 120, 562–566, 2001.

Sathyamoorthy, V. and DasGupta, B.R., Separation, purification, partial characterization and comparison of the heavy and light chains of botulinum neurotoxin types A, B and E, *J. Biol. Chem*., 260, 10461–10466, 1985.

Schiavo, G., Benfenati, F., Poulain, B., Rosetto, O., Polverino de Laureto, P., DasGupta, B.R., and Montecucco, C., Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin, *Nature*, 359, 832–835, 1992.

Schiavo, G., Rosetto, O., Catsicas, S., Polverino de Laureto, P., DasGupta, B.R., Benfenati, F., and Montecucco, C., Identification of the nerve terminal targets of botulinum neurotoxin serotypes A, D, and E, *J. Biol. Chem*., 268, 23784–23787, 1993.

Shapiro, R.L., Hatheway, C., and Swerdlow, D.L., Botulism in the United States: a clinical and epidemiological review, *Ann. Intern. Med*., 129, 221–228, 1998.

Simpson, L., The origin, structure and pharmacological activity of botulinum toxin, *Pharmacol. Rev*., 33, 155–188, 1981.

Simpson, L., Identification of the major steps in botulinum toxin action, *Annu. Rev. Pharmacol. Toxicol*., 44, 167–193, 2004.

Snydman, D.R., Foodborne diseases, in *Mechanism of Microbial Diseases*, Schaechter, M., Medoff, G., and Schlessinger, D., Eds., Williams and Wilkins, Baltimore, 1989.

Spika, J.S., Shaffer, N., Hargrett-Bean, N.,Collin, D.S., MacDonald, K.L., and Blake, P.A., Risk factors for infant botulism in the United States, *Am. J. Dis. Child*., 143, 828–832, 1989.

Sugiyama, H., Clostridium botulinum neurotoxin, *Microbiol. Rev*., 44, 419–448, 1980.

Sugiyama, H. and Mills, D.C., Intraintestinal toxin in infant mice challenged intragastrically with *Clostridium botulinum* spores, *Infect. Immun*., 21, 59–63, 1978.

Vasconcelos, D., Barnewall, R., Babin, M., Hunt, R., Estep, J. Nielsen, C., Carnes, R., and Carney, J., Pathology of inhalation anthrax in cynomolgus monkeys (Macaca fascicularis), *Lab. Investig*., 83, 1201–1209, 2003.

Williamson, L.C. and Neale, E.A., Syntaxin and 25-kDa synaptosomal-associated protein: differential effects of botulinum neurotoxins C1 and A on neuronal survival, *J. Neurosci. Res*., 52, 569–583, 1998.

Yamasaki, S., Baumeister, A., Binz, T., Blasi, J., Link, E., Cornille, F., Roques, B., Fykse, E.M., Sudhof, T.C., and Jahn, R., Cleavage of members of the synaptobrevin/VAMP family by types D and F botulinal neurotoxins and tetanus toxin, *J. Biol. Chem*., 269, 12764–12772, 1994.

39 Inhalation Ricin: Aerosol Procedures, Animal Toxicology, and Therapy

Robert W. Wannemacher and Jamie B. Anderson

CONTENTS

39.1 INTRODUCTION

Ricin is an extremely potent toxin (Fodstad et al., 1976) derived from the castor bean, *Ricinus communis, Euphorbiaceae,* with an estimated human lethal dose as low as 1 μg/kg. It is a potential biological warfare and terrorist threat to military and civilian personnel. No effective antidote currently exists for toxic exposure to ricin.

Ricin is one of four plant toxins, including abrin, modeccin, and viscumin, which have similar structure and mechanism of action. Their properties have been reviewed by Olsnes and Pihl (1982a). Ricin remains in the residue mash of castor beans from which oils and other materials have been extracted by cold-pressing seeds of the plant (Brugsch, 1960). Highly purified ricin is commercially available. The toxin is synthesized as a single polypeptide in maturing castor beans where it accumulates in the storage granules of the seeds. The toxin consists of two dissimilar polypeptide chains held together by a disulfide bond, which joins cysteinyl residues near the carboxy terminus of the A-chain and the amino terminus of the B-chain. One of these polypeptides (32 kDa, designated the A-chain) is a potent inhibitor of protein synthesis. The other (34 kDa, the B-chain) is a galactose- or an *N*-acetylgalactosamine-binding lectin (Lin et al., 1971; Funatsu et al., 1978; Lord et al., 1987).

When administered parenterally to animals, ricin is one of the most toxic compounds known. The toxic consequences of ricin are attributed to the biological activity of the A-chain, whereas the B-chain function is to bind the toxin to cell surface receptors. During endocytosis, the A-chain of ricin is transferred to the cytosol where ribosome inactivation occurs. This results in an inhibition

of protein synthesis by inhibiting elongation factor-2 (EF-2)-dependent GTPase activity of the ribosome (Lin et. al, 1971; Nicolson et al., 1975; Olsnes, and Pihl, 1976; Funatsu et al., 1978; Lord et al., 1987; Endo et al., 1988; Stirpe et al., 1988; Preijers et al., 1989).

When the A-chain of ricin is separated from the B-chain and administered parenterally to animals, it has little or no toxicity (Olsnes and Pihl, 1976, 1982b; Lord et al., 1987; Soler-Rodriguez et al., 1992). It has been combined with cell-specific antibodies to form immunotoxins that are being evaluated in phase I and II clinical studies in cancer patients (Mendelsohn, 1991; Byers and Baldwin, 1992; Vitetta et al.,, 1993; Ghetie and Vitetta, 1994). The A-chain of ricin is a glycoprotein that is rapidly cleared from circulation (Wawrzynczak et al, 1991; Trown et. al., 1991). When the A-chain of ricin toxin is deglycosylated (dgRTA), it is cleared from the circulation at a slower rate than the native chain (Blakey, and Thorpe, 1986; Foxwell et al., 1987).

39.2 AEROSOL PROCEDURES

39.2.1 Methods

There are various methods and equipment used in research for aerosol delivery. Changes in either the method or equipment used to generate and deliver the aerosol can affect the properties of the aerosol such as the particle size which can alter the location of ricin deposition within the animal (Doebler et al, 1995; Roy et al., 2003; Phalen, 1984). These variations in location of deposition may potentially have an impact on the pathogenesis in the case of ricin where the target organ may also be the initial site of deposition (Xu et al., 1995; Rauber and Heard, 1985; Fodstad et al., 1984; Baluna and Sausville, 1996; Balint, 2000). Therefore, methods and equipment must be carefully reviewed when evaluating research data. Data must also be interpreted in light of which animal model was used in the research. For example, the use of various strains of inbred and outbred mice have yielded different LD_{50} levels, different times to death, and different pathology (Wilhelmsen,1997).

Inhalation aerosols may be generated and studied by using whole-body exposure chambers, nose-only and head only exposure systems, or closed-loop metabolism chambers. The aerosol chamber is designed to accommodate the type of exposure method by allowing the animals head, snout, or whole body to be exposed to the generated aerosol. Aerosols can be generated using wet-droplet dispersions or dry-powder agent preparations (Baron et al., 2001). Wet preparation of agent is normally used and is the method of preparation for the studies cited in the following sections of this chapter. Wet dispersion of aerosols is normally generated with a collision nebulizer (Neb) and the aerosol concentration is normally sampled using all-glass impingers (AGIs) or impactors although other types of generators and collection devices are available. The concentration of the sampled aerosol may be determined by protein assay, but other methods may be used, such as labeling the ricin with fluorescent dye or radioisotope tags and analyzing the sample for such activity. After the amount of ricin in the collected sample is determined, the aerosol concentration (Aero conc.) can be calculated as follows (Jemski and Phillips, 1964):

$$\text{Aero conc.}_{\mu g/ml} = (\text{AGI conc.}_{\mu g/ml} \times \text{AGI volume.}_{ml}) / (\text{exposure time}_{min} \times \text{AGI flow rate}_{ml/min})$$

The animal's minute volume (MV) is either determined by plethysmography just prior to or during the exposure. It may also be empirically determined using Guyton's formula as follows (Guyton, 1947):

$$MV = 2.1\ (\text{weight}_{gm})^{0.75}$$

The total amount of ricin the animal inhaled from the aerosol exposure can be calculated with the following formula (Jemski and Phillips, 1964):

$$\text{Total dose} = \text{Aerosol conc.}_{\mu g/ml} \times \text{Minute volume}_{ml} \times \text{Exposure time}_{min}$$

The efficiency of the system for delivering the toxin is reflected in the spray factor (SF). The SF is the ratio between the number of organisms in the aerosol and the number of organisms from the suspension from which the aerosol was generated. The SF can be derived from the following formulas (Miller and Gremillion, 1963).

$$SF = \text{Microorganisms per liter of aerosol/ microorganism per liter of liquid suspension}$$
$$SF = [(\text{AGI conc.}_{\mu g/ml} \times \text{AGI volume.}_{ml}) / (\text{exposure time}_{min} \times \text{AGI total flow rate}_{ml/min})] / \text{nebulizer conc.}_{\mu g/ml}$$

The concentration of suspension from which the aerosol was generated is known as the starting concentration (Cst) and can be calculated as shown below. The historical spray factor (hSF) can be determined by averaging the SFs of previous sprays that used the same methods and starting concentrations (Miller and Gremillion, 1963).

$$Cst = \text{Aerosol concentration/spray factor}$$
$$Cst = [(\text{Dose/animal}_{\mu g}) / (MV_{ml} \times \text{time}_{min})] / hSF$$

39.3 ANIMAL TOXICOLOGY

39.3.1 Mice and Rats

A well-characterized mouse inhalation model (Hewetson et. al., 1996) was developed to study toxicity of aerosolized ricin. An inbred strain of mice (BALB/c) has been used in most of the ricin toxicity studies. The median lethal dose (LD_{50}) of ricin by whole-body aerosol route of exposure was 11.2 μg/kg, with 95% confidence limits of 10.0 to 12.8 μg/kg. Similar results (12.1 and 14.8 μg/kg) were obtained using a different source of ricin in whole-body aerosol exposure to BALB/c mice (Wilhelmsen, 1997). However, there appears to a strain susceptible to ricin toxicity by aerosol exposure (Wilhelmsen, 1997). The most susceptible strain was BXSB ($LD_{50} \approx 2.8$ μg/kg) and the least susceptible strain was BALB/c. Other strains and the outbred stock were intermediated in susceptibility (LD_{50} NIH Swiss ≈4.9 μg/kg; LD_{50} CBA/J, C57BL/6J, C2H/HeJ ≈5.3 μg/kg; LD_{50} A/J ≈8.2 μg/kg; LD_{50} C3H/HeN ≈9.0 μg/kg).

The onset of illness, morbidity, and death is very rapid in mice. While we are dedicated to reducing distress in all animals, it is difficult to identify terminally ill mice that should be euthanized without jeopardizing the integrity of the study. Time to death is dose dependent and can vary from 3 to 12 days for aerosol exposure. On day 1 after aerosol challenge, the lungs of the mice exposed to a lethal dose of ricin had perivascular edema, alveolar flooding, acute peribronchovasculitis, acute bronchitis, acute alveolitis, perivascular cuffing, and eosinophilia. The bronchiolar alveolar lavage (BAL) fluid had a marked increase in the albumin concentration on day 1 after exposure to a lethal dose of aerosolized ricin. An elevation in the albumin concentration in the BAL fluid is considered to be a biochemical marker of lung injury. In contrast, in mice injected with a lethal i.p. dose of ricin, the only pathological lesion was necrosis in some of the rapidly dividing tissues such as lymphoid tissue and intestinal cells. From the observation in the mouse model, it can be concluded that inhaled ricin is a very severe lung toxin.

Similar conclusions were indicated from ricin inhalation studies in the rat (Assard et al., 1993; Fritz et al., 1993; Langford et al., 1993; Griffiths et al., 1994, 1995). The lesions induced by acute inhaled ricin intoxication in rats were confined to the lung and consisted of intraalveolar edema, acute alveolitis, and diffuse necrosis of the epithelium lining the lower respiratory tract. Intoxicated rats died in 29–32 h postexposure to inhaled ricin. Death was attributed to rapid pulmonary flooding that could no longer compensate for the dynamic accumulation of edema fluid. In Fisher rats exposed to a sublethal dose of inhaled ricin (Steele et al., 1995), light microscopic changes were first noted at 16 h postexposure. By 24 h, there was extensive debris within alveoli, admixed with few granulocytes, and

detached type II pneumocytes. Also, segmental and single-cell necrosis of bronchiolar was observed at 24 h postexposure. Pulmonary edema was evident by 32 h postexposure. Between 16 and 48 h postexposure to inhaled ricin, macrophages were not observed within alveoli. Macrophages were also absent in bronchoalveolar fluid after exposure to inhaled ricin (Assard et al., 1993). Thus, an intriguing hypothesis was proposed in which alveolar macrophages were postulated to mediate pulmonary damage and alveolar edema after exposure to lethal doses of inhaled ricin.

In contrast, the lesions induced by lethal parenteral doses of ricin in laboratory rodents, rabbits, and ruminants include lymphocytic necrosis of lymphoid organs; hemorrhages, necrosis, hyaline droplets, and fibrin thrombi in the liver; hemorrhages and necrosis in the adrenal glands; tubular hyaline changes in the kidney; degeneration of the heart muscle; and congestion and hemorrhages of the gastrointestinal tract (Flexner, 1897; Waller et al., 1966; Bingen et al., 1987; Griffiths et al., 1987).

39.3.2 Non-Human Primate (NHP)

Because there are no reported observations on the toxicity of ricin in humans, studies were initiated in NHP models. A pilot study (Pitt, 1995) was done on toxicity of inhaled ricin in rhesus monkeys *(Macaca mulatta).* Because of the prevalence of naturally occurring viral infections in rhesus monkeys, it was decided to use African green *(Chlorocebus aethiops)* monkeys in future studies. The estimate of the median lethal dose determinations for the two species of monkeys was by the staircase method of statistical analysis. The African green monkeys (LD_{50} 5.8 μg/kg) appeared to be slightly more susceptible to the lethal affects of inhaled ricin than the rhesus monkeys (LD_{50} 15 μg/kg). At an inhaled dose of ricin ranging from 21.0 to 41.8 μg/kg, the rhesus monkeys died in 36 to 40 h. The most consistent gross and microscopic lesions were confined to the respiratory system. All rhesus monkeys exposed to inhaled ricin had multifocal to coalescing fibrinopurulent pneumonia, diffuse necrosis, acute inflammation of airways, and nearly diffuse alveolar flooding, with peribrovascular edema (Wilhelmsen and Pitt,1996). They also had purulent traceitis, fibrinopurulent pleuritis, and purulent mediastinal lymphadenitis. All of which led to serve respiratory distress and death.

As with mice, the onset of illness, morbidity, and death in the monkey is very rapid. In the African green monkeys, a dose-related (3–96 μg/kg) time-to-death (20–72 h) was observed for six nontreated animals (Wannemacher et al., 2004). Clinical signs included elevated body temperature (2–3°C) on day after exposure, decrease in food intake, slow to sluggish activity, antisocial to depressed behavior, and rapid and labored breathing. All the monkeys on gross examination had lungs with hemorrhage, congestion, and edema that was bilateral, multifocal, and moderate with serosanguinous pleural effusion. The primary histological lesions were restricted to the lungs and consisted of varying severity of an acute to subacute, fibrinohemorrhagic pneumonia with edema filling alveoli and/or expanding of the adventitial tissue surrounding bronchioles and medium arterioles. Multifocally, there were areas of necrosis that involved alveolar septa and bronchiolar mucosa.

Three of the monkeys on the median lethal dose study were exposed to lower doses (0.8–7.7 μg/kg) of inhaled ricin and survived for 12 weeks postexposure at which time they were euthanized and necropsied. All had the following clinical signs that were dose-related in severity, including elevated body temperature, decrease in food intake, slow to sluggish activity, antisocial to depressed behavior, loss of body weight, elevated serum LDH, decreased serum albumin, and chest x-rays that indicated areas of increased lung opacity and alveolar consolidation. At necropsy, the lungs of all three monkeys were pink and of normal consistency. Histology lung lesions were dose related and varied from pulmonary architecture that was within normal limits to the lungs that had moderate interstitial fibrosis, mild interstitial and periateriolar edema, and lymphangiectasia.

The lesions of acute inhaled ricin intoxication in NHP closely resembled those reported in mice, rats, rabbits, and ruminants, which suggests that similar lung lesions could be seen in humans exposed to inhaled ricin.

39.4 TREATMENT/PREVENTION OF INHALED RICIN INTOXICATION

39.4.1 Mice and Rats

39.4.1.1 Efficacy of Therapeutics against Inhaled Ricin

Approximately 100 compounds have been screened in an *in vitro* cell system for ricin therapy. A few of these were protective in the cell system but none were efficacious in preventing or treating ricin intoxication in a mouse model. At the present time, the only treatment for ricin intoxication is supportive therapy for respiratory distress.

39.4.1.2 Efficacy of Vaccines against Inhaled Ricin

The first-generation vaccine was a formalin-inactivated ricin toxoid. This vaccine did protect vaccinated mice from parental or inhaled exposure to ricin (Hewetson et al, 1995). Although this vaccine was efficacious, some concerns were raised about its safety, the possibility of reversion to native toxin, and manufacturing procedures. Therefore, a second-generation vaccine was recommended that overcame some of the concerns with ricin toxoid. The A-chain of ricin contains an epitope that is highly antigenic and induces neutralizing antibodies (Lemley et al., 1994). Thus, a subunit vaccine was selected as a second-generation candidate.

Because chemically deglycosylated ricin A-chain (dgRTA) is cleared from the circulation at a slower rate than the native chain (Blakey and Thorpe, 1986; Foxwell et al., 1987), it was evaluated for efficacy in vaccinated rats and mice against lethal intoxication from parenteral or inhaled ricin challenge (Hewetson et al., 1996). The dgRTA stimulated high-neutralizing serum antibodies that protected the animals from lethal effects of parenteral and inhaled ricin challenge and reduced the severity of lung injury. Approximately 140,000 doses of the dgRTA vaccine were produced under cGMP procedures as a lyophilized product, which were stored under GLP condition at –20°C. This product was tested periodically for potency, which continued to remain highly efficacious over seven 7 years of storage. However, although this dgRTA vaccine was shown to be safe in short- and long-term studies in mice, this vaccine still contained an active enzyme site, which if transported into a cell, would be cytotoxic. In addition, the current manufacturing process did not meet cGMP requirements for a licensed vaccine and dgRTA tented form aggregates when reconstituted in water. Thus, a third-generation vaccine effort began during which a recombinant ricin toxin A-chain (rRTA) mutant was produced in which the enzymatic site was inactivated.

The development of this third-generation vaccine involved computer modeling of rRTA mutants, synthesis of synthetic genome, expression of the rRTA mutant in *Escherichia coli,* and purification of the rRTA protein (Olson et al., in press). Once purified, the mutants of rRTA were tested for alteration in enzyme activity and the ability to protect against ricin challenge. Five mutants of rRTA were produced that had no measurable enzymatic activity and were evaluated for efficacy in a mouse model against lethal i.p. and aerosolized ricin challenge. While all five of the recombinant candidates offered some protection against i.p. and aerosolized ricin, one vaccine candidate, with deletions at positions 33–44 and 199–206 in the ricin A-chain (1–33/44–198 rRTA), at 10-μg/mouse protected 100% of mice that were boosted three times (0, 4, and 8 weeks) and challenged with aerosolized ricin (Table 39.1). This product was very soluble in phosphate-buffered saline (PBS) and did not form aggregates. Therefore, the 1–33/44–198 rRTA was selected as the lead recombinant ricin vaccine candidate. However, even though the 1–33/44–198 rRTA protected the mice against aerosolized ricin, the serum anti-ricin IgG concentrations were approximately one tenth those obtained with a similar amount of dgRTA. The incorporation of an adjuvant (0.2% Alhydrogel) with 1–33/44–198 rRTA increased the anti-ricin IgG concentrations to one half or equal to those observed with dgRTA, while it had no effect on anti-ricin IgG concentration of the nonrecombinant vaccine.

Table 39.1 Summary of Survival and Preexposure Antibody Concentrations of Mice Vaccinated with (1-33/44-198 rRTA) ± Adjuvant against Aerosol Exposure to Lethal Doses of Ricin Toxin[a]

Antigen	Survival (alive/total)	Anti-Ricin IgG, mg/ml
1-33/44-198 rRTA	10/10	0.15 ± 0.04[b]
1-33/44-198 rRTA + 0.2% Alhydrogel	10/10	0.81 ± 0.12
Chemically dgRTA	10/10	0.95 ± 0.15
Phosphate-buffered saline	0/10	<0.01

[a] Average whole-body exposure to between 5 and 10 LD_{50}s of ricin toxin.
[b] Average ± standard error.

While vaccinated mice did survive an aerosol challenge of at least 100 LD_{50} of inhaled ricin, some lung damage was present in these survivors. Mice vaccinated with dgRTA and those that survived challenge with aerosolized ricin toxin at 3 months after the last immunization injection were euthanized and necropsied on days 14, 28, 56, and 112 postchallenge. The lungs were examined histologically to determine the long-term sequelae of ricin toxin challenge in mice vaccinated with the candidate ricin toxin vaccine. Changes attributable to ricin challenge gradually resolved at each subsequent examination time point. There was moderate infiltration of the perivascular interstitium by lymphocytes, eosinophils, plasma cells, and macrophages (perivasculitis); moderate goblet cell metaplasia of bronchiolar epithelium; minimal to mild infiltration of alveoli by inflammatory cells (alveolitis); and minimal alveolar histiocytosis in all mice at day 14 postchallenge. One mouse also exhibited mild bronchiolo-alveolar fibroplasia at day 14. The histopathological changes were reduced in incidence and severity, and eosinophils were no longer a prominent feature of the perivasculitis at day 28. By days 56 and 112, perivasculitis and goblet cell metaplasia were generally minimal; mild alveolitis and minimal alveolar histiocytosis were present in only one mouse. Fibroplasia was not observed. Thus, in vaccinated mice, this is was a self-limiting lung injury with no long-term effects.

When ricin toxoid vaccine with and with out liposomal encapsulation was delivered intratracheally or by subcutaneous injection in rats, it elicited a good immune response and protected them from lethal intratracheal instillation of ricin (Griffiths et al., 1997, 1998). Recombinant ricin A-chain (RTA) given subcutaneously but not intratracheally protected rats from lethal intratracheal instillation of ricin (Griffiths et al., 1998). Intratracheal instillation of a liposomal formulation of RTA vaccine improved the immune response, lung protection, and survival of rats challenged with a lethal dose of instillation of ricin (Griffiths,, et al., 1998, 1999).

39.4.1.3 Efficacy of Antiricin Antibody against Inhaled Ricin

Affinity-purified mouse antiricin IgG in doses up to 100 μg were given to mice by intranasal instillation. When these mice were challenged with aerosolized ricin toxin, all the high-dose mice died with severe injury to one lobe of the lung (Poli et al., 1996). The study was repeated with administration of the antibody by small-particle aerosol. Each group of mice was challenged with a lethal dose of aerosolized ricin toxin at 1 hour after antibody exposure. Survival data are summarized in Table 39.2. Fourteen of 16 mice treated with aerosolized antiricin IgG survived with minimal lung damage. The two nonsurvivors had obstructions due to proximal airway epithelial damage. In contrast, all 19 mice treated with inhaled, nonspecific IgG died with severe lung injury after exposure to aerosolized ricin toxin. These studies clearly demonstrated that localized antiricin can protect the lung from injury by inhaled ricin toxin.

Table 39.2 Protection of Mice from Inhaled Ricin Toxin with Aerosolized Antiricin IgG

Group	Inhaled antibody dose, μg/mouse	14 day survival
Control, nonspecific IgG	54	0/19
Anti-ricin toxin IgG	24	8/8
Anti-ricin toxin IgG	82	6/8

In a second study, mice were pretreated with an intravenous (i.v.) injection of 100 μg of affinity-purified mouse, antiricin IgG or nonspecific mouse IgG (control IgG) at 24 h before exposure to 3–6 LD_{50} of inhaled ricin. Other groups of mice were treated i.v. with 100 μg of affinity-purified mouse, antiricin IgG or normal mouse IgG at 1, 6, 24, 48, and 72 h after exposure to 3–6 LD_{50} of inhaled ricin. All the mice pretreated with antiricin survived the challenge with inhaled ricin, whereas all pretreated with control IgG died.

An i.v. injection of antiricin IgG completely protected the mice 1 to 6 h after exposure to inhaled ricin. By 24 h postexposure to inhaled ricin, antiricin IgG failed to protect any of the mice for from lethality but it did extend survival time from 3 to 6 days. Thus, a short window of opportunity exists for treating intoxication from inhaled ricin with heterogeneous antiricin IgG.

39.5 RISK FOR INHALED RICIN

The potential use of biological agents as offensive or terrorist weapons has recently been reviewed in a number of articles (Cadule, 1997; Christopher et al., 1997; Franz and Jaxx, 1997; Simon, 1997; Zilinskas, 1997). Approximately 10 or more countries are thought to have development programs for biological weapons. Because of its relatively high toxicity and its extreme ease of production, ricin toxin, code-named Compound W, was considered for weaponization by the United States during its offensive Biological Warfare Program. The U.S. Chemical Warfare Program began studying ricin as a weapon near the end of World War I. Work in collaboration with the British resulted in development of the W bomb in World War II. The weapon was tested but apparently never used in battle (Cookson and Nottingham, 1969). Ricin toxin was used in the highly publicized assassination of Bulgarian defector Georgi Markov (Crompton and Gail, 1980). Because ricin intoxication is a relatively uncommon occurrence in human medicine, no concerted effort was made to produce specific therapies or prophylactic measures until the early 1990s, when ricin was perceived to be a significant biological warfare threat. In recent years, ricin toxin has become a favorite tool of extremist individuals or groups who seek to harm others, including the recent sample found in the mail of the senate building. The major threat from ricin is powdered material that could be inhaled to cause lung injury and/or death.

ACKNOWLEDGMENTS

The authors thank LTC Charles B. Millard and Drs. Leonard A. Smith and M. Louise M. Pitt for many fruitful and enlightening discussions; LTC Gary M. Zaucha and MAJ Randall L. Rietcheck for their pathology support; CPT Curtis Klages for his radiology support; MAJ Pedro Rico, CPT Sarah B. Hinds, and staff of the Veterinary Medicine Division for animal care and technical support; Drs. John F. Hewetson and Changhong Y. Lindsay for the serology support; and Mr. Richard E. Dinterman for his excellent technical support in these studies.

The research described herein was sponsored by the Medical Biological Defense Research Program, U.S. Army Medical Research and Materiel Command. Research Project 02-4-2S-066.

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

REFERENCES

Assard, A., Wilhelmsen, C., Pitt, L., Swauger, J., Kokes, J., Parker, G., and Franz, D., Ricin, a plant toxin, induces acute lung injury and high permeability pulmonary edema in rats (Abstr.), *Am. Rev Respir. Dis.*, 147, A361m 1993.

Baron, P.A. and Willeke, K., *Aerosol Measurement*, 2nd ed.,, John Wiley and Sons, New York, 2001k, pp. 644–650, 1053–1063.

Balint, G.A., Ultrastructural study of liver cell damage induced by ricin, *Exp. Toxicol Pathol.*, 52, 413–417, 2000.

Baluna, R. and Sausville, E.A., Decreases in levels of serum fibrinonectin predict the severity of vascular leak syndrome in patients treated with A chain-containing immunotoxins, *Clin. Cancer Res.*, 2, 1705–1712, 1996.

Bingen, A., Creppy, E.E., Gut, J.P., Direimer, G., and Kirn, A., The Kupffer cell the first target in ricin-induced hepatitis, *J. Submicrosc. Cytol.*, 19, 247–256, 1987.

Blakey, D.C. and Thorpe, P.E., Effect of chemical deglycosylation on the in vivo fate of ricin A-chain, *Cancer Drug Deliv.*, 3, 189–196, 1986.

Brugsch, H.G., Toxic hazards (the castor bean), *N. Engl. J. Med.*, 262, 1039–1040, 1960.

Byers, V.S. and Baldwin, R.W., Targeted kill: from umbrellas to monoclonal antibodies, *J. Clin. Immunol.*, 12, 391–405, 1992.

Cadule, L.C., III, The biological warfare threat, in *Textbook of Military Medicine*, Zajtchuk, R. and Bellamy, R.F., Eds., Office of the Surgeon General, Department of the Army, United States of America, 1997, pp. 451–466.

Cookson, J. and Nottingham, J., *A Survey of Chemical and Biological Warfare*, Monthly Review Press, New York, 1969, 6, 1969.

Christopher, G.W., Ciesalk, T.J., Pavlin, J.A., and Eitzen, E.N., Jr., Biological warfare. A historical perspective, *JAMA*, 278, 412–417, 1997.

Crompton, R. and Gail, D., Georgi Markov: Death in a pellet, *Med. Leg. J.*, 48, 51–62, 1980.

Doebler, J.A., Wiltshire, N.D., Mayer, T.W., Estep, J.E., Moeller, R.B., Traub, R.K., Brommfield, C., Calamato, C.A., Thompson, W.L., and Pitt, M.L., The distribution of [125I]ricin in mice following aerosol inhalation exposure, *Toxicology*, 98, 137–149, 1995.

Endo, Y., Tsurugi, K., and Lambert, J.M., The site of action of six different ribosome-inactivating proteins from plants on eukaryotic ribosomes: the RNA N-glycosidase activity of the proteins, *Biochem. Biophys. Res. Commun.*, 150, 1032–1036, 1988.

Flexner, S., The histological changes produced by ricin and abrin intoxication, *J. Exp. Med.*, 2, 187–216, 1897.

Fodstad, O., Olsnes, S., and Pihl, A., Toxicity, distribution and elimination of the cancerostatic lectins abrin and ricin after parenteral injection into mice, *Br. J. Cancer*, 34, 418–425, 1976.

Fodstad, O., Kvalheim, G., Godal, A., Lotsberg, J., Aamdal, S., Host, H., and Phil, A., Phase I study of the plant protein ricin, *Cancer Res.*, 44, 862–865, 1984.

Foxwell, B.M., Blakey, D.C., Brown, A.N., Donovan, T.A., and Thorpe, P.E., The preparation of deglycosylated ricin by recombination of glycosidase-treated A- and B-chains: effects of deglycosylation on toxicity and in vivo distribution, *Biochim. Biophys. Acta*, 923, 59–65, 1987.

Franz, D.R. and Jaxx, N.K., Ricin toxin, in *Textbook of Military Medicine*, Zajtchuk, R. and Bellamy, R.F., Eds., Office of The Surgeon General, Department of the Army, United States of America, 1997, pp. 631–642.

Fritz, D.L., Connolly, B., Pitt, M., and Langford, M.J., Ultrastructural findings in rats finding in rats following lethal ricin inhalation (Abstr.), *Vet. Pathol.*, 30, 482, 1993.

Funatsu, G., Mise, T., Matsuda, H., and Funatsu, M., Isolation and characterization of two constituent polypeptide chains of ricin E, *Agric. Biol. Chem.*, 42, 851–859, 1978.

Ghetie, M.-A. and Vitetta, E.S., Recent developments in immunotoxin therapy, *Curr. Opin. Immunol.*, 6, 707–714, 1994.

Griffiths, C.D., Allenby, A.C., Bailey, S.C., Hambrook, J.L., Rice, P., and Upshall, D.G., The inhalation toxicity of ricin purified 'in house' from seeds of *Ricinus communis var zanzibariensis*, Chemical and Biological Defense Establishment Technical Paper 742, Ministry of Defense, Porton Down, Salisbury, UK, *Vaccine*, 15, 1933–1939, 1994.

Griffiths, C.D., Bailey, S.C., Hambrook, J.L., and Keyte, M.P., Local and systemic response against ricin toxin promoted by toxoid or peptide vaccines alone or in liposomal formulations, *Vaccine*, 16, 530–535, 1998.

Griffiths, C.D., Leek, M.D., and Greek, D.G., The toxic plant proteins ricin and abrin induce apoptotic changes in mammalian lymphoid tissue and intestine, *J. Pathol.*, 50, 221–229, 1987.

Griffiths, C.D., Philips, S.C., and Bailey, S.C., Comparison of the quality of protection elicited by toxoid and peptide liposomal vaccine formulations against ricin as assessed by markers of inflammation, *Vaccine*, 17, 2562–2568, 1999.

Griffiths, C.D., Rice, P., Allenby, A.C., Bailey, S.C., and Upshall, D.G., Inhalation toxicology and histopathology of ricin and abrin, *Inhalation Toxicol.*, 7, 269–288, 1995.

Guyton, A.C., Measurement of the respiratory volume of laboratory animals,. *Am. J. Physiol.*, 150, 70–77, 1947.

Hambrook, J.L., Keyte, M., Jayasekera, P., Miles, J., and Williamson, E., Liposomally-encapsulated ricin toxoid vaccine delivered intratracheally elicits a good immune response and protects against a lethal pulmonary dose of ricin, *Vaccine*, 15, 1933–1939, 1997.

Hewetson, J.F., Assaad, A., Teters, D.R., Kotes, J.M., Washington, E., Tammariello, R., and Wade, J., Immune response and protection of rats and mice from ricin aerosol exposure after vaccination with deglycosylated ricin A chain (Abstr.), *Fundam. Appl. Toxicol.*, 30 Suppl. (The Toxicologist), 59, 1996.

Hewetson, J.F., Rivera, V.R., Lemely, P.V., Pitt, M.L., Creasia, D.A., and Thompson, W.L., A formalinized toxoid for protection of mice from inhaled ricin, *Vaccine Res.*, 4, 179–187, 1995.

Jemski, J.V. and Phillips, G.B., Aerosol Challenge in Animals, U.S. Army Medical Research Institute of Infectious Disease Technical manuscript 144, Fort Detrick, MD, 1964, pp. 22–31.

Langford, M.J., Pitt, M.L., and Jaax, N.K., Pulmonary lesions in adult male rats exposed to an acute lethal dose of aerosolized ricin (Abstr.), *Vet. Pathol.*, 30, 479, 1993.

Lemley, P.V., Amanatides, P., and Wright, D.C., Identification and characterization of a monoclonal antibody that neutralizes ricin toxicity *in vitro* and *in vivo, Hybridoma*, 13, 417–421, 1994.

Lin, J.Y., Liu, K., Chen, C.-C. and Tung, T.-C., Effect of crystalline ricin on the biosynthesis of protein, RNA and DNA in experimental tumor cells. *Cancer Res.*, 31, 921–924, 1971.

Lord, J.M., Gould, J., Griffiths, D., O'Hare, M., Prior, B., Richardson, P.T., and Roberts, L.M., Ricin: cytotoxicity, biosynthesis and use in immunoconjugates, *Prog. Med. Chem.*, 24, 1–28, 1987.

Mendelsohn, J., Immunotoxins: prospects and problems, *J. Clin. Oncol.*, 9, 2088-2090, 1991.

Miller, O.T. and Gremillion, G.G., Respiratory exposure of animals to microorganisms with the Henderson Apparatus, USAMRIID technical manuscript 39, 1963.

Nicolson, G.L., Lacorbiere, M., and Hunter, T.R., Mechanism of cell entry and toxicity of an affinity-purified lectin from *Ricinus communis* and its differential effects on normal virus-transformed fibroblasts, *Cancer Res.*, 35, 144–155, 1975.

Olsnes, S. and Pihl, A., Abrin, ricin and their associated agglutinins, in *The Specificity and Action of Animal, Bacterial and Plant Toxins: Receptors and Recognition*, Series B, Vol. 1, Cuatrecasas, P., Ed., Chapman and Hall, London, 1976, pp. 129–173.

Olsnes, S. and Pihl, A., Toxic lectins and related proteins, in *Molecular Action of Toxins and Viruses*, van Heiningen, G., Ed., Elsevier Biomedical Press, Amsterdam, 1982, pp. 51–105.

Olson, M.A., Carra, J.H., Roxas-Duncan, V., Wannemacher, R.W., Smith, L.A., and Millard, C.B., Finding a new vaccine in the ricin protein fold, *PEDS*, 17, 391–397, 2004.

Pitt, M.L.M., Efficacy of Formalinized Ricin Toxoid in Nonhuman Primates, U.S. Army Medical Research Institute of Infectious Disease Report, 1995.

Phalen, R.F., *Inhalation Studies: Foundation and Techniques*, CRC Press, Boca Raton, FL, pp. 38.

Poli, M.A., Rivera, V.R., Pitt, M.L., and Vogel, P., Aerosolized specific antibody protects mice from lung injury associated with aerosolized ricin exposure, *Toxicon*, 34, 1037–1044, 1996.

Preijers, F.W., De Witte, T., Wessels, J.M., De Gast, G.C., Van Leeuwen, E., et al., Autologous transplantation of bone marrow purged in vitro with anti-CD7-(WT1-) ricin A immunotoxin in T-cell lymphoblastic leukemia and lymphoma, *Blood*, 74, 1152–1158, 1989.

Rauber, A. and Heard, J., Caster bean toxicity re-examined: A new perspective, *Vet. Hum. Toxicol.*, 27, 498–502, 1985.

Roy, J.C., Hale, M., Hartings, J., Pitt, L., and Dunibo, S., Impact of inhalation exposure modality and particle size on the respiratory deposition of ricin in BALB/c mice, *Inhalation Toxicol.*, 15, 619–638, 2003.

Simon, J.D., Biological terrorism. Preparing to meet the threat, *JAMA*, 278, 428–430, 1997.

Soler-Rodriguez, A.M., Uhr, J.W., Richardson, J., and Vitetta, E.S., The toxicity of chemically deglycosylated ricin A-chain in mice, *Int. J. Immunopharmacol.*, 14, 281–291, 1992.

Steele, K., Davis, K., Pitt, L., and Langford, M., Pulmonary lesions in rats exposed to a sublethal dose of inhaled ricin (Abstr.), *Vet. Pathol.*, 32, 600, 1995.

Stirpe, F., Bailey, S., Miller, S.P., and Bodley, J.W., Modification of ribosomal RNA by ribosome-inactivating proteins from plants, *Nucleic Acids Res.*, 16, 1349–1357, 1988.

Trown, P.W., Reardan, D.T., Carroll, S.F., Stoudemire, J.B., and Kawahata, R.T., Improved pharmacokinetics and tumor localization of immunotoxins constructed with the M_r 30,000 form of ricin A chain, *Cancer Res.*, 51, 4219–4225, 1991.

Vitetta, E.S., Thorpe, P.E. and Uhr, J.W., Immunotoxins: Magic bullets or misguided missilesM *Trends Pharmacol. Sci.*, 14, 148–154, 1993.

Walker, G.R., Ebner, K.E., Scroggs, R.A., Das Gupa, B.R., and Corcoran, J.B., Studies on the toxic action of ricin, *Proc. Soc. Exp. Biol. Med.*, 121, 685–691, 1966.

Wannemacher, R.W., Dinterman, R., Hewetson, J., Pitt, M., Tammariello, R., Rietcheck, R., Klages, C., and Millard, C., Use of African green monkeys (*Chlorocebus aethiops)* model top determine pathophysiological response to inhaled ricin toxin and efficacy of ricin vaccines (Abstr.), *The Toxicologist-Supplement Tox. Sci.*, 78 (S-1), 793, 2004.

Wawrzynczak, E.J., Cumber, A.J., Henry, R.V. and Parnell, G.D., Comparative biochemical, cytotoxic and pharmacokinetic properties of immunotoxins made with native ricin A chain, ricin A_1 chain and recombinant ricin A chain, *Int. J. Cancer*, 47, 130–135, 1991.

Wilhelmsen, C.L. and Pitt, M.L.M., Lesions of acute inhaled ricin intoxication in rhesus monkeys, *Vet. Pathol.*, 33, 296–302, 1996.

Wilhelmsen, C., Inhaled ricin dose ranging and pathology in inbred strains of mice, USAMRIID Technical Report, 1997.

Xu, I.L.L., McVicar, D.W., Ben-Baruch. A,, Kunhs, D.B., Johnston, J., Oppenheim. J.J., and Wang, J.M., Oncocyte chemotactic protein-3 (MCP3) interacts with multiple leukocyte receptors: binding and signaling of MCP3 through shared as well as unique receptors on monocytes and neutrophils, *Eur. J. Immunology*, 25, 2612–2617, 1995.

Zilinskas, R.A., Iraq's biological weapons. The Pat as Future? *JAMA,* 278, 418–424, 1997.

40 Toxicity, Mechanisms, Prophylaxis, and Therapy of Inhaled Biological Toxins

Brian M. Paddle

CONTENTS

40.1 INTRODUCTION

The aim of this chapter is to consider the issue of biological toxins as inhalational hazards and ultimately to suggest experimental approaches for identifying possible therapeutic and prophylactic agents for protection against them. The term 'biological toxin' generally refers to nonreplicating toxic organic materials produced by living organisms. Such a definition could however be extended to include materials similar to those produced by living organisms but made by *in vitro* means.

The consideration of aerosolized biological toxins as an inhalation threat of any kind has, in the past, largely been the domain of those with an interest in 'biological toxins' that are or have been considered as Biological Warfare (BW) agents (Hamilton, 1996; Franz, 1997; USAMRIID, 1998; Ellison, 1999; Madsen, 2001; Henghold II, 2004). More recently it has also become a public health issue for people living or working in the vicinity of, for example, toxic marine dinoflagellate blooms (Smith and Music, 1998; Van Dolah, 2000; Pierce et al., 2003; Miller and Belas, 2003; Abraham et al., 2005) or aflatoxin (molds) contaminated grain storage areas (Kelly et al., 1997; Bunger et al., 2004).

This discussion begins with a short perspective on the relative toxicity of selected toxins and the aerosol hazard followed by an outline of their molecular nature and cellular sites of action. Then there is a brief summary of the defensive components and mechanisms within the lungs that could be involved in the initial response to the inhalation of biological toxins. Finally consideration is given to what might be effective future therapy and prophylaxis against such toxins, with a bias toward molecular species for use as extracellular scavengers. In this context, it is also appropriate to consider certain inhaled pathogenic microorganisms (e.g., *Bacillus anthracis*) whose secreted exotoxins are largely responsible for the pathogenicity (Mock and Mignot, 2003). Targeting these toxins could be especially important where the organism is antibiotic resistant.

40.2 THE HAZARD OF AEROSOLIZED TOXINS

Biological toxins that have been aerosolized may make contact with the skin, be inhaled into the lungs, or find their way into the gut—either as a result of deposition in the major airways or following ingestion of contaminated food and water. They may have direct toxic effects on and/or initiate defensive responses at all of these sites. The lungs particularly, because of the large surface area provided by the cells forming the interface between the inhaled air and the circulating blood, provide a potentially extremely important portal of entry for toxin absorption into the body. Thus the lethal or incapacitating effects of an inhaled toxin may not only result from lung damage *per se* but also be the result of transfer of the toxin into the circulation and to internal organs.

How to estimate the hazard due to the inhalation of a particular aerosolized toxin remains a major point for discussion and resolution.

40.2.1 Measures of Toxicity

From the military and public health points of view, both the incapacitating ability as well as lethality of a toxin should be considered. Calculations of the ED_{50} and LD_{50} have been used as indicators of toxicity. The term ED_{50} refers to the concentration (e.g., μg inhaled, injected, or absorbed per kg of animal mass) of a specific material that will cause a particular effect (e.g., incapacitation) in 50% of the population. The LD_{50} refers to the concentration that is lethal to 50% of the population. The LD_{50} can be defined precisely whereas an incapacitating effect is more difficult to define precisely.

An examination of dose–response statistics (e.g., plots of relative frequency of animals dying versus different dose levels on a log scale) indicates that the use of any simple comparison of the LD_{50}s of different toxins has limitations. This is true even if they were obtained in studies on the same animal species under precisely the same conditions. The use of the LD_{50} *per se* does not take into account the dosage range over which deaths occurred. The latter is reflected in the slope of the cumulative

TABLE 40.1 Selected Toxins Listed According to LD_{50}s (i.v. Delivery in Mice unless Otherwise Stated)[a]

Toxin	LD_{50}, μg/kg	Natural source
Botulinum toxins***	0.001 (Gill, 1982)	Bacterium (*Clostridium botulinum*)
Tetanus toxin***	0.001 (Li et al., 1994)	Bacterium (*Clostridium tetani*)
Diphtheria toxin***	0.01–0.1 (Gill, 1982)	Bacterium (*Corynebacterium diphtheriae*)
Maitotoxins (1 or 2)**	0.05–0.1 i.p. (Holmes and Lewis, 1994; Murata et al., 1994)	Dinoflagellates (e.g., *Gambierdiscus toxicus*)
Palytoxin**	0.05–0.15 i.p. (Kaul et al., 1974; Baden, 1983; Haberman et al., 1989)	Dinoflagellates (e.g., *Ostreopsis siamensis*)
Clostridium perfringens ϵ-toxin**	0.1 (Gill, 1982; Sakurai, 1995)	Bacteria (*Clostridium perfringens* types B and D)
Batrachotoxin**	0.1, 2.0 s.c. (Myers et al., 1978)	South American frogs (genus, *Phyllobates*) and New Guinea birds (e.g., genus, *Pitohui*) (Dumbacher et al., 2000)
Shiga toxin**	0.45, 0.002 rabbit (Eiklid and Olsnes, 1983), 1.5 i.p. (Yutsudo et al., 1986)	Bacterium (*Shigella dysenteriae*)
Ciguatoxin**	0.25 i.p. (Legrand, 1998), 0.35 (Yasumoto and Satake, 1996), 0.45 i.p. (Baden, 1983)	Dinoflagellates (e.g., *Gambierdiscus toxicus*)
Abrins**	0.5–0.7 (Fodstad et al., 1976), 3.3 inh rat (Griffiths et al., 1992), 0.05 rabbit (Olsnes and Pihl, 1982)	Plant (*Abrus precatorius Leguminosae*)
Textilotoxin**	1 (Su et al., 1983)	Snake-elapid (*Pseudonaja textilus*)
Taipoxin**	2–3 (Fohlman et al., 1976; Crosland, 1991)	Snake-elapid (*Oxyuranus scutellatus*)
Ricin**	2.8–3.3 (Fodstad et al., 1976), 0.15 hum, 3.7 inh rat (Griffiths et al., 1992)	Plant (*Ricinus communis*)
Clostridium perfringens* α-toxins	3–5 (Gill, 1982; Sakurai, 1995), 10–30, 3.3–10 rat, ~2 i.trac. rat (Allenby et al., 1994)	Bacteria (*Clostridium perfringens*)
Saxitoxin*	3–4, 10 i.p. (Wiberg and Stephenson, 1960), 3.0 i.p. (Baden, 1983), 1.2 i.tr. (Naseem and Creasia, 1994)	Dinoflagellates (e.g., *Alexandrium toxicus, Gonyaulax catenella, Gonyaulax tamarensis*)
Tetrodotoxin*	8–10 (2 rabbits), 8 i.p. (Baden, 1983)	Marine and other bacteria (e.g., *Listonella anguillarum, Pseudoalteromonas tetraodonis, Vibrio alginolyticus*)
α-Tityustoxins* (K^+ channel acting)	9 (in: Franz, 1997), 3.5 i.c.v. (Lecomte et al., 1999)	Scorpion, Brazilian (e.g., *Tityus serrulatus*)
α-Conotoxin (G or M)*	~11 (Ashcom and Stiles, 1997)	Marine snail (e.g., *Conus geographus, Conus magus*)

(*Continued*)

TABLE 40.1 Selected Toxins Listed According to LD_{50}s (i.v. Delivery in Mice unless Otherwise Stated)[a] (Continued)

Toxin	LD_{50}, μg/kg	Natural source
Staphylococcal enterotoxin B*	13 i.p. (with LPS), 29 inh. monkey (LeClaire et al., 1996), (Humans est. LD_{50} 0.02 inh., ED_{50} 0.0004 inh. (in Ulrich et al., 1997)	Bacterium (*Staphylococcus aureus*)
Anatoxin −A(s)*	20 (Matsunaga et al., 1989)	Blue-green algae (e.g., *Anabaena flosaquae, Anabaena lemmermannii*)
Microcystins (e.g., -LR)*	36–122 (also i.p., inh) (in Dawson, 1998)	Freshwater cyanobacteria (*Microcystis aeruginosa, Anabaena oscillatoria*)
Aconite (Aconitine?)*	(64 rat) (Gutierrez et al., 1987)	Plant (*Aconitum napellus L, Aconitum ferox*)
Tricothecene mycotoxins (T-2)*	4 mg/kg (Ueno, 1984) 0.24 mg/kg inh. (Creasia et al., 1987), 3-4.5 mg/kg GP inh. (Marrs et al., 1986), 0.05 mg/kg rat inh., 0.4 mg/kg GP inh. (Creasia et al., 1990)	Molds (e.g., *Fusarium tricinctum*)
Aflatoxins (e.g., B1, G, and M)*	(B_1 9–10 mg/kg, i.p.) (Buchi et al., 1973)	Fungi (e.g., *Aspergillus flavis, Aspergillus parasiticus*)

* Moderately toxic.

** Highly toxic.

*** Most toxic.

[a] **Bold type:** Toxins for which aerosol studies have been reported in the open literature.

dose–response curve at say the 50% response level. The greater the slope, the narrower the range of concentration of the toxin over which deaths can occur (WHO, 1970; Ballantyne et al., 1995).

In general, human toxicity has to be inferred from animal experiments and how this can best be achieved is still being investigated (Bide et al., 1997, 2000). For aerosols it is the inhalation toxicity that is of prime concern. Calculations for a particular toxin based on the aerosol concentration and the duration of the exposure are generally expressed as an LCt_{50} in $mg \cdot min \cdot m^{-3}$ units. If an approximate comparison with LD_{50}s obtained by i.v. (intravascular) or other injection routes is required, the LCt_{50} can be converted to an LD_{50} using the respiratory volume and rate and body weight parameters. Even so, animal LD_{50}s for inhaled toxins are not generally available and may be different from LD_{50}s obtained by i.v., s.c. (subcutaneous), or i.p. (intraperitoneal) injection or even by intratracheal instillation (see Table 40.1). If the target organ is the lung then defining the actual dose reaching and remaining in the alveoli, for example (or being transferred to the gut), in inhalation studies is a further problem. If the aerosolized toxin can enter the circulation then it becomes even more difficult to calculate the lethal dose.

Despite these reservations, and the lack of information from a variety of species (including humans), as well as variations in results reported from different species (see Table 40.1), the tendency is to use the available LD_{50} data on toxins as a rough guide to their relative human toxicity (Bide et al., 2000). Table 40.1 includes toxins either known (or thought) to have been weaponized, or associated with weapons research. Where possible the toxins in this list have been arranged in descending order of lethality based on mice LD_{50}s following an i.v. injection. Data for other animals and/or alternative routes of challenge are as noted. Toxins for which aerosol studies in animals have been reported in the open literature are in **bold** print.

The effect of the test species and of the route of administration on toxicity data is well illustrated in Table 40.1. Staphylococcal enterotoxin B (SEB), which (in the presence of lipopolysaccharide) is only moderately toxic after injection (i.p.) into mice, is in the most toxic category according to its aerosol LD_{50} in primates. According to its ED_{50} as measured in primates it is even more effective as an incapacitating agent. Shiga toxin, according to LD_{50} studies carried out in rabbits, is then also in the most toxic category. Intravenous studies of the abrins have shown as much as a 100-fold variation in toxicity, depending on the test animal species. The enhanced toxicity of abrin over ricin was not apparent in a study of their inhalation toxicity and it appears that humans are considerably more sensitive to i.v. ricin than mice. The data in Table 40.1 also suggest that *Clostridium perfringens* α-toxin and saxitoxin, which are in the moderately toxic category using i.v. injection, are at least as toxic as ricin when instilled intratracheally. *C. perfringens* ε-toxin is already in the highly toxic category.

What these findings illustrate is that the toxicity data currently available from animal studies are generally insufficient to decide the relative inhalational toxicity for humans. Nevertheless calculations based on LD_{50}s have been used to suggest that the above toxins can be classified into three toxicity groups (USAMRIID, 1998). These are:

- Most toxic*** (aerosol toxicity < 0.025 μg/kg),
- Highly toxic** (aerosol toxicity between 0.025 and 2.5 μg/kg), and
- Moderately toxic* (aerosol toxicity > 2.5 μg/kg)

40.2.2 Risk Assessment

Although risk assessment is a very necessary and evolving scientific discipline it is said to be an imprecise science (Derelanko, 1995). No doubt this is due to the large number of factors that need to be taken into account and for which data may be unavailable or difficult to obtain. According to Derelanko (1995) it is due to this uncertainty and the desire to protect human populations that risk assessments generally tend to be conservative and overestimate risk.

A risk assessment analysis (a) to ascertain the likelihood that humans will be adversely affected by biological toxins disseminated into the air as finely divided powders or aerosols, and (b) to characterize the nature of the effects that may be experienced, would include not only an evaluation of the toxic properties (dose–response assessment) of the toxins but also of the conditions and extent of exposure (exposure assessment). The exposure assessment would include such factors as the stability of the toxins in the environment, meteorological or other environmental conditions, the magnitude, duration and frequency of exposure and subsequent calculation of the dose, nature of exposure (oral, dermal, inhalational), and the characteristics of the population exposed (e.g., general health and fitness, age, and sex). More precise details of the methods used in risk analysis can be found in the general literature (Derelanko, 1995; McClellan, 1995; Byrd and Cothern, 2000).

Military analysts have used a plot of aerosol LD_{50}s versus the amount that needs to be used to obtain lethal effects over an area of 100 km^2 to determine which toxins may be considered for use as BW agents (USAMRIID, 1998). The risk assessment would need to take into account the real possibility that deliberately disseminated toxins may be relatively impure. Such toxin aerosols may include materials of lower toxicity or be a mixture of toxins, or contain other materials that would enhance the overall toxicity.

In an urban environment, in the enclosed spaces provided by buildings, smaller quantities of an aerosolized toxin would be required to reach hazardous levels. Aerosols might be disseminated within buildings via air-conditioning systems, or generated by showering with contaminated water. In the latter case a risk assessment analysis carried out with a volatile contaminant has indicated that the quantity of it absorbed during inhalation of shower air may be the same as that absorbed by fluid ingestion over the same period (Derelanko, 1995).

40.2.3 Toxins as Public Health Issues

Apart from the BW perspective, some of the toxins in Table 40.1 are also of increasing interest as potential public health risks. Toxins from marine bacteria or marine dinoflagelates (which are part of the food chain for various species) may be present as contaminants in seafood or as components of seaspray.

The cyanobacteria or blue-green algae that are frequently found in marine, brackish, or fresh waters can produce significant quantities of natural toxins. These include the anatoxins and mycrocystins, as well as saxitoxin, lyngbyatoxin, and debromo-aplysiatoxin, and it has been suggested that during 'blooms' humans could also be exposed to their toxic affects via the aerosol route (Van Dolah, 2000; Fleming and Stephan, 2001; Osborne et al., 2001).

The presence of paralytic shellfish poisons (PSPs) in commercially important shellfish is of great concern among coastal communities. Saxitoxin is the most important of this group (Schantz, 1986) and marine bacteria have been isolated which produce the similarly toxic tetrodotoxin (Kodama et al., 1996).

Marine dinoflagellates have been discovered that produce analogs of the highly toxic palytoxin (Usami et al., 1995). The equally toxic and stable maitotoxins have also been harvested and identified from marine dinoflagellates (Holmes and Lewis, 1994). There are other polyether dinoflagellate toxins, not so toxic as the above, but which may be incapacitating at concentrations significantly lower than their LD_{50}s. Examples are the ciguatoxins and the brevetoxins. The latter are known to be present in naturally formed aerosols along coastal areas at the time of red tides (Pierce, 1986; Pierce et al., 2003). The symptoms of brevetoxin poisoning mimic many of the physiological consequences of acetylcholine esterase inhibition (Abraham et al., 2005).

Another dinoflagellate species *(Pfiesteria piscicida)* remains under scrutiny as a public health risk (Miller and Belas, 2003) because of reports that it may produce exotoxins that can be naturally aerosolized and cause a range of incapacitating effects in humans. These include narcosis, respiratory distress with asthmalike symptoms, severe stomach cramping, nausea, eye irritation with reddening and blurred vision, and autonomic and central nervous system dysfunction. The latter include sudden rages and personality changes, reversible cognitive impairment, and short-term memory loss. The toxin(s) involved—which have not yet been characterized (Swinker et al., 2002)—may also induce immune system suppression (Glasgow et al., 1995; Levin et al., 2003).

Finally, although civilian exposure to mycotoxins (T-2, aflatoxins) from molds is mostly by ingestion, it also occurs by the dermal and inhalational routes (Peraica et al., 1999) and there is evidence that mycotoxins may be involved in certain cases of lung disease (Bunger et al., 2004).

40.3 MOLECULAR NATURE AND CELLULAR ACTION OF TOXINS

The molecular nature and cellular interaction of inhaled biological toxins will determine their fate, the lungs defensive response, and cell damage.

40.3.1 Molecular Characteristics

Although the toxins listed in Table 40.1 exhibit a great variety of molecular types, they can be collected into a number of chemical categories (Table 40.2). Generally the most toxic are the larger-molecular-weight protein toxins produced by pathogenic bacteria and some plants and present in the venom of certain snakes (Table 40.2: Categories 1 and 3).

The bacterial and plant toxins (Table 40.2: Category 1) have a subunit structure in which the different subunits play separate and clearly defined roles in the intoxication process. Thus they have one or more membrane-binding B subunits (in the case of the Botulinum toxins—the heavy chain has this role), as well as internalization and cytosol toxicity sequences (A subunits)

TABLE 40.2 Toxin Categories by Molecular Type

Category	Type	Examples
1	Proteins that are a combination of (a) one or more subunits for membrane binding and translocation of the whole toxin into the cell, with (b) another subunit that is enzymatically active within the cytosol	Botulinum toxins, tetanus toxin, diphtheria toxin, shiga toxin, abrin, ricin (60–150 kDa). [Anthrax lethal toxin = lethal factor plus protective antigen]
2	Polycyclic (polyether) macromolecules active at the cell membrane	Maitotoxin, palytoxin, ciguatoxin, (brevetoxin) (0.9–3.4 kDa)
3	Proteins with enzymatic (phospholipase) activity that act on the cell membrane	Textilotoxin, taipoxin, *C. perfringens* α-toxin. (43–88 kDa)
4	Complex heterocyclic molecules that act at the cell membrane	Saxitoxin, tetrodotoxin, batrachotoxin, aconitine, anatoxin-A(s) (300–650 Da)
5	Peptides with several disulfide cross-bridges that act at the cell membrane	Tityustoxin K-2α, α-conotoxin. (3.9 and ~1.5 kDa, respectively)
6	Complex heterocyclic molecules whose major toxic effects are intracellular	Aflatoxins, tricothecene mycotoxins (300–460 Da)
7	Peptides with intracellular activity	Microcystins (~1 kDa)
8	Proteins with superantigen activity	SEB (28.4 kDa)

(Olsnes and Pihl, 1982; Fujii et al., 1992; London, 1992; Krieglstein et al., 1994; Sandvig and van Deurs, 1996). Each toxin's A subunits have a specific intracellular catalytic (enzymatic) activity (Collier, 1975; Endo et al., 1987, 1988; Fujii et al., 1992; Montecucco and Schiavo, 1993; Brigotti et al., 2002).

The snake toxins (Table 40.2, Category 3) also have a subunit structure in which one or more units have phospholipase A2 activity (Fohlman et al., 1976; Harris and Maltin, 1982; Pearson et al., 1993; Lipps, 2000). It is not clear what role other subunits that may also be present play. Since these toxins can act destructively on cell membranes there is no requirement for an internalization mechanism. *C. perfringens* α-toxins appear to have no specific subunit structure but are also active on cell membranes by virtue of their phospholipase C and sphingomyelinase activities (Titball, 1993; Sakurai, 1995).

Staphylococcus enterotoxin B (Table 40.2: Category 8) is a single-chain protein that has no enzymic activity (Warren et al., 1974). Its incapacitating activity as a superantigen is dependent on cross-linking receptor sites on two cell types. The amino acid sequence of *C. perfringens* ε-toxin (Table 40.1) is not suggestive of any enzyme activity (Knight et al., 1990) but determines that it will form membrane pores.

The largest of the nonprotein toxins are the polycyclic polyether compounds produced by certain dinoflagellates (Table 40.2: Category 2) (Moore and Bartolini, 1981; Murata et al., 1994; Yasumoto and Satake, 1996). These are a highly toxic and stable group of toxins. The largest of them—maitotoxin—is composed of an aliphatic chain that includes 32 cyclic ether rings (Murata et al., 1994). Also in the same molecular weight range are: (a) the small, folded, disulfide cross-linked peptide toxins (Table 40.2: Category 5) from the venoms of scorpions (Becerril et al., 1997) and certain marine snails (Gehrmann et al., 1998) and (b) the stable cyclic peptides (Table 40.2: Category 7) from freshwater cyanobacteria (blue-green algae) (Namikoshi et al., 1992).

The remaining smaller toxins (Table 40.2: Categories 4 and 6) are all complex heterocyclic compounds (Woodward, 1964; Tokuyama et al., 1969; Schantz, 1986; Merck Index, 1989a, 1989b; Carmichael, 1994) and, with the exception of batrachotoxin, are all in the moderately toxic group.

40.3.2 Sites of Cell Membrane Interaction

The initial step in the overall action of a toxin with a cell is its interaction with the cell membrane. The organ and cell specificity of a toxin's membrane interaction will determine the nature and extent of any direct action on the cells present in the lungs. Where known for the above toxins (Tables 40.1 and 40.2), the possible sites of this interaction are listed in Table 40.3.

Many of the toxins of Category 1 in Table 40.3 (aconitine, batrachotoxin, ciguatoxin, α-conotoxins, maitotoxin, palytoxin, saxitoxin, tetrodotoxin, and tityustoxin) alter ion-channel mechanisms and interfere with ion-channel function by binding with the channel proteins of excitable tissues (Lombet et al., 1987; Tosteson et al., 1991; Werkman et al., 1993; Lipkind and Fozzard, 1994; Gutierrez et al., 1997; Wang and Wang, 1998; Arias and Blanton, 2000; Rao and Sidkar, 2000).

As a result of Ca^{2+} influx the maitotoxins cause hormone and neurotransmitter release as well as phosphoinositide breakdown in a variety of cells (Gusovsky et al., 1989). The opening of conductance channels and subsequent depolarization due to palytoxin may lead to the influx of Ca^{2+} into synaptic nerve terminals, mast cells, vascular endothelial cells, and smooth and striated muscle cells, causing the release of neurotransmitters, histamine, and vasoactive factors, as well as muscle contraction (Frelin and Van Renterghem, 1995). The toxic symptoms due to ciguatoxin poisoning are the result of a general increase in membrane permeability to Na^+ (Bidard et al., 1984), whereas those for batrachotoxin are due to a selective and irreversible increase in Na^+ permeability in muscle and nerve cells (Wang and Wang, 1998). The blockade of voltage-gated Na^+ channels by saxitoxin (and tetrodotoxin) leads to the failure of propagation of action potentials along nerves and a decreased neurotransmitter release (Benton et al., 1998).

The α-conotoxins inhibit neuron-stimulated skeletal muscular contraction by binding to the nicotinic ion-channel receptors and blocking the binding of acetylcholine and hence the influx of Na^+ that is essential for membrane depolarization (Marshall and Harvey, 1990). The scorpion toxin,

TABLE 40.3 Membrane Binding Sites of Selected Toxins

Membrane Site	Toxin
1. Integral cell membrane proteins:	
• Voltage-gated ion channels (Na^+, K^+, Ca^{2+})	Batrachotoxin, palytoxin, ciguatoxin, maitotoxin? saxitoxin, tetrodotoxin, tityustoxin K-α, aconitine.
• Ligand-gated ion channels (n-AChR)	α-conotoxin (G or M)
• Ion transport ATPases	Palytoxin
• Membrane proteins involved in synaptic vesicle exocytosis (e.g., synaptotagmin)	Botulinum toxin, tetanus toxin
• Enzymes (e.g., AChase)	Anatoxin-A(s)
• Others	Staphylococcal enterotoxin B, diphtheria toxin
2. Carbohydrate residues (e.g., galactose) of glycoproteins and glycolipids	Abrins, ricin
3. Glycolipids (Gb3 or globotriosyl ceramide, gangliosides, such as GT1b)	Shiga toxin, botulinum toxin, tetanus toxin
4. Glycoproteins	*Clostridium perfringens* ε-toxin
5. Specific phospholipids (phosphatidylcholine, sphingomyelin)	Textilotoxin, taipoxin
6. Nonspecific phospholipids	*Clostridium perfringens* α-toxin
7. Undefined or incompletely defined	Microcystins (LR?), aflatoxins (B, G, and M), tricothecene mycotoxins (T2), diphtheria toxin, tetanus toxin, textilotoxin, taipoxin (also anthrax toxins)

α-tityustoxin (K-α), enhances transmitter release from central and peripheral noradrenergic nerve terminals as well as cholinergic and sensory nerves by binding to voltage-gated K^+ channels without occluding the pores (Rogowski et al., 1994).

Of the other toxins binding to membrane proteins, anatoxin-A(s) irreversibly inhibits acetylcholinesterase (Cook et al., 1988) resulting in an overstimulation of muscarinic and nicotinic receptors. Specific membrane proteins are also one of two receptor components involved in the initial binding of botulinum and tetanus toxins to nerve endings (Nishiki et al., 1994; Li and Singh, 1998; Herreros et al., 2000). The second membrane receptor component for the botulinum toxins and tetanus toxin is a specific ganglioside prevalent in nerve endings and containing galactose and sialic acid moieties (Kozaki et al., 1998; Fotinou et al., 2001).

The plant lectins (abrins, ricin) and some bacterial protein toxins (e.g., Shiga or shiga-like toxins) (Categories 2 and 3) bind to specific carbohydrate residues on membrane glycoproteins or glycolipids to initiate entry into the cell and exert their toxic action (Wu et al., 2001; Sandvig et al., 1978; Jacewicz et al., 1986). The relatively simple receptor for the plant lectins is unlikely to be cell or organ specific, however the more complex receptors for the A-B type bacterial toxins are not necessarily expressed by all cell types or in all organs *in situ.* Variations of organ receptor specificity may explain the differences of biological effects between species.

Evidence has been accumulating that the receptors utilized by bacterial A-B type toxins are mobilized within the plasma membrane in lipid microdomains known as lipid rafts—characterized by a critical high cholesterol and glycosphingolipid content (Herreros et al., 2001). The lateral aggregation of these lipid rafts not only allows the juxtaposition of specific proteins for cell signaling (Simons and Toomre, 2000) and endocytosis (Nabi and Le, 2003), but also allows for high-affinity binding through clustered receptors for the multiple B-chains for a toxin like shiga toxin (LaFont et al., 2004). It also enables the formation of the protective antigen heptamers that are critical for the internalization of both anthrax lethal factor and edema factor (Abrami et al., 2003).

Staphylococcal enterotoxin B is different from the other protein toxins in Table 40.3 with respect to its membrane-binding sites. To trigger its superantigen activity it needs to simultaneously bind to protein receptors on two different types of cells involved in the adaptive immune response. Thus it crosslinks MHC class II molecules on B cells (lymphocytes), macrophages or dendritic cells with specific receptors on T cells (also lymphocytes) (Li et al., 1998).

The toxins of Categories 4, 5, and 6 (Table 40.3) react with membrane glycoproteins or phospholipids to form pores or cause a loss of membrane structure and function. *C. perfringens* ε-toxin causes a rapid swelling in canine kidney cells due to its heptamerization within the membrane and the consequent formation of a large pore complex (Petit et al., 2001; Miyata et al., 2002). It may also bind to membranes in a wider range of cell types (Shortt et al., 2000). The main physiological effect of taipoxin and textilotoxin is the gradual reduction to a complete stop of the evoked and spontaneous release of acetylcholine from motor nerve terminals. Death is by respiratory failure (Simpson et al., 1993; Dodds et al., 1997). *C. perfringens* α-toxin degrades most of the phospholipids in mammalian cell membranes (Sakurai, 1995).

40.3.3 Intracellular Sites of Action

For many toxins the toxic activity is localized to an intracellular site rather than at the cell membrane. Sometimes the binding of one component of the toxin to membrane sites leads to an assisted entry and release of a component with a toxic and highly specific enzymatic activity into the cell (see section 40.3.2).

The intracellular catalytic activity of the A subunits of the abrins, diphtheria toxin, ricin, and shiga toxin lead to an inhibition of protein synthesis (Van Ness et al., 1980a, 1980b; Endo et al., 1987, 1988) and, in some cases, damage to DNA (Brigotti et al., 2002). For the abrins, ricin and shiga toxin, cell death due to inhibition of protein synthesis is the result of the *N*-glycosidase activity of the A chain in removing a specific adenine (A4324) from the 28S rRNA of the 60S ribosome subunit (Endo et al., 1987, 1988). The A chain of diphtheria toxin ADP-ribosylates elongation

factor 2 to cause inhibition of protein synthesis and cell death (Van Ness et al., 1980a, 1980b). The botulinum toxins and tetanus toxin disrupt neurotransmitter release from peripheral and central nerve endings, respectively, through the intracellular Zn-protease activity of their light chains on various synaptic vesicle proteins (Montecucco and Schiavo, 1994, 1995; Ahnert-Hilger and Bigalke, 1995).

There are other toxins (e.g., the tricothecene mycotoxins, mycrocystins, and aflatoxins), which, besides having an incompletely defined method of entering the cell, also exhibit overall a less specific intracellular toxic activity. The tricothecene mycotoxins (e.g., T2) have multiple effects on intracellular membranes and associated enzyme activity (Khachatourians, 1990) as well as being potent inhibitors of protein, DNA and RNA synthesis (Ueno et al., 1973; Rosenstein and Lafarge-Frayssinet, 1983). The mycrocystins inhibit enzymes that effect cell structural proteins, leading to apoptotic cell death (Zambrano and Canelo, 1996) and aflatoxins have a more general cytotoxic action possibly due to epoxide formation damage to cell macromolecules (Baertschi et al., 1988; Choy, 1993; Jennings et al., 1994).

40.4 LUNG RESPONSE TO INHALATION OF TOXINS

Despite an enormous literature on pulmonary defense mechanisms (Sibille and Reynolds, 1990; Toews, 1993; Crystal et al., 1997; Stockley, 1997; Nicod 1999), and the effects of inhaled toxicants (inorganic and organic particulates, aerosols, and gases) and inhaled allergens (pollens) or microorganisms (Gordon and Amdur 1991; Cross et al., 1997; Schraufstatter and Cochrane 1997; Li and Holian, 1998; Bouhafs and Jarstrand, 1999), there is relatively little detailed information on the consequences of inhaling biological toxins (LeClaire et al., 1996a, 1996b; Wilhelmsen and Pitt, 1996; Brown and White, 1997; Park and Simpson, 2003; Abraham et al., 2005).

In particular we have little information on the role of the lung pathology versus other organ pathology in the lethal or incapacitating effects of these toxins. Further there is a lack of information on whether and, if so, how biological toxins are transferred from the lungs via the blood or lymph to other body organs. It is more usual to find information regarding toxins, either released directly into the respiratory tract from resident microorganisms (Hadfield et al., 2000; Garcia et al., 2002), or being transferred systemically to the lungs from microorganisms elsewhere in the body (Lyons et al., 2004). Importantly there is little information available in the open literature as to what cellular effects or mode of action individual biological toxins have in the lungs. This is irrespective of whether the source of the toxin is from direct inhalation or from systemic release. The question arises as to whether a toxin has a direct cytotoxic or cytostatic effect on particular lung cells or whether the major effect is the induction of neurohumoral inflammatory processes? Perhaps both these factors are responsible for any resultant tissue damage (Yoshida et al., 2003)?

The general lack of information as to the mechanism of action of particular inhaled biological toxins no doubt contributes to the fact that there are few, if any, proposed therapeutic drugs (apart from antibody preparations) that are available to counter their lethal or incapacitating effects.

40.4.1 Pertinent Anatomical and Functional Aspects

Some factors that would affect aerosolized biological toxin delivery to particular lung sites include:

- Particle size and density,
- The inhaled dose,
- Respiratory breath profiles,
- Total ventilation,
- Airway mechanics, and
- The degree of reaction with constituents of mucus and epithelial lining fluid.

With respect to the first of these factors, it is the equivalent aerodynamic diameter that actually controls whether the deposition in the lungs is due to sedimentation or impaction rather than to diffusion. For spherical particles the equivalent aerodynamic diameter is a function of the geometric diameter and the particle density relative to water. Diffusion becomes predominant when the particle diameter is less than 0.5 to 1.0 μm (Schlesinger, 1995).

Further, as inhaled air is humidified in the upper respiratory tract, particulates—particularly those that are hydrophilic—are likely to be enveloped in moisture and assume new dimensional and aerodynamic characteristics (Gordon and Amdur, 1991). This will also affect how deeply they are carried with the air stream into the airways and where they impact or land.

The next consideration would be the effect of various inhaled toxins on specific lung components or cells. The consequences would depend on:

- The chemical nature of the toxin,
- The amount of toxin deposited at the particular anatomic site along the respiratory tract,
- The period during which the toxin acts,
- The relative sensitivity to damage of the cells at risk in that location,
- The effectiveness of the local extracellular and intracellular defense mechanisms, and
- The sensitivity, intensity and time course of neurohumoral inflammatory–immune processes.

The measured toxin-induced effects on the lung may represent not only direct toxicity of the inhaled agent, but also pathophysiological lung 'defense' responses involving secreted neurohumoral and immunoinflammatory substances. The direct responses of respiratory tract cells to toxic substances are often dose dependent. Small doses may cause up-regulation of defense mechanisms and large doses cell death through apoptotic or necrotic mechanisms (Komatsu et al., 2000; Kirby, 2004). In a toxin whose lethal or incapacitating activity depends on an enzymatic property the deciding parameter may be more related to the time over which it is allowed to act.

Opposing the influx of noxious contaminants in inhaled air are the various components of the lung's defense system.

40.4.1.1 The Nasopharyngeal Region

In the nasopharynx, mechanical barriers and reflex mechanisms like sneezing, coughing, and the ciliary action of epithelial cells in the mucosa largely operate. In general, larger particles (>10 μm) are either filtered out in the nose or impacted on the nasal and oral pharynx. They are then cleared by coughing or sneezing. Diversion of the laminar airflow in the larger bronchi at dichotomous branching points may deflect 3- to 10-μm particles carried in the airstream against the mucosal surface where they can stick. These are then moved toward the pharynx by ciliary action. At the level of the larynx they are either swallowed or expectorated (Schlesinger, 1995).

40.4.1.2 The Tracheobronchial Region

The mucus on the luminal surface of the conducting airways contains other elements of the immunodefense system. These include immunoglobulins (Igs) (antibodies)—especially IgA, cells such as surface macrophages and lymphocytes, and local mediators such as histamine and possibly neuropeptides (Reynolds, 1997). The IgA in the mucus is predominantly polymeric in form and known as secretory IgA or S-IgA. It consists of two monomers of IgA held together, tail to tail, by their Fc regions with a further two specialized glycoproteins (the secretory protein and the J-chain) (Roitt et al., 1993a). Besides having antibody activity against a variety of microorganisms that can colonize or infect the airways (Janeway and Travers, 1994; Reynolds, 1997) there has long been evidence that, at least at other mucosal surfaces, S-IgAs can effectively neutralize bacterial exotoxins (Delmas et al., 1989; Keren et al., 1989; Janeway and Travers, 1994). It is therefore possible that they would be active in this fashion in the conducting airways.

40.4.1.3 The Pulmonary Region

Final arborizations of the conducting airways merge from the respiratory bronchioles, from which the alveolar ducts and alveoli extend to create the air-exchange surface that can be reached by small particles <2–3 μm in diameter (Gordon and Amdur, 1991; Schlesinger, 1995; Reynolds, 1997).

In the alveoli the air-exchange barrier between the lung and the blood is only about two attenuated cells (~1.0–1.5 μm) thick (Weibel, 1997). In an adult the aggregate alveolar surface area is estimated to be about the area of a tennis court or ~140 m^2 (Reynolds, 1997; Weibel, 1997) and therefore at least 70 times the surface area of the skin. It is thus extremely important to devise therapeutic and prophylactic measures to protect the cells of this surface from the effects of noxious substances, including biological toxins.

Because the flow velocity of air molecules ceases when the large cross-sectional area of the alveoli units is reached, very small particles (0.5 to <3 μm) in the inhaled air may not immediately impact against the alveoli surface. However the dynamic expansion and closure of the alveoli with respiration may compress and force these particles against the alveolar walls so that they become enmeshed in the alveolar film (Reynolds, 1997)—otherwise impact simply results from diffusion (Gordon and Amdur, 1991).

Once such particles are captured in this way, appropriate components of the host defense system in the alveoli are needed to deal with them. A number of immune (and nonimmune) substances that can function as opsonins to coat particulates and aid their removal are present within the surfactant-rich lipoprotein film. IgG, which constitutes ~5% of the total protein content of normal bronchoalveolar lavage (BAL) fluid, seems to be the predominant immunoglobulin in the alveoli and also seems to be the most clearly identified with this opsonic activity (Janeway and Travers, 1994; Reynolds, 1997). The resultant complexes can then be more readily recognized and taken up (phagocytosed) by the scavenger alveolar macrophages (Bezdicek and Crystal, 1997) that exist just beneath or within the alveolar film, and by other inflammatory cells, particularly polymorphonuclear neutrophils that may be on the alveolar surface.

40.4.2 The Lung Inflammatory Response

Inflammation is defined as a fundamental pathological process involving cytological and chemical reactions occurring in affected blood vessels and adjacent tissues in response to an injury or abnormal stimulation caused by a physical, chemical, or biological agent (Stedman's Medical Dictionary, 2000). Inhaled toxicants frequently induce an inflammatory response in the lungs (Gordon and Amdur, 1991). The cells involved include epithelial, interstitial, and endothelial cells, as well as mast cells and a wide spectrum of resident inflammatory/immune effector cells such as alveolar macrophages, neutrophils, and lymphocytes, and to an extent, lung nervous system components (Gordon and Amdur, 1991; Reynolds, 1997).

Apart from any direct cytotoxic or cytostatic action, many of the biological toxins that have been considered here (Table 40.1) would be expected, on the basis of their molecular size (>5–10 kDa) and nature (e.g., foreign protein), to trigger an immune response in the lung. Lung inflammatory/immune processes initiated by the action of inhaled toxins would induce the generation of a host of mediator substances. Complex interactions would occur between the endogenous lung cells, the resident and recruited inflammatory/immune effector cells, elements of the nervous system, and the various mediators elaborated by these cells. The mediators themselves are capable of interacting synergistically (or antagonistically) to modulate the intensity of overall inflammatory responses. Early mediators that induce and organize the inflammatory response include the proinflammatory cytokines—Il-1 and tumor necrosis factor alpha (TNF-α)—which are central to the response of the host (Wewers et al., 1997). [Cytokines are low-molecular-weight (8–30 kDa) proteins—usually single-chain glycoproteins—that are produced locally in small amounts by certain cells to act on other cells.]

40.4.2.1 Epithelial Cells

The general pathological response to an inhaled toxicant is said to be epithelial cell injury and the triggering of acute inflammatory-immune processes (Cross et al., 1997). The epithelial cells from the lining of the luminal surface of the lungs actively participate in the host defense (Rennard et al., 1997). This may be a direct or indirect response to a foreign substance (Robbins and Rennard, 1997). The response is indirect if the inhaled foreign substance directly activates macrophages to secrete Il-1 and/or TNF-α that subsequently stimulate the epithelial cells (Adler et al., 1994). Epithelial cells are capable of producing and responding to a wide variety of cytokines and growth factors that form a complex network regulating the inflammatory response (Nicod, 1999). Their secretory products recruit inflammatory cells. Thus, from membrane phospholipids and fatty acids, they release the arachidonic acid derivative, 15-HETE, which is an active neutrophil chemoattractant (Holtzman, 1992). Il-8, which is also a neutrophil chemoattractant, is released in response to a variety of bacterial products and other cytokines such as Il-1 and TNF-α (Adler et al., 1994). An observation with potentially important therapeutic implications is that corticosteroids, commonly used to treat airway inflammation (Barnes and Adcock, 1997), are potent inhibitors of Il-8 mRNA transcription (Kwon et al., 1994). Lung epithelial cells also release the cytokine Il-16, which is a lymphocyte chemoattractant factor (Center et al., 1997), and growth factors like granulocyte macrophage-colony stimulating factor (GM-CSF) that can maintain recruited inflammatory cells such as dendritic cells and macrophages in an active state (Sallusto and Lanzavecchia, 1994).

40.4.2.2 Dendritic Cells

Dendritic cells, which are derived from bone marrow stem cells, are widely distributed in the normal lung including an intimate association with the epithelial cell basement membrane. They are considered the most potent antigen-presenting cells capable of initiating a primary immune response (Nicod, 1999). Lung dendritic cells can release a number of cytokines, including Il-1, Il-6, Il-8, Il-12, and TNF-α that could modify the functional properties of T cells (Hance, 1997).

40.4.2.3 Macrophages

Lung macrophages belong to the mononuclear phagocyte system, a family of cells derived from the monoblast series of bone marrow precursors (Roitt et al., 1993b). They play a central role in maintaining normal lung structure and function. They do this through a variety of mechanisms, including their ability to phagocytize, to express specific cell surface receptors (e.g., that recognize the Fc portion of most classes of immunoglobulins) and to synthesize and release a broad range of mediators (Bezdicek and Crystal, 1997). Macrophages can also be a danger to the lung, because the mediators possess the capacity to injure normal structures. There is no doubt that the alveolar macrophage (AM) has a central role in mediating lung injury. However it is not always clear whether it results from the AM responding in normal fashion to exogenous stimuli, or whether it represents a loss of control of these cells (Bezdicek and Crystal, 1997).

Lung macrophages appear to play a central role in the lung's biological responses to inhaled toxicants (Sibille and Reynolds, 1990; Cross et al., 1997). Macrophages in lung-lining fluids are among the first lung cells to encounter inhaled particulates and they may respond by ingesting and destroying (or trying to destroy) these toxicants and/or by secreting cytokines that recruit other phagocytic cells (e.g., neutrophils and eosinophils) into the lung. Since the Fc receptors on macrophages have a high avidity for antibodies bound to antigen and a relatively low affinity for free immunoglobulin molecules (Janeway and Travers, 1994), the presence in the respiratory tract of antibodies to inhaled toxins would increase receptor-mediated uptake of the latter by these cells (Lombry et al., 2004). The associated rise in AM numbers also appears to be a very important element in the response of the lungs to toxicants, especially to particulates. Alveolar macrophages represent 85% of the cells retrieved by bronchoalveolar lavage (Nicod, 1999). They are normally

the only phagocytic cells present within the lower respiratory tract. They can initiate inflammation by the release of Il-1α and Il-1β or TNF-α that in turn induce a cascade of events including the release of other cytokines (e.g., Il-8 from epithelial cells and Il-6) and growth factors. Activation of AMs also results in the activation of several potentially toxic reactive oxygen and nitrogen species (e.g., O_2^- and NO). At the same time, however, macrophages have the capacity to control inflammation by the release of an inhibitor of Il-1 or TNF-α in the form of Il-1 receptor antagonist or TNF-soluble receptors (Cross et al., 1997).

Although several cell types can produce TNF-α and Il-1, the monocyte/macrophage is the principal source of both. Likewise, though a variety of stimuli may trigger production of each, gram-negative bacterial endotoxin (LPS) is the most important stimulus for both. However, exotoxins such as the Staphyloccocal enterotoxins from gram-positive organisms can also serve as potent stimuli for the production of both cytokines (Andersson et al., 1992). Functionally, TNF-α has dichotomous effects, depending on its concentration. In low concentration, it serves to protect the host. In high concentrations, it induces pathophysiological host alterations that are indistinguishable from bacterial sepsis (White and Das, 1997). Also recently it has been shown, for certain endothelial cells, that a raised level of TNF-α and Il-1 may cause an increased sensitivity to the Shiga-like toxins because of an up-regulation of the cell surface receptor (the ganglioside Gb3) (Stricklett et al., 2002). Conversely, a raised level of interferon-γ (IFN-γ) has been found to substantially reduce microvascular endothelial cell sensitivity to Shiga toxin (Yoshida et al., 2003).

40.4.2.4 Lymphocytes

Other cells, which are found in the alveolar walls and on the epithelial surface from the trachea to the alveoli, are the lymphocytes. In the alveoli they comprise about 10% of the total number of resident mobile cells and of these the T lymphocytes are the dominant species (Nicod, 1999). Although T lymphocytes carry antigen receptors, these are of a type that require the antigen to be processed (i.e., partially degraded) first. Thus the response of T lymphocytes to foreign substances is indirect. The processing can be carried out by B lymphocytes, macrophages or dendritic cells that interact directly with antigens (Delmas et al., 1989). These cells then present the processed antigen to the T lymphocytes and so initiate the humoral response and neutralizing antibody production. Raised levels of Il-1, TNF-α, and Il-6 cause the proliferation of lymphocytes in the lung (Wewers et al., 1997; White and Das, 1997).

40.4.3 Direct Actions on Lung Cells

In general, evidence for the action of individual biological toxins (Table 40.1) on lung cells *in situ*, particularly those of the alveoli, is lacking. The problem is even more acute if one is searching for evidence regarding the mechanism of such an action. Table 40.4 summarizes some of the available information regarding the interactions of lung cells both *in vitro* and *in vivo* with particular toxins.

Much further investigation is required to determine whether lung damage is likely to be initiated by a direct cytotoxic action of the toxin on lung cells, or the recognition of the toxin as a foreign agent and the initiation of an immune defensive response. It is well known (see section 40.4.2.1) that inhaled foreign substances can directly stimulate airway epithelial cells to release inflammatory modulators such as the cytokines Il-1, Il-6, Il-8, and TNF as part of the lungs defensive response (Robbins and Rennard, 1997). However, the very large surface area presented to the inhaled air by all the alveolar epithelial cells is generally considered solely as a physical barrier with gas and solute transport properties (Lubman et al., 1997). Whether alveolar epithelial cells and the associated capillary endothelial cells also might liberate inflammatory mediators as a direct response to foreign substances, or simply respond to inflammatory cytokines from other direct responders such as macrophages, is not clear (Krakauer, 2002).

TABLE 40.4 Known Direct Effects of Biological Toxins on Lungs or Lung Cells

Toxin	Lung Cell	Toxicity/Effect
Abrins	Endothelial and epithelial cells	Inhalation leads to an acute necrosis/apoptosis of lower respiratory tract cells (Griffiths et al., 1995; Hughes et al., 1996)
Aflatoxins (B1, G, and M)	Epithelial cells (tracheal, bronchial and alveolar)	Converted to carcinogenic epoxide in upper airway cells (Ball et al., 1995; Van Vleet et al., 2002)
	Alveolar macrophages	Cytotoxic (Liu et al., 2002)
Botulinum toxin A	Alveolar epithelial cells	Displays specific binding and transcytosis across monolayers (Park and Simpson, 2003)
Botulinum toxin B	Endothelial cells	Cleaves key membrane proteins (VAMP as well as SNARE, SNAP, etc.) known to mediate different aspects of vesicle formation and docking and/or fusion induction (Schnitzer et al., 1995)
C .perfringens α-*toxin*	Nonspecific	If inhaled would degrade surfactant and cell membranes (Saint-Joanis et al., 1989). More toxic in rats after intratracheal instillation (Allenby et al., 1994)
Diphtheria toxin	Epithelial and other cells	Effects lung–blood barrier → edema (Todar, 2002)
Maitotoxin	Tracheal epithelial cells	↑ influx of extracellular Ca^{2+} (Venant et al., 1994)
Palytoxin	Bronchial epithelial cells	Cytotoxic and growth inhibitory (known potent inhibitor of protein synthesis) (Bonnard et al., 1988)
Ricin	Alveolar macrophages	Apoptotic cell death *in vivo* (Brown and White, 1997)
	Alveolar epithelial cells and capillary endothelial cells	Ultrastructural and necrotic changes *in vivo* accompanied by interstitial edema, inflammation and microvascular thrombosis (Brown and White, 1997)
	Airway epithelial cells	Inhalation in mice causes necrosis, accompanied by severe interstitial edema and inflammation (Poli et al., 1996)
	Pulmonary endothelial cells	Cytotoxic (Hughes et al., 1996)
		After inhalation, mostly retained in respiratory and gastrointestinal tracts (Wilhelmson and Pitt, 1996; Doebler et al., 1995)
Saxitoxin	Alveolar macrophages	Induces thromboxane B2 and arachidonic acid release (both involved in acute lung injury) (Naseem et al., 1989)
Shiga toxin (and Shiga-like or Vero toxins)	Microvascular endothelial cells	Inhibits protein synthesis, cytotoxic to cells. Interferon-gamma pretreatment suppresses cytotoxic action, but not protein synthesis inhibition (Yoshida et al., 2003)
	Epithelium cells and vascular endothelial cells	Shiga-like toxin-binding sites present. Cytotoxicity involves apoptosis (Uchida et al., 1999)

(*Continued*)

TABLE 40.4 Known Direct Effects of Biological Toxins on Lungs or Lung Cells (Continued)

Toxin	Lung Cell	Toxicity/Effect
Staphylococcal enterotoxin B	Dendritic cells, macrophages, and small airway epithelial cells	Dendritic cells, alveolar macrophages, and small airway epithelial cells express class II MHC complexes (Holt et al., 1994; Cunningham et al., 1997). Inhaled aerosol causes T-cell-mediated toxic shock (Hermann et al., 1991; Neumann et al., 1997)
Taipoxin and textilotoxin	Nonspecific	May degrade surfactant and cause acute lung injury (Arbibe et al., 1998)

40.5 PROPHYLAXIS AND THERAPY AGAINST TOXINS

40.5.1 Vaccination

For many of the toxins that may be a significant threat, vaccination is considered to be the prime means of preventing casualties. The resultant antibodies, notably the IgG antibodies expressed into the alveolar lining fluid and the IgA antibodies secreted into the mucus lining the lung airways, would bind the toxins. This would not only prevent them from acting at possible receptor sites, but also facilitate their uptake by macrophages and other cells for elimination. However, effective vaccines are not yet available for all the protein or peptide toxins that may be considered as a BW threat, let alone the macromolecular polyether toxins and the small, complex heterocyclic toxins.

Vaccination requires not only that the antigen is capable of inducing an appropriate immune response, but also that it is not toxic at the concentration used. For the more lethal toxins this means that they must be presented in the vaccine in a chemically inactivated or otherwise structurally altered or truncated form. The latter must be both nontoxic and yet at the same time capable of stimulating the production of neutralizing antibodies that specifically recognize the original toxin.

Most recent effort to date in developing effective therapy and prophylaxis against toxins that may be a potential BW threat has been directed at the botulinum toxins (because of their extreme toxicity) (Holley et al., 2000; Chaddock et al., 2002), SEB (because of its incapacitating activity) (LeClaire et al., 2002), and ricin (because of its availability and high toxicity) (Smallshaw et al., 2002). Nevertheless, at this time, there is still no acceptable vaccine for SEB and ricin and few adjunctive therapeutic or prophylactic drugs for any of the other toxins. Most licensed toxoid vaccines consist of partially purified toxin preparations obtained from culture supernatants of bacteria such as *C. diphtheriae, C. tetani, or B. anthracis* that have been formaldehyde-treated to detoxify them for vaccine formulation. The controlled-use pentavalent botulinum toxoid vaccine is produced in a similar fashion.

40.5.1.1 Future Developments

The next generation of toxoid vaccines are likely to include purified recombinant nontoxic mutants or fractions of the particular holotoxin such as those being developed for the *C. perfringens* α- and ε-toxins (Oyston et al., 1998) and the botulinum toxins (Chaddock et al., 2002; Smith et al., 2004). In the short term, improved anthrax vaccines will consist primarily of recombinant PA (Leppla et al., 2002; Laird et al., 2004). Recombinant nontoxic ricin A-chain mutants (Smallshaw et al., 2002) and Shiga toxin 1 variants mutated in the A-chain (Ishikawa et al., 2003) have been shown to be highly protective against their respective toxins in animal studies. It has also been suggested that human commensals (nonpathogenic bacteria) such as certain *Lactobacilli* (Shaw et al., 2000; Grangette et al., 2001) or *Streptococcus* species (Medaglini et al., 2001) be used as vectors to deliver the appropriate nontoxic antigens *in vivo*.

Besides the availability of suitable vaccines, the decision to use the protective effects of immunization also requires a prior knowledge of the particular threat(s) and time for the body to make its own protective antibodies—from 1 to 3 months. Further, some vaccines currently in use require multiple injections, often weeks apart. The logistical burden of assuring that troops are given booster immunizations at the correct time could be overwhelming in a fast moving buildup of hostilities (Franz, 1997). The development of single-shot DNA vaccinations could vastly improve the situation (Lewis and Babiuk, 1999; Hermanson et al., 2004).

40.5.2 Passive Antibody Therapy or Prophylaxis

In a scenario involving the use of biological weapons, not only might the availability of suitable vaccines and the immunization status of the population be a problem but, even where prior vaccination has been carried out, it is possible that the effectiveness of immunization might be compromised by the relative levels of the inhaled agent versus the individuals' level of circulating and local antibodies (Leppla et al., 2002). In any case the availability of adjunctive therapeutic and prophylactic drugs such as antibodies would be critically important.

The use of serum-derived polyclonal antibodies from animal sources—as exemplified in the case of the trivalent equine botulinum antitoxin—is accompanied by the risk of hypersensitivity, serum sickness, or overt anaphylaxis, especially after repeated injections (Arnon et al., 2001). As well as these problems the serum half-life in humans of antibodies sourced from animals is also reduced over that of their human counterpart. Thus although the injection of rhesus monkeys with immunopurified antibodies generated in chickens against the whole SEB toxin has been found to be protective against a lethal aerosol SEB challenge—even if treatment is delayed for up to 4 h after exposure (LeClaire et al., 2002)—it seems likely that their use in humans could have similar problems.

As a solution to the antianimal immune responses, polyclonal antibodies may be obtained from animals made transgenic for the human immunoglobulin genes (Houdebine, 2000; Ishida et al., 2002). However very stringent purification procedures of these human antibodies are still required to eliminate possible contamination by animal serum proteins and pathogenic microorganisms, including prions (Bregenholt and Haurum, 2004).

Several recent reports have discussed the use of humanized or human antibodies or specific antibody fragments (e.g., Fab's) as a rapidly deployed passive immunotherapy or prophylaxis against exposure to toxins (Casadevall, 2002; Brekke and Sandlie, 2003; Bregenholt and Haurum, 2004). The ability to produce human antibodies from transgenic mice has enabled the production of human monoclonal antibodies from mouse hybridomas (Brüggemann et al., 1989; Wagner et al., 1994; Ishida et al., 2002). A panel of human monoclonal antibodies that are each highly effective against Shiga toxin 1 and 2, and have potential therapeutic applications, have been developed in this way (Tzipori et al., 2004).

In some cases the combination of several neutralizing monoclonal antibodies can have a synergistic protective activity. For instance, it has recently been shown that the neutralizing potency of recombinant monoclonal antibodies against botulinum toxin type A was very markedly increased by using a mixture of three selected antibodies against nonoverlapping epitopes (Nowakowski et al., 2002). Whether one or more monoclonal antibodies against a specific target can be found with sufficient affinity or avidity to equal or out-perform their polyclonal counterpart still appears to be a critical question. In this context, a high-affinity chimeric, deimmunized monoclonal antibody against anthrax protective antigen has recently been reported that is under development for both prophylactic and therapeutic use against inhalational anthrax because of the relatively low dose required to protect rabbits when given before or after spore challenge (Mohamed et al., 2005). However in the same context it is also now possible to produce recombinant human polyclonal antibodies that combine the strengths of the target-specific antibodies of the human immune system with methods that enable large quantities of a well-characterized and safe product to be produced (Bregenholt and Haurum, 2004).

With respect to the smaller molecular weight and polyether macromolecular toxins (Table 40.2), it is possible that by methods such as the use of hapten-carrier protein conjugates (Baier et al., 2000; Xu et al., 2005), or of a mixture of defined synthetic epitopes (Alvarenga et al., 2002), prophylactic or therapeutic antibodies may be prepared for these also.

40.5.2.1 Antibody Inhalation

Other than intramuscular or intravenous injection of antibodies for passive immunoprophylaxis or –therapy, another possible method of delivery is as a component of an inhaled aerosol (Vogel et al., 1996). The inhalation of drugs is a well-established means of treating localized diseases within the lungs and there is now increasing recognition that the lung is also an ideal site for the noninvasive delivery of therapeutic molecules, including immunoglobulins, to the systemic circulation (Bot et al., 2000; Fu et al., 2002). Much work has been carried out in an attempt to engineer suitably sized and structured carrier particles for the delivery of drugs to the lower respiratory tract by the inhalational route (Newhouse and Corkery, 2001; Labiris and Dolovich, 2003).

The so-called large (>5 μm), low density or porous particles (Bot et al., 2000; Fu et al., 2002; Labiris and Dolovich, 2003) can be tailored to reach the alveoli upon inhalation where they remain for longer than their smaller (<5 μm) nonporous counterparts, apparently because of their size which enables them to avoid phagocytic clearance (Edwards et al., 1997). Thus immunoglobulins delivered via such particles in this way would be available for a longer period for both antigen complex formation within the alveolae, as well as for systemic delivery.

Immunoglobulins can be absorbed across the alveolar epithelial barrier by trancytosis involving specialized FcRn receptors (Spiekermann et al., 2002; Kim and Malik, 2003). Absorption into the bloodstream may then occur through leaky junctions between capillary endothelial cells and via lymphatics (Newhouse and Corkery, 2001), although endothelial cells also carry the FcRn receptor (Lobo et al., 2004). The serum lifetime of an absorbed IgG is controlled by many factors including the relative affinities for its specific target and the FcRn receptor, its susceptibility to proteolysis and the number and location of the catabolic sites (Lobo et al., 2004).

Assuming that the inhaled aerosolized particles containing antibodies or antibody fragments do not in themselves cause a local immune reaction (Bot et al., 2000), passive antibody therapy could be more effective against some inhaled toxins than others. Apart from the binding characteristics of the antibodies, the effectiveness would depend on the rapidity of the inhaled toxin's action and any associated immune response. For instance it is likely that a superantigen like SEB would cause a more immediate direct immune response than other protein toxins. The smaller peptide toxins would presumably be less immunogenic.

With regard to the onset of toxicity, the phospholipase toxins—provided they have rapid access to the cell membrane—would be expected to be both rapidly acting and cell damaging and therefore induce a rapid inflammatory reaction. Toxins acting at the cell membrane to alter ion transport would be expected to be rapidly acting in this regard, but not necessarily in causing an immune response. Protein toxins requiring membrane binding, internalization and release of the catalytic component into the cytosol for their activity would be expected to take a longer time to induce a cytotoxic action and cell damage. Information as to how long passive antibody therapy could be delayed after initial contact with a particular toxin, and still be effective, would be an important requirement. It may be more worthwhile to consider inhaled aerosolized antibody use as a rapid prophylactic measure. In this case it would be more important to examine how long protective levels of antibody remain in the lungs after aerosol usage.

Problems encountered with the use of antibodies in either of these ways would include the need for prior knowledge of the use and identity of the threat toxin, the availability of appropriate (neutralizing) antibodies and the possibility of adverse reactions. The latter may include an exacerbating complement driven immune response due to high levels of antibody-antigen complexes. Macrophages would be stimulated to secrete TNF-α and Il-1 as well as the macrophage inflammatory protein—leading

eventually to lung injury (Ward, 1996). This is less likely to be a problem if Fab's can be used rather than whole antibodies.

40.5.3 Prophylactic or Therapeutic Drugs

The action of drugs that have been found to antagonize the action of toxins on cells, tissues or in whole-animal studies may be direct (due to interaction with the toxin itself or its sites of action), or indirect (at some other site) (see Paddle, 2003).

As the molecular structure of individual toxins, their sites of activity, and the dynamics of their mode of action become better understood, the combination of computerized three-dimensional molecular modeling and chemical synthesis skills should allow us to more easily tailor toxin antagonists to our needs (Olson and Cuff, 1999; Arad et al., 2000; Kitov et al., 2000; Tanaka et al., 2001).

40.5.3.1 Multivalent Ligands

A common property of a number of the protein synthesis inhibiting A–B type toxins (e.g. Botulinum toxins, Tetanus toxin, Shiga toxin, the abrins and ricin) is the affinity of the B chains for particular carbohydrates or glycolipids and glycoproteins at the cell surface (see Table 40.3). Thus it would be expected that macromolecular species with say numerous galactose moieties at their surface would compete with these cell surface receptors for ricin (Dawson et al., 1999). Recently, multivalent water-soluble carbohydrate ligands that specifically bind Shiga toxins with high affinity, and neutralize their activity on cells, have been produced (Kitov et al., 2000; Mulvey et al., 2003). A carbosilane-based dendrimer with branches terminated by the trisaccharide moiety of the Gb3 receptor has also been prepared. This binds the Shiga-like toxin produced by *E. coli O157:H7* with a high affinity and prevents its lethal action (Nishikawa et al., 2002).

Also of interest in this context is the demonstrated use of oligosaccharide-derivatized dendritic polymers to bind cholera toxin and prevent its adherence to cell surface ganglioside (GM1) receptors (Page and Roy, 1997; Thompson and Schengrund, 1998). Further, botulinum toxin A has been reported to have a greater binding affinity for a dendritic polymer with six residues of the oligosaccharide portion of brain GT1b ganglioside attached to it than for the whole ganglioside (Schengrund et al., 1997).

Since dendritic polymers (dendrimers) (Vogtle et al., 2000) can also be derivatized with peptide sequences (Mitchell et al., 1999) it is possible that diphtheria toxin (Brooke and Cha, 2000) and SEB (Arad et al., 2000) could be prevented from acting at their cell surface receptor sites. In fact, once the cell surface receptors are known in detail, this approach could be applicable to the neutralization of many more toxins. It is conceivable that solutions of such derivatized polymers (either against individual toxins or a mixture of toxins) could be used therapeutically or prophylactically as injections or as inhaled aerosol sprays.

40.5.3.2 Enzyme Inhibitors

Almost all of the protein toxins in Table 40.1 have enzyme activity (or potential enzyme activity) that is the basis of their intracellular toxicity. The enzymatic A chains of shiga toxin, diphtheria toxin, the abrins and ricin, and especially the light chains of the botulinum toxins and tetanus toxin, have extremely specific intracellular target sites. Blockade of this enzyme activity could be protective against their action. However questions that would need to be asked include:

(a) whether the inhibitor crosses the cell membrane, and
(b) what the fate of the toxin/inhibitor complex is inside the cell.

Taipoxin, textilotoxin, and *C. perfringens* α-toxin have phospholipase activity and act on cell membranes to produce highly active signal molecules. Since endogenous phospholipase A2 enzymes

play an important role in the acute inflammatory response in the lung there has been considerable clinical interest in phospholipase inhibitors. Some of these may be effective against the activity of taipoxin and textilotoxin.

40.5.3.3 Aptamers

An entirely different approach would be to use selected oligonucleotides—aptamers—to bind toxins and neutralize their activity. Aptamers are derived from a very large chemically synthesized library of random base sequence oligonucleotides of ssDNA or RNA type. For sequences with a randomized length of 40 bases and with a constant mixture of 4 alternative nucleotides available at each position, the maximum theoretical diversity is ~ 10^{24}. In practice the sample may contain 10^{13}–10^{15} different sequences. The required sequences may be selected by repeating a cycle of affinity binding to the target molecule of interest, selective elution, and PCR amplification (SELEX process [Ellington and Szostak, 1990; Tuerk and Gold, 1990]). Cloning methods can then make sufficient purified material available for sequence analysis and hence chemical synthesis. A considerable number of aptamer sequences that bind to large and small target molecules of many kinds have been reported (Famulok, 1999; Famulok et al., 2000), and therefore their use should be applicable to most toxins of interest in the current context. However, despite this and their potential high affinity and specificity to the target molecule, as well as their low level of toxicity and immunogenicity, there are still only a few reports of aptamers for toxin binding or neutralization in the open literature (e.g., Hesselberth et al., 2000; Hirao et al., 2000; Bruno and Kiel, 2002).

Initial testing of the therapeutic or prophylactic effectiveness of potential neutralizing IgG antibodies, derivatized dendrimers, aptamers, receptor site binding antagonists, or enzyme inhibitors could be carried out using appropriate cell lines and a range of cytotoxicity, cell proliferation, and function tests.

40.5.3.4 Generic Agents

It would also be a great practical advantage if prophylactic or therapeutic drugs could be found which were not only, (a) stable in storage, (b) useable in the field, (c) rapid in action, but also (d) effective against more than one toxin. Clearly, finding drugs with the latter property is likely to be the most difficult task given the variety of toxins and their receptor sites. However, further studies of the molecular character of the toxins, their receptor sites, and cellular and tissue responses may lead to the categorization of generic characteristics or pathways critical to the discovery and design of such drugs.

A recent case in point stems from earlier findings that bacteria may secrete their toxins in a form that requires proteolytic clipping for them to be effective. For several toxins this is carried out by furin-like proteases at the target cell surfaces (Klimpel et al., 1992; Tsuneoka et al., 1993; Garred et al., 1995). Thus finding small peptides that would bind to the highly negatively charged active site of this enzyme and remain inhibitory was of high interest. In fact positively charged polyarginine-containing peptides such as nona-D-arginine amide have been found to be potent and noncleavable inhibitors of this enzyme (Kacprzak et al., 2004). The smaller hexa-D-arginine has been found to be protective against anthrax toxemia *in vivo* (Sarac et al., 2004).

Also it is noteworthy that about a third of the toxins considered here act at voltage-gated ion channel sites (see Table 40.3). The latter play a critical role in the electrical activity of neuronal and muscle cells and in controlling the secretion of neurotransmitters and hormones by regulating Ca^{2+} entry. The responsible ion-channel proteins that span the cell membrane have no physiologically relevant agonist-binding site, as there are on ligand-gated ion channels. However, there are various toxin-identified sites that are important in enabling a change in conformation from the conducting to the nonconducting state, as well as a direct block of ion movement. Although the channels are specific for a particular ion, the different proteins have common functional elements. Thus channel opening or closing (gating) is controlled by conserved helical voltage-sensitive regions of the protein that contain repeated positively charged lysine or arginine moieties (Catterall, 1988).

According to Denyer et al. (1998), these ion-channel states provide unique opportunities for drug discovery, enabling state-dependent molecules to be developed that, for example, only bind to nonconducting (inactivated) channels. The overall effect would be to target drugs to tissues exhibiting abnormal electrical activity, while leaving normal channels in active tissues unaffected.

Techniques that could be used to measure the ability of drugs to counter the activity of toxins at ion channels include functional approaches applied to whole cells in high-density (e.g., 96-well) formats. In the absence of electrophysiological measurements, which have been difficult to incorporate into such formats, ion-channel function may be monitored by using fluorescent-ion indicators (Haugland, 1999b), which can be used to directly measure changes in intracellular concentrations of specific ions.

Alternatively fluorescent potentiometric probes (Haugland 1999c) offer an indirect method of detecting the translocation of these ions across cell membranes. Increases and decreases in membrane potential play a central role in many physiological/cellular processes—including cell signaling and ion-channel gating. Potentiometric probes are important for studying these processes as well as for cell viability assessment. In particular slow response probes could be used to detect changes in average membrane potential of nonexcitable cells that would occur for example in the presence of changes to channel permeability due to toxin binding.

Cell survival can also be related to ion channel activity, and colorimetric or fluorescence-based cell viability assays using mammalian cell lines have been developed for ion-channel targets (Manger et al., 1993; Haugland 1999a).

Another possibility for the use of generic agents is in the alteration or delay of the tissue immune response to toxins (e.g., SEB or ricin) by modulating the action of mediators such as TNF-α and Il-1 (Takx-Köhlen, 1992). Despite the vast amount of information that has been accumulated regarding the armamentarium of the alveolar macrophage (AM), there is little known about how to suppress its release of secretory products. Glucocorticoids are widely used. Inhaled corticosteroids decrease TNF-α and Il-1 production by AM. Currently therapeutic strategies are directed toward removing or suppressing AM stimuli and/or acting at a more distal site in the consequent series of events. The latter may be achieved by suppressing the actions of the secretory products of AM, either by directly interacting with the mediator itself (e.g., antioxidants to suppress injury from oxidants) or by preventing the target cell from responding.

40.6 CONCLUSIONS AND FUTURE DIRECTIONS

The cellular and molecular biology of the lungs and their response to biological toxins should be a prime focus for therapy and prophylaxis investigations. Although the evaluation of possible therapeutic or prophylactic agents must include testing with a suitable animal model, initial screening could be carried out using cultured normal human lung cells.

Cell culture offers a number of advantages for drug evaluation over the use of live animals or of isolated tissues. Thus it offers:

- Human cells for testing.
- Increased control of the medium surrounding the cells.
- Sufficient material for experiments through *in vitro* propagation of cells if the starting material is limited (e.g., for ethical reasons), and
- Cryopreservation of cell lines to permit storage for use later.

Future research strategies with regard to therapy and prophylaxis against inhaled biological toxins, should include the use of cultured normal human lung cells (e.g., alveolar and small airway epithelial cells, endothelial cells, macrophages, and dendrocytes) and an appropriate array of cytotoxicity, cell proliferation, and cell function tests. These cells and test capabilities can be used to evaluate the

effectiveness of antibodies, antibody fragments, substituted dendrimers, aptamers, and other drugs as neutralizing agents for selected biological toxins.

This overall approach has the added benefit that it is also applicable to studies directed at the treatment of infections from those pathogenic bacteria (e.g., *B. anthracis, C. diphtheriae, V. cholerae*) where much of the symptomatology is mediated by secreted exotoxins (e.g., anthrax lethal toxin (Hanna, 1999), diphtheria toxin, and cholera enterotoxin). A further benefit of considering the use of derivatized dendritic polymers or aptamers in any studies is the possibility that these could be designed to bind to a wide range of bacteria and viruses and block their activity also.

ACKNOWLEDGMENTS

The author would like to acknowledge the necessary and most helpful support of PSL Library and Publications staff in the collection of material for the preparation of this review. The perspective placed on this gathered material represents the personal viewpoint of the author and does not necessarily reflect that of the Australian Defence Science and Technology Organization.

REFERENCES

Abraham, W.M., Bourdelais, A.J., Sabater, J.R., Ahmed, A., Lee, T.A., Serebriakov, I., and Baden, D.G., Airway responses to aerosolised brevetoxins in an animal model of asthma, *Am. J. Respir. Crit. Care Med.*, 171, 26–34, 2005.

Abrami, L., Liu, S., Cosson, P., Leppla, S.H., and Gisou van der Goot, F., Anthrax toxin triggers endocytosis of its receptor via a lipid raft-mediated clathrin-dependent process, *J. Cell Biol.*, 160, 321–328, 2003.

Adler, K.B., Fischer, B.M., Wright, D.T., Cohn, L.A., and Becker, S., Interactions between respiratory epithelial cells and cytokines: relationships to lung inflammation, *Ann. N. Y. Acad. Sci.*, 725, 128–145, 1994.

Ahnert-Hilger, G. and Bigalke, H., Molecular aspects of tetanus and botulinum neurotoxin poisoning, *Prog. Neurobiol.*, 46, 83–96, 1995.

Allenby, A.C., Brown, J.F., Clarke, J.L., Dewey, J., Griffiths, G.D., Mode, N.K., Rubidge, T., Scawin, J.W., Titball, R.W., and Upshall, D.G., The production, purification and toxicity of Clostridium perfringens alpha-toxin cloned into Escherichia coli, in *Minutes of The Technical Cooperation Program*, *Subgroup E – Technical Panel 1 Meeting of 3rd–5th May*, *1994*, Chemical & Biological Defence Establishment, Annex C (U), Porton Down, Salisbury, UK, 1994.

Alvarenga, L.M., Diniz, C.R., Granier, C., and Chavez-Olortegui, C. Induction of neutralizing antibodies against Tityus serrulatus scorpion toxins by immunization with a mixture of defined synthetic epitopes, *Toxicon*, 40, 89–95, 2002.

Andersson, J., Nagy, S., Bjork, L., Abrams, J., Holm, S., and Andersson, U., Bacterial toxin-induced cytokine production studied at the single-cell level, *Immunol. Rev.*, *127*, 69–96, 1992.

Arad, G., Levy, R., Hillman, D., and Kaempfer, R., Superantigen antagonist protects against lethal shock and defines a new domain for T-cell activation, *Nat. Med.*, 6, 414–421, 2000.

Arbibe, L., Koumanov, K., Vial, D., Rougeot, C., Faure, G., Havet, N., Longacre, S., Vargaftig, B.B., Bereziat, G., Voelker, D.R., Wolf, C., and Touqui, L., Generation of lyso-phospholipids from surfactant in acute lung injury is mediated by type-II phospholipase A2 and inhibited by a direct surfactant protein A-phospholipase A2 protein interaction, *J. Clin. Investig.*, 102, 1152–1160, 1998.

Arias, H.R. and Blanton, M.P., Alpha conotoxins, *Int. J. Biochem. Cell Biol.*, 32, 1017–1028, 2000.

Arnon, S.S., Schecter, R., Inglesby, T.V., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Fine, A.D., Hauer, J., Layton, M., Lillibridge, S., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T.M., Russell, P.K., Swerdlow, D.L., and Tonat, K., Botulinum toxin as a biological weapon. Medical and public health management, *JAMA*, 285, 1059–1070, 2001.

Ashcom, J.D. and Stiles, B.G., Characterization of alpha-conotoxin interactions with the nicotinic acetylcholine receptor and monoclonal antibodies, *Biochem. J.*, 328 (Pt 1), 245–250, 1997.

Baden, D.G., Marine food-borne dinoflagellate toxins, *Int. Rev. Cytol.*, 82, 99–150, 1983.

Baertschi, S.W., Raney, K.D., Stone, M.P., and Harris, T.M., Preparation of the 8,9-epoxide of the mycotoxin aflatoxin B_1: the ultimate carcinogenic species, *J. Am. Chem. Soc.*, 110, 7929–7931, 1988.

Baier, W., Loleit, M., Fischer, B., Jung, G., Neumann, U., Weiss, M., Weckesser, J., Hoffman, P., Bessler, W.G., and Mittenbuhler, K., Generation of antibodies directed against the low-immunogenic peptide-toxins microcystin-LR/RR and nodularin, *Int. J. Immunopharmacol.*, 22, 339–353, 2000.

Ball, R.W., Huie, J.M., and Coulombe, R.A.J., Comparative activation of aflatoxin B1 by mammalian pulmonary tissues, *Toxicol. Lett.*, 75, 119–125, 1995.

Ballantyne, B., Marrs, T.C., and Turner, P., Fundamentals of toxicology, in *General and Applied Toxicology*, abr. ed., Ballantyne, B., Marrs, T., and Turner, P., Eds., Macmillan Press Ltd., New York, 1995, pp. 3–38.

Barnes, P.J. and Adcock, I.M., Glucocorticoid receptors, in *The Lung: Scientific Foundations*, 2nd ed., Crystal, R.G., West, J.B., Weibel, E.R., Barnes, P.J., Eds., Lippincott-Raven, Philadelphia, 1997, pp. 37–55.

Becerril, B., Marangoni, S., and Possani, L.D., Toxins and genes isolated from scorpions of the genus tityus [Review], *Toxicon*, 35, 821–835, 1997.

Benton, B.J., Keller, S.A., Spriggs, D.L., Capacio, B.R., and Chang, F.C., Recovery from the lethal effects of saxitoxin: a therapeutic window for 4-aminopyridine (4-AP), *Toxicon*, 36, 571–588, 1998.

Bezdicek, P. and Crystal, R.G., Pulmonary macrophages, in *The Lung: Scientific Foundations*, 2nd ed., Crystal, R.G., West, J.B., Weibel, E.R., and Barnes, P.J., Eds., Lippincott-Raven: Philadelphia, 1997, pp. 859–875.

Bidard, J.N., Vijverberg, H.P., Frelin, C., Chungue, E., Legrand, A.M., Bagnis, R., and Lazdunski, M., Ciguatoxin is a novel type of Na+ channel toxin, *J. Biol. Chem.*, 259, 8353–8357, 1984.

Bide, R.W., Armour, S.J., and Lee, E., Estimation of Human Toxicity from Animal Inhalation Toxicity Data: 1. Minute Volume-Body Weight Relationships Between Animals and Man, Report DRES-SR 673 (U), Defence Research Establishment, Suffield, Canada, 1997.

Bide, R.W., Armour, S.J., and Yee, E., Allometric respiration/body mass data for animals to be used for estimates of inhalation toxicity to young adult humans, *J. Appl. Toxicol.*, 20, 273–290, 2000.

Bonnard, C., Lechner, J.F., Gerwin, B.I., Fujiki, H., and Harris, C.C., Effects of palytoxin or ouabain on growth and squamous differentiation of human bronchial epithelial cells in vitro, *Carcinogenesis*, 9, 2245–2249, 1988.

Bot, A.I., Tarara, T.E., Smith, D.J., Bot, S.R., Woods, C.M., and Weers, J.G., Novel lipid-based hollow-porous microparticles as a platform for immunoglobulin delivery to the respiratory tract, *Pharm. Res.*, 17, 275–283, 2000.

Bouhafs, R.K. and Jarstrand, C., Lipid peroxidation of lung surfactant by bacteria, *Lung*, 177, 101–110, 1999.

Bregenholt, S. and Haurum, J., Pathogen-specific recombinant human polyclonal antibodies: biodefence applications, *Expert Opin. Biol. Ther.*, 4, 387–396, 2004.

Brekke, O.H. and Sandlie, I., Therapeutic antibodies for human diseases at the dawn of the twenty-first century, *Nat. Rev. Drug Discov.*, 2, 52–62, 2003.

Brigotti, M., Alfieri, R., Sestili, P., Bonelli, M., Petronini, P.G., Guidarelli, A., Barbieri, L., Stirpe, F., and Sperti, S., Damage to nuclear DNA induced by Shiga toxin 1 and ricin in human endothelial cells, *FASEB J.*, 16, 365–372, 2002.

Brooke, J.S. and Cha, J.H., Molecular characterization of key diphtheria toxin: receptor interactions, *Biochem. Biophys. Res. Commun.*, 275, 374–381, 2000.

Brown, R.F. and White, D.E., Ultrastructure of rat lung following inhalation of ricin aerosol, *Int. J. Exp. Pathol.*, 78, 267–276, 1997.

Brüggemann, M., Caskey, H.M., Teale, C., Waldmann, H., Williams, G.T., Surani, M.A., and Neuberger, M.S., A repertoire of monoclonal antibodies with human heavy chains from transgenic mice, *Proc. Natl. Acad. Sci. USA*, 86, 6709–6713, 1989.

Bruno, J.G. and Kiel, J.L., Use of magnetic beads in selection and detection of biotoxin aptamers by electrochemiluminescence and enzymatic methods, *Biotechniques*, 32, 178–183, 2002.

Buchi, G., Spitzner, D., Paglialunga, S., and Wogan, G.N., Synthesis and toxicity evaluation of aflatoxin P, *Life Sci.*, 13, 1143–1149, 1973.

Bunger, J., Westphal, G., Monnich, A., Hinnendahl, B., Hallier, E., and Muller, M., Cytotoxicity of occupationally and environmentally relevant mycotoxins, *Toxicology*, 202, 199–211, 2004.

Byrd, D.M. and Cothern, C.R., *Introduction to Risk Analysis: A Systematic Approach to Science Based Decision Making*, Government Institutes. A division of ABS Groups Inc., MD, 2000.

Carmichael, W.W., The toxins of cyanobacteria, *Sci. Am.*, 270, 64–72, 1994.

Casadevall, A., Passive antibody administration (immediate immunity) as a specific defense against biological weapons, *Emerg. Infect. Dis.*, 8, 833–841, 2002.

Catterall, W.A., Structure and function of voltage-sensitive ion channels, *Science*, 242, 50–61, 1988.

Center, D.M., Kornfeld, H., and Cruikshank, W.W., Interleukin-16, *Int. J. Biochem. Cell. Biol.*, 29, 1231–1234, 1997.

Chaddock, J.A., Herbert, M.H., Ling, R.J., Alexander, F.C., Fooks, S.J., Revell, D.F., Quinn, C.P., Shone, C.C., and Foster, K.A., Expression and purification of catalytically active, non-toxic endopeptidase derivatives of Clostridium botulinum toxin type A. *Protein Expr. Purif.*, 25, 219–228, 2002.

Choy, W.N., A review of the dose-response induction of DNA adducts by aflatoxin B1 and its implications to quantitative cancer-risk assessment, *Mutat. Res.*, 296, 181–198, 1993.

Collier, R.J., Diphtheria toxin: mode of action and structure, *Bacteriol. Rev.*, 39, 54–85, 1975.

Cook, W.O., Beasley, V.R., Dahlem, A.M., Dellinger, J.A., Harlin, K.S., Carmichael, W.W., Comparison of effects of anatoxin-a(s) and paraoxon, physostigmine and pyridostigmine on mouse brain cholinesterase activity, *Toxicon*, 26, 750–753, 1988.

Creasia, D.A., Thurman, J.D., Jones, L.J., Nealley, M.L., York, C.G., Wannemacher, R.W., and Bunner, D.L., Acute inhalation toxicity of T-2 mycotoxin in mice, *Fundam. Appl. Toxicol.*, 8, 230–235, 1987.

Creasia, D.A., Thurman, J.D., Wannemacher, R.W., and Bunner, D.L., Acute inhalation toxicity of T-2 mycotoxin in the rat and guinea pig, *Fundam. Appl. Toxicol.*, 14, 54–59, 1990.

Crosland, R.D., Effect of drugs on the lethality in mice of the venoms and neurotoxins from sundry snakes, *Toxicon*, 29, 613–631, 1991.

Cross, C.E., Eiserich, J.P., and Halliwell, B., General biological consequences of inhaled environmental toxicants, in *The Lung: Scientific Foundations*, 2nd ed., Crystal, R.G., West, J.B., Weibel, E.R., Barnes, P.J., Eds., Lippincott-Raven, Philadelphia, 1997, pp. 2421–2437.

Crystal, R.G., West, J.B., Weibel, E.R., and Barnes, J.B., Eds., *The Lung: Scientific Foundations*, 2nd ed., Lippincott-Raven, Philadelphia, 1997.

Cunningham, A.C., Zhang ,J.G., Moy, J.V., Ali, S., and Kirby, J.A., A comparison of the antigen-presenting capabilities of class II MHC-expressing human lung epithelial and endothelial cells, *Immunology*, 91, 458–463, 1997.

Dawson, R.M., The toxicology of microcystins, *Toxicon*, 36, 953–962, 1998.

Dawson, R.M., Paddle, B.M., and Alderton, M.R., Characterization of the asialofetuin microtitre plate-binding assay for evaluating inhibitors of ricin lectin activity, *J. Appl. Toxicol.*, 19, 307–312, 1999.

Delmas, A., Gruaz, G., Pedoussaut, S., Pierre, P., Rivaille, P., and Vaerman, J.P., Neutralization of cholera toxin by rat IgA secretory antibodies induced by a free synthetic peptide, *Biochem. Biophys. Res. Commun.*, 159, 707–712, 1989.

Denyer, J., Worley, J., Cox, B., Allenby, G., and Banks, M., HTS approaches to voltage-gated ion channel drug discovery, *Drug Discovery Today*, 3, 323–332, 1998.

Derelanko, M.J., Risk assessment, in *CRC Handbook of Toxicology*, Derelanko, M.J. and Hollinger, M.A., Eds., CRC Press Inc, New York, 1995, pp. 591–676.

Dodds, D.C., Omeis, I.A., Cushman, S.J., Helms, J.A., and Perin, M.S., Neuronal pentraxin receptor, a novel putative integral membrane pentraxin that interacts with neuronal pentraxin 1 and 2 and taipoxin-associated calcium-binding protein 49, *J. Biol. Chem.*, 272, 21488–21494, 1997.

Doebler, J.A., Wiltshire, N.D., Mayer, T.W., Estep, J.E., Moeller, R.B., Traub, R.K., Broomfield, C.A., Calamaio, C.A. Thompson, W.L., and Pitt, M.L., The distribution of [^{125}I]ricin in mice following aerosol inhalation exposure, *Toxicology*, 98, 137–149, 1995.

Dumbacher, J.P., Spande, T.F., and Daly, J.W., Batrachotoxin alkaloids from passerine birds: a second toxic bird genus (Ifrita kowaldi) from New Guinea, *Proc. Natl. Acad. Sci. USA*, 97, 12970–12975, 2000.

Edwards, D.A., Hanes, J., Caponetti, G., Hrkach, J., Ben-Jebria, A., Eskew, M.L., Mintzes, J., Deaver, D., Lotan, N., and Langer, R., Large porous particles for pulmonary drug delivery, *Science*, 276, 1868–1871, 1997.

Eiklid, K. and Olsnes, S., Animal toxicity of Shigella dysenteriae cytotoxin: evidence that the neurotoxic, enterotoxic, and cytotoxic activities are due to one toxin,. *J. Immunol.*, 130, 380–384, 1983.

Ellington, A.D. and Szostak, J.W., In vitro selection of RNA molecules that bind specific ligands, *Nature*, 346, 818–822, 1990.

Ellison, D.H., *Handbook of Chemical and Biological Warfare Agents*, CRC Press Inc., New York, 1999, pp. 184–191.

Endo, Y., Mitsui, K., Motizuki, M., and Tsurugi, K., The mechanism of action of ricin and related toxic lectins on eukaryotic ribosomes. The site and the characteristics of the modification in 28 S ribosomal RNA caused by the toxins, *J. Biol. Chem.*, 262, 5908–5912, 1987.

Endo, Y., Tsurugi, K., Yutsudo, T., Takeda, Y., Ogasawara, T., and Igarashi, K., Site of action of a Vero toxin (VT2) from Escherichia coli O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins,. *Eur. J. Biochem.*, 171, 45–50, 1988.

Famulok, M., Oligonucleotide aptamers that recognize small molecules, *Curr. Opin. Struct. Biol.*, 9, 324–329, 1999.

Famulok, M., Mayer, G., and Blind, M., Nucleic acid aptamers-from selection in vitro to applications in vivo,. *Acc. Chem. Res.*, 33, 591–599, 2000.

Fleming, L.E. and Stephan, W., Blue-Green Algae, Their Toxins and Public Health Issues, Report to the Florida Harmful Algal Bloom Taskforce, NIEHS Marine and Freshwater Biomedical Sciences Center, University of Miami, Miami, FL, 2001.

Fodstad, O. Olsnes, S., and Pihl, A., Toxicity, distribution and elimination of the cancerostatic lectins abrin and ricin after parenteral injection into mice, *Br. J. Cancer*, 34, 418–425, 1976.

Fohlman, J., Eaker, D., Karlsoon, E., and Thesleff, S., Taipoxin, an extremely potent presynaptic neurotoxin from the venom of the Australian snake taipan (Oxyuranus s. scutellatus). Isolation, characterization, quaternary structure and pharmacological properties, *Eur. J. Biochem.*, 68, 457–469, 1976.

Fotinou, C., Emsley, P., Black, I., Ando, H., Ishida, H., Kiso, M., Sinha, K.A., Fairweather, N.F.,and Isaacs, N.W., The crystal structure of tetanus toxin Hc fragment complexed with a synthetic GT1b analogue suggests cross-linking between ganglioside receptors and the toxin,. *J. Biol. Chem.*, 276, 32274–32281, 2001.

Franz, D.R., Defense against toxin weapons, in *Textbook of Military Medicine. Part 1: Medical Aspects of Chemical and Biological Warfare*, Zajtchuk R. and Bellamy, R.F., Eds., Office of the Surgeon General, Department of the Army, USA, 1997, pp. 603–619.

Frelin, C. and Van Renterghem, C., Palytoxin. Recent electrophysiological and pharmacological evidence for several mechanisms of action, *Gen. Pharmacol.*, 26, 33–37, 1995.

Fu, J., Fiegel, J., Krauland, E., and Hanes, J., New polymeric carriers for controlled drug delivery following inhalation or injection, *Biomaterials*, 23, 4425–4433, 2002.

Fujii, N., Kimura, K., Yokosawa, N., Tsuzuki, K., and Oguma, K., The zinc-protease specific domain in botulinum and tetanus neurotoxins, *Toxicon*, 30, 1486–1488, 1992.

Garcia, J.G.N., Wang, P., Schaphorst, K.L., Becker, P.M., Borbiev, T., Liu, F., Birukova, A., Jacobs, K., Bogatcheva, N., and Verin, A.D., Critical involvement of p38 MAP kinase in pertussis toxin-induced cytoskeletal reorganization and lung permeability, *FASEB J.*, 16, 1064–1076, 2002.

Garred, O., van Deurs, B., and Sandvig, K., Furin-induced cleavage and activation of Shiga toxin, *J. Biol. Chem.*, 270, 10817–10821, 1995.

Gehrmann, J., Alewood, P.F., and Craik, D.J., Structure determination of the three disulfide bond isomers of alpha-conotoxin GI: a model for the role of disulfide bonds in structural stability, *J. Mol. Biol.*, 278, 401–415, 1998.

Gill, D.M., Bacterial toxins: a table of lethal amounts, *Microbiol. Rev.*, 46, 86–94, 1982.

Glasgow, H.B.J., Burkholder, J.M., Schmechel, D.E., Tester, P.A., and Rublee, P.A., Insidious effects of a toxic estuarine dinoflagellate on fish survival and human health, *J. Toxicol. Environ. Health*, 46, 501–522, 1995.

Gordon, T. and Amdur, M.O., Responses of the respiratory system to toxic agents, in *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 4th ed., Klaassen, C.D., Amdur, M.O., and Doull, J., Eds., McGraw-Hill, New York, 1991, pp. 383–406.

Grangette, C., Muller, A., Goudercourt, D., Geoffroy, M.C., Turneer, M., and Mercenier, A., Mucosal immune responses and protection against tetanus toxin after intranasal immunization with recombinant Lactobacillus plantarum, *Infect. Immun.*, 69, 1547–1553, 2001.

Griffiths, G.D., Lindsay, D., Rice, P., and Upshall, D.G., The toxicology of ricin and abrin toxins. *In vivo* and *in vitro* studies, in Minutes of the Technical Cooperation Program, Subgroup E – AG37 Meeting of 21–22 September, 1992, Defence Research Establishment Suffield, Alberta, Canada; Annex C (U), 1992.

Griffiths, G.D., Rice, P., Allenby, A.C., Bailey, S.C., and Upshall, D.G., Inhalation toxicology and histopathology of ricin and abrin toxins, *Inhalation Toxicol.*, 7, 269–288, 1995.

Gusovsky, F., Yasumoto, T., and Daly, J.W., Maitotoxin, a potent, general activator of phosphoinositide breakdown, *FEBS Lett.*, 243, 307–312, 1989.

Gutierrez, B., Vilumara, A., and Farre, A.J., Inhibition of aconitine-induced mortality in the conscious rat: a screening test for antiarrhythmic drugs, *Methods Find Exp. Clin. Pharmacol.*, 9, 307–310, 1987.

Gutierrez, D., Diaz, de L., and Vaca, L., Characterization of the maitotoxin-induced calcium influx pathway from human skin fibroblasts, *Cell Calcium*, 22, 31–38, 1997.

Habermann, E., Hudel, M., and Dauzenroth, M.E., Palytoxin promotes potassium outflow from erythrocytes, HeLa and bovine adrenomedullary cells through its interaction with Na^+, K^+ -ATPase, *Toxicon*, 27, 419–430, 1989.

Hadfield, T.L., McEvoy, P., Polotsky, Y., Tzinserling, V.A., and Yakovlev, A.A., The pathology of diphtheria, *J. Infect. Dis.*, 181, S116–S120, 2000.

Hamilton, M.G., *Toxins. The Emerging Threat*, Defence Research Establishment Suffield, Canada, Special Publication, SSP 184 (U), 1996.

Hance, A.J,. Accessory-cell-lymphocyte interactions, in *The Lung: Scientific Foundations*, 2nd ed., Crystal, R.G., West, J.B., Weibel, E.R., Barnes, P.J., Eds., Lippincott-Raven, Philadelphia, 1997, pp. 821–839.

Hanna, P., Lethal toxin actions and their consequences, *J. Appl. Microbiol.*, 87, 285–287, 1999.

Harris, J.B. and Maltin, C.A., Myotoxic activity of the crude venom and the principal neurotoxin, taipoxin, of the Australian taipan, Oxyuranus scutellatus, *Br. J. Pharmacol.*, 76, 61–75, 1982.

Haugland, R.P., Assays for Cell Viability, Proliferation and Function, in *Handbook of Fluorescent Probes and Research Chemicals*, 7th ed., Chap. 15, Molecular Probes Inc., Eugene, OR, 1999a.

Haugland, R.P., Indicators for Na^+, K^+, Cl^- and other anions, in *Handbook of Fluorescent Probes and Research Chemicals*, 7th ed., Chap. 22, Molecular Probes Inc., Eugene, OR, 1999b.

Haugland, R.P., Probes for membrane potential, in *Handbook of Fluorescent Probes and Research Chemicals*, 7th ed., Chap. 23, Molecular Probes Inc., Eugene, OR, 1999c.

Henghold, W.B. II, Other biologic toxin bioweapons: ricin, staphylococcal enterotoxin B, and trichothecene mycotoxins, *Dematol. Clin.*, 22, 257–262, 2004.

Herman, A., Kappler, J.W., Marrack, P., and Pullen, A.M., Superantigens: mechanism of T-cell stimulation and role in immune responses, *Annu. Rev. Immunol.*, 9, 745–772, 1991.

Hermanson, G., Whitlow, V., Parker, S., Tonsky, K., Rusalov, D., Ferrari, M., Lalor, P., Komai, M., Mere, R., Bell, M., Brenneman, K., Mateczun, A., Evans, T., Kaslow, D., Galloway, D., and Hobart, P., A cationic lipid-formulated plasmid DNA vaccine confers sustained antibody-mediated protection against aerosolized anthrax spores, *Immunology*, 101, 13601–13606, 2004.

Herreros, J., Lalli, G., and Schiavo, G., C-terminal half of tetanus toxin C is sufficient for neuronal binding and interaction with a putative protein receptor, *Biochem. J.*, 347 (Pt 1), 199–204, 2000.

Herreros, J. Ng, T., and Schiavo, G., Lipid rafts act as specialized domains for tetanus toxin binding and internalisation into neurons, *Mol. Biol. Cell*, 12, 2947–2960, 2001.

Hesselberth, J.R., Miller, D., Robertus, J., and Ellington, A.D., In vitro selection of RNA molecules that inhibit the activity of ricin A-chain, *J. Biol. Chem.*, 275, 4937–4942, 2000.

Hirao, I., Madin, K., Endo, Y., Yokoyama, S., and Ellington, A.D., RNA aptamers that bind to and inhibit the ribosome-inactivating protein, pepocin, *J. Biol. Chem.*, 275, 4943–4948, 2000.

Holley, J.L., Elmore, M., Mauchline, M., Minton, N., and Titball, R.W., Cloning, expression and evaluation of a recombinant sub-unit vaccine against Clostridium botulinum type F toxin, *Vaccine*, 19, 288–297, 2000.

Holmes, M.J. and Lewis, R.J., Purification and characterisation of large and small maitotoxins from cultured Gambierdiscus toxicus, *Nat. Toxins*, 2, 64–72, 1994.

Holt, P.G., Haining, S., Nelson, D.J., and Sedgwick, J.D., Origin and steady-state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways, *J. Immunol.*, 153, 256–261, 1994.

Holtzman, M.J., Arachidonic acid metabolism in airway epithelial cells, *Annu. Rev. Physiol.*, 54, 303–329, 1992.

Houdebine, L.M., Transgenic animal bioreactors, *Transgenic Res.*, 9, 305–320, 2000.

Hughes, J.N., Lindsay, C.D., and Griffiths, G.D., Morphology of ricin and abrin exposed endothelial cells is consistent with apoptotic cell death, *Hum. Exp. Toxicol.*, 15, 443–451, 1996.

Ishida, I., Tomizuka, K., Yoshida, H., Tahara, T., Takahashi, N., Ohguma, A., Tanaka, S., Umehashi, M., Maeda, H., Nozaki, C., Halk, A., and Lonberg, N., Production of human monoclonal and polyclonal antibodies in TransChromo animals, *Cloning Stem Cells*, 4, 91–102, 2002.

Ishikawa, S., Kawahara, K., Kagami, Y., Isshiki, Y., Kaneko, A., Matsui, H., Okada, N., and Danbara, H., Protection against Shiga toxin 1 challenge by immunization of mice with purified mutant Shiga toxin 1, *Infect. Immun.*, 71, 3235–3239, 2003.

Jacewicz, M., Clausen, H., Nudelman, E., Donohue, R., Keusch, G.T., Pathogenesis of shigella diarrhea. XI. Isolation of a shigella toxin-binding glycolipid from rabbit jejunum and HeLa cells and its identification as globotriaosylceramide, *J. Exp. Med.*, 163, 1391–1404, 1986.

Janeway, C.A., Jr. and Travers, P., *Immunobiology: The Immune System in Health and Disease*, Blackwell Scientific Publications, Oxford, 1994, pp. 8.20–8.28.

Jennings, G.S., Heck, R., Oesch, .F, and Steinberg, P., Metabolism and cytotoxicity of aflatoxin B1 in cultured rat hepatocytes and nonparenchymal cells: Implications for tumorigenesis, *Toxicol. Appl. Pharmacol.*, 129, 86–94, 1994.

Kacprzak, M.M., Peinado, J.R., Than, M.E., Appel, J., Henrich, S., Lipkind, G., Houghten, R.A., Bode, W., and Lindberg, I., Inhibition of furin by polyarginine-containing peptides. Nanomolar inhibition by nona-D-arginine, *J. Biol. Chem.*, 279, 36788–36794, 2004.

Kaul, P.N., Farmer, M.R., and Ciereszko, L.S., Pharmacology of palytoxin. Most potent marine toxin, *Proc. West. Pharmacol. Soc.*, 17, 294–301, 1974.

Kelly, J.D., Eaton, D.L., Guengerich, F.P., and Coulombe, R.A., Aflatoxin B-1 activation in human lung, *Toxicol. Appl. Pharmacol.*, 144, 88–95, 1997.

Keren, D.F., Brown, J.E., McDonald, R.A., and Wassef, J.S., Secretory immunoglobulin A response to Shiga toxin in rabbits: kinetics of the initial mucosal immune response and inhibition of toxicity in vitro and in vivo, *Infect. Immun.*, 57, 1885–1889, 1989.

Khachatourians, G.G., Metabolic effects of trichothecene T-2 toxin, *Can. J. Physiol. Pharmacol.*, 68, 1004–1008, 1990.

Kirby, J.E., Anthrax lethal toxin induces human endothelial cell apoptosis, *Infect. Immun.*, 72, 430–439, 2004.

Kim, K.J. and Malik, A.B., Protein transport across the lung epithelial barrier, *Am. J. Physiol.*, 284, L247–L259, 2003.

Kitov, P.I., Sadowska, J.M., Mulvey, G., Armstrong, G.D., Ling, H., Pannu, N.S., Read, R.J., and Bundle, D.R., Shiga-like toxins are neutralized by tailored multivalent carbohydrate ligands, *Nature*, 403, 669–672, 2000.

Klimpel, K.R., Molloy, S.S., Thomas, G., and Leppla, S.H., Anthrax toxin protective antigen is activated by a cell-surface protease with the sequence specificity and catalytic properties of furin, *Proc. Natl. Acad. Sci. USA*, 89, 10277–10281, 1992.

Knight, P.A., Queminet, J., Blanchard, J.H., and Tilleray, J.H., In vitro tests for the measurement of clostridial toxins, toxoids and antisera. II. Titration of Clostridium perfringens toxins and antitoxins in cell culture, *Biologicals*, 18, 263–270, 1990.

Kodama, M., Sato, S., Sakamoto, S., and Ogata, T., Occurrence of tetrodotoxin in Alexandrium tamarense, a causative dinoflagellate of paralytic shellfish poisoning, *Toxicon*, 34, 1101–1105, 1996.

Komatsu, N., Nakagawa, M., Oda, T., and Muramatsu, T., Depletion of intracellular NAD^+ and ATP levels during ricin-induced apoptosis through the specific ribosomal inactivation results in the cytolysis of U937 cells, *J. Biochem.*, 128, 463–470, 2000.

Kozaki, S., Kamata, Y., Watarai, S., Nishiki, T., and Mochida, S., Ganglioside GT1b as a complementary receptor component for Clostridium botulinum neurotoxins, *Microb. Pathog.*, 25, 91–99, 1998.

Krakauer, T., Stimulant-dependent modulation of cytokines and chemokines by airway epithelial cells: Cross talk between pulmonary epithelial and peripheral blood mononuclear cells, *Clin. Diagn. Lab. Immunol.*, 9, 126–131, 2002.

Krieglstein, K.G., DasGupta, B.R., and Henschen, A.H., Covalent structure of botulinum neurotoxin type A: Location of sulfhydryl groups, and disulfide bridges and identification of C-termini of light and heavy chains, *J. Protein Chem.*, 13, 49–57, 1994.

Kwon, O.J., Au, B.T., Collins, P.D., Baraniuk, J.N., Adcock, I.M., Chung, K.F., and Barnes, P.J., Inhibition of interleukin-8 expression by dexamethasone in human cultured airway epithelial cells, *Immunology*, 81, 389–394, 1994.

Labiris, N.R. and Dolovich, M.B., Pulmonary drug delivery. Part II: The role of inhalant delivery devices and drug formulations in the therapeutic effectiveness of aerosolised medications, *Br. J. Clin. Pharmacol.*, 56, 600–612, 2003.

LaFont, F., Abrami, L., and Gisou van der Goot, F., Bacterial subversion of lipid rafts, *Curr. Opin. Microbiol.*, 7, 4–10, 2004.

Laird, M.W., Zukauskas, D., Johnson, K., Sampey, G.C., Olsen, H., Garcia, A., Karwoski, J.D., Cooksey, B.A., Choi, G.H., Askins, J., Tsai, A., Pierre, J., and Gwinn, W., Production and purification of Bacillus anthracis protective antigen from Escherichia coli, *Protein Expr. Purif.*, 38, 145–152, 2004.

LeClaire, R.D., Hunt, R.E., Bavari, S., Estep, J.E., Nelson, G.O., and Wilhelmson, C.L., Potentiation of inhaled Staphylococcal enterotoxin B-induced toxicity by lipopolysaccharide in mice, *Toxicol. Pathol.*, 24, 619–626, 1996a.

LeClaire, R.D., Kell, W., Bavari, S., Smith, T.J., and Hunt, R.E., Protective effects of niacinamide in staphylococcal enterotoxin-B-induced toxicity, *Toxicology*, 107, 69–81, 1996b.

LeClaire, R.D., Hunt, R.E., and Bavari, S., Protection against bacterial superantigen Staphylococcal enterotoxin B by passive vaccination, *Infect. Immun.*, 70, 2278–2281, 2002.

Lecomte, C., Ferrat, G., Fajloun, Z., Van Rietschoten, J., Rochat, H., Martin, E., Darbon, H., and Sabatier, J.M., Chemical synthesis and structure-activity relationships of Ts kappa, a novel scorpion toxin acting on apamin-sensitive SK channel, *J. Pept. Res.*, 54, 369–376, 1999.

Legrand, A.M., Ciguatera toxins: origin, transfer, through the food chain and toxicity to humans, in *Proceedings of the 8th International Conference on Harmful Algae*, *Vigo*, *Spain*, *25–29 June*, *1997*, Ruguera, J., Blanco, M.L., Fernandez, M.L., and Wyatt, T., Eds., Xunta de Galicia, Spain, and The Intergovernmental Oceanographic Commission (IOC), 1998, pp. 39–43.

Leppla, S.H., Robbins, J.B., Schneerson, R., and Shiloach, J., Development of an improved vaccine for anthrax, *J. Clin. Investig.*, 110, 141–144, 2002.

Levin, E.D., Blackwelder ,W.P., Glasgow, H.B., Jr., Burkholder, J.M., Moeller, P.D., and Ramsdell, J.S., Learning impairement caused by a toxin produced by Pfiesteria piscicida infused into the hippocampus of rats, *Neurotoxicol. Teratol.*, 25, 419–426, 2003.

Lewis, P.J. and Babiuk, L.A., DNA vaccines: a review, *Adv. Virus Res.*, 54, 129–188, 1999.

Li, H., Llera, A., Tsuchiya, D., Leder, .L, Ysern, X., Schlievert, P.M., Karjalainen, K., and Mariuzza, R.A., Three-dimensional structure of the complex between a T cell receptor beta chain and the superantigen staphylococcal enterotoxin B, *Immunity*, 9, 807–816, 1998.

Li, L. and Holian, A., Acrolein: a respiratory toxin that suppresses pulmonary host defense, *Rev. Environ. Health*, 13, 99–108, 1998.

Li, L. and Singh, B.R., Isolation of synaptotagmin as a receptor for types A and E botulinum neurotoxin and analysis of their comparative binding using a new microtiter plate assay, *J. Nat. Toxins*, 7, 215–226, 1998.

Li, Y., Foran, P., Fairweather, N.F., de Paiva, A., Weller, U., Dougan, G., and Dolly. J.O., A single mutation in the recombinant light chain of tetanus toxin abolishes its proteolytic activity and removes the toxicity seen after reconstitution with native heavy chain, *Biochemistry*, **33,** 7014–7020, 1994.

Lipkind, G.M. and Fozzard, H.A., A structural model of the tetrodotoxin and saxitoxin binding site of the Na+ channel, *Biophys. J.*, 66, 1–13, 1994.

Lipps, B.V., Isolation of subunits, alpha, beta and gamma of the complex taipoxin from the venom of Australian taipan snake (Oxyuranus s. scutellatus): characterization of beta taipoxin as a potent mitogen, *Toxicon*, **38,** 1845–1854, 2000.

Liu, B.H., Yu, F.Y., Chan, M.H., and Yang, Y.L., The effects of mycotoxins, fumonisin B1 and aflatoxin B1, on primary swine alveolar macrophages, *Toxicol. Appl. Pharmacol.*, 180, 197–204, 2002.

Lobo, E.D., Hansen, R.J., Balthasar, J.P., Antibody pharmacokinetics and pharmacodynamics, *J. Pharm. Sci.*, 93, 2645–2668, 2004.

Lombet, A., Bidard, J.N., and Lazdunski, M., Ciguatoxin and brevetoxins share a common receptor site on the neuronal voltage-dependent Na^+ channel, *FEBS Lett.*, 219, 355–359, 1987.

Lombry, C., Edwards, D.A., Preat, V., and Vanbever, R., Alveolar macrophages are a primary barrier to pulmonary absorption of macromolecules, *Am. J. Physiol. Lung Cell. Mol. Physiol.*, 286, L1002–L1008, 2004.

London, E., Diphtheria toxin: membrane interaction and membrane translocation, *Biochim. Biophys. Acta*, 1113, 25–51, 1992.

Lubman, R.L., Kim, K.J., and Crandall, E.D., Alveolar epithelial barrier properties, in *The Lung: Scientific Foundations*, 2nd ed., Crystal, R.G., West, J.B., Weibel, E.R., and Barnes, P.J., Eds., Lippincott-Raven, Philadelphia, 1997, 585–602.

Lyons, C.R., Lovchik, J., Hutt, J., Lipscomb, M.F., Wang, E., Heninger, S., Berliba, L., and Garrison, K., Murine model of pulmonary anthrax: Kinetics of dissemination, histopathology, and mouse strain susceptibility, *Infect. Immun.*, 72, 4801–4809, 2004.

Madsen, J.M., Toxins as weapons of mass destruction. A comparison and contrast with biological-warfare and chemical-warfare agents, *Clin. Lab. Med.*, 21, 593–605, 2001.

Manger, R.L., Leja, L.S., Lee, S.Y., Hungerford, J.M., and Wekell, M.M., Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels: semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins, *Anal. Biochem.*, 214, 190–194, 1993.

Marrs, T.C., Edginton, J.A., Price, P.N., and Upshall, D.G., Acute toxicity of T2 mycotoxin to the guinea-pig by inhalation and subcutaneous routes, *Br. J. Exp. Pathol.*, 67, 259–268, 1986.

Marshall, I.G. and Harvey, A.L., Selective neuromuscular blocking properties of alpha-conotoxins in vivo, *Toxicon*, 28, 231–234, 1990.

Matsunaga, S., Moore, R.E., and Niemczura, W.P., Anatoxin-a(s), a potent anticholinesterase from Anabaena flos-aquae, *J. Am. Chem. Soc.*, 111, 8021–8023, 1989.

McClellan, R.O., Risk assessment for inhaled toxicants, in *Concepts in Inhalation Toxicology*, 2nd ed., McClellan, R.O. and Henderson, R.F., Eds., Taylor and Francis, Washington, DC, 579–638, 1995.

Medaglini, D., Ciabattini, A., Spinosa, M.R., Maggi, T., Marcotte, H., Oggioni, M.R., and Pozzi, G., Immunization with recombinant Streptococcus gordonii expressing tetanus toxin fragment C confers protection from lethal challenge in mice, *Vaccine*, 19, 1931–1939, 2001.

Merck Index, 11th ed., Merck and Co. Inc., Rahway, NJ, 1989a, pp. 18–19.

Merck Index., 11th ed., Merck and Co. Inc., Rahway, NJ, 1989b, p. 30.

Miller, T.R. and Belas, R., Pfiesteria piscicida, P. shumwayae, and other Pfiesteria-like dinoflagellates, *Res. Microbiol.*, 154, 85–90, 2003.

Mitchell, J.P., Roberts, K.D., Langley, J., Koentgen, F., and Lambert, J.N., A direct method for the formation of peptide and carbohydrate dendrimers, *Bioorg. Med. Chem. Lett.*, 9, 2785–2788, 1999.

Miyata, S., Minami, J., Tamai, E., Matsushita, O., Shimamoto, S., and Okabe, A.. Clostridium perfringens ε-toxin forms a heptameric pore within the detergent-insoluble microdomains of Madin-Darby canine kidney cells and rat synaptosomes, *J. Biol. Chem.*, 277, 39463–39468, 2002.

Mock, M. and Mignot, T., Anthrax toxins and the host: a story of intimacy, *Cell Microbiol.*, 5, 15–23, 2003.

Mohamed, N., Clagett, M., Li, J., Jones, S., Pincus, S., D'Alia, G., Nardone, L., Babin, M., Spitalny, G., and Casey, L., A high-affinity monoclonal antibody to anthrax protective antigen passively protects rabbits before and after aerosolized Bacillus anthracis spore challenge, *Infect. Immun.*, 73, 795–802, 2005.

Montecucco, C. and Schiavo, G., Tetanus and botulism neurotoxins: a new group of zinc proteases, *Trends Biochem. Sci.*, 18, 324–327, 1993.

Montecucco, C. and Schiavo, G., Mechanism of action of tetanus and botulinum neurotoxins, *Mol. Microbiol.*, 13, 1–8, 1994.

Montecucco, C. and Schiavo, G., Structure and function of tetanus and botulinum neurotoxins, *Q. Rev. Biophys.*, 28, 423–472, 1995.

Moore, R.E. and Bartolini, G., Structure of palytoxin, *J. Am. Chem. Soc.*, 103, 2491–2494, 1981.

Mulvey, G.L., Marcato, P., Kitov, P.I., Sadowska, J., Bundle, D.R., and Armstrong, G.D., Assessment in mice of the therapeutic potential of tailored, multivalent Shiga toxin carbohydrate ligands, *J. Infect. Dis.*, 187, 640–649, 2003.

Murata, M., Naoki, H., Matsunaga, S., Satake, M., and Yasumoto, T., Structure and partial stereochemical assignments for maitotoxin, the most toxic and largest natural non-biopolymer, *J. Am. Chem. Soc.*, 116, 7098–7107, 1994.

Myers, C.W., Daly, J.W., and Malkin, B., A dangerously toxic new frog (*Phyllobates*) used by Emberá Indians of Western Columbia, with discussion of blowgun fabrication and dart poisoning, *Bull. Am. Museum Nat. Hist.*, 161, 311–365, 1978.

Nabi, I. and Le, P.U., Caveolae/raft-dependent endocytosis, *J. Cell Biol.*, 161, 673–677, 2003.

Namikoshi, M., Sivonen, K., Evans, W.R., Carmichael, W.W., Sun, F., Rouhiainen, L., Luukkainen, R., and Rinehart, K.L., Two new L-serine variants of microcystins-LR and -RR from Anabaena SP. strains 202 A1 and 202 A2, *Toxicon*, 30, 1457–1464, 1992.

Naseem, S.M., Hines, H.B., and Creasia, D.A., Effect of toxins on arachidonic acid metabolism in rat cultured pulmonary alveolar macrophages, *Biochem. Int.*, 19, 583–592, 1989.

Naseem, S.M. and Creasia, D.A., Oxidation of saxitoxin: detection and biological activity of its reaction products, *Biochem. Mol. Biol. Int.*, 33, 177–186, 1994.

Neumann, B., Engelhardt, B., Wagner, H., and Holzmann, B., Induction of acute inflammatory lung injury by staphylococcal enterotoxin B, *J. Immunol.*, 158, 1862–1871, 1997.

Newhouse, M.T. and Corkery, K.J., Aerosols for systemic delivery of macromolecules, *Respir. Care Clin. North Am.*, 7, 261–275, 2001.

Nicod, L.P., Pulmonary defence mechanisms, *Respiration*, 66, 2–11, 1999.

Nishikawa, K., Matsuoka, K., Kita, E., Okabe, N., Mitzuguchi, M., Hino, K., Miyazawa, S., Yamasaki, C., Aoki, J., Takashima, S., Yamakawa, Y., Nishijima, M., Terunuma, D., Kuzahara, H., and Natori, Y., A therapeutic agent with oriented carbohydrates for treatment of infections by Shiga toxin-producing Escherichia coli O157:H7, *Proc. Natl. Acad. Sci. USA*, 99, 7669–7674, 2002.

Nishiki, T., Kamata, Y., Nemoto, Y., Omori, A., Ito, T., Takahashi, M., and Kozaki, S., Identification of protein receptor for Clostridium botulinum type B neurotoxin in rat brain synaptosomes, *J. Biol. Chem.*, 269, 10498–10503, 1994.

Nowakowski, A,. Wang, C., Powers, D.B., Amersdorfer, P., Smith, T.J., Montgomery, V.A., Sheridan, R., Blake, R., Smith, L.A., and Marks, J.D., Potent neutralization of botulinum neurotoxin by recombinant oligoclonal antibody, *Proc. Natl. Acad. Sci. USA*, 99, 11346–11350, 2002.

Olsnes, S. and Pihl, A., Toxic lectins and related proteins, in *Molecular Action of Toxins and Viruses*, Cohen, P., van Heyningen, S., Eds., Elsevier Biomedical Press, Amsterdam, The Netherlands, 1982, pp. 51–105.

Olson, M.A. and Cuff, L., Free energy determinants of binding the rRNA substrate and small ligands to ricin A-chain, *Biophys. J.*, 76, 28–39, 1999.

Osborne, N.J.T., Webb, P.M., and Shaw, G.R., The toxins of Lyngbya majuscula and their human and ecological health effects, *Environ. Int.*, 27, 381–392, 2001.

Oyston, P.C., Payne, D.W., Havard, H.L., Williamson, E.D., and Titball, R.W., Production of a non-toxic site-directed mutant of Clostridium perfringens epsilon-toxin which induces protective immunity in mice, *Microbiology*, 144 (Pt 2), 333–341, 1998.

Paddle, B.M., Therapy and prophylaxis of inhaled biological toxins, *J. Appl. Toxicol.*, 23, 139–170, 2003.

Page, D. and Roy, R., Synthesis and biological properties of mannosylated starburst poly(amidoamine) dendrimers, *Bioconjug. Chem.*, 8, 714–723, 1997.

Park, J.B. and Simpson, L.L., Inhalational poisoning by Botulinum toxin and inhalation vaccination with its heavy chain component, *Infect. Immun.*, 71, 1147–1154, 2003.

Pearson, J.A., Tyler, M.I. Retson, K.V., and Howden, M.E., Studies on the subunit structure of textilotoxin, a potent presynaptic neurotoxin from the venom of the Australian common brown snake (Pseudonaja textilis). 3. The complete amino-acid sequences of all the sub-units, *Biochim. Biophys. Acta*, 1161, 223–229, 1993.

Peraica, M., Radiç, B., Luciç, A., and Pavloviç, M., Toxic effects of mycotoxins in humans, *Bull*, *World Health Org.*, 77, 754–766, 1999.

Petit, L., Maier, E., Gibert, M., Popoff, M.R., and Benz, R., Clostridium perfringens epsilon toxin induces a rapid change of cell membrane permeability to ions and forms channels in artificial lipid layers, *J. Biol. Chem.*, 276, 15736–15740, 2001.

Pierce, R.H., Red tide (Ptychodiscus brevis) toxin aerosols: a review, *Toxicon*, 24, 955–965, 1986.

Pierce, R.H., Henry, M.S., Blum, P.C., Lyons, J., Cheng, Y.S., Yazzie, D., and Zhou, Y., Brevetoxin concentrations in marine aerosol: human exposure levels during a Karenia brevis harmful algal bloom, *Bull. Environ. Contam. Toxicol.*, 70, 161–165, 2003.

Poli, M.A., Rivera, V.R., Pitt, M.L., and Vogel, P., Aerosolized specific antibody protects mice from lung injury associated with aerosolized ricin exposure, *Toxicon*, 34, 1037–1044, 1996.

Rao, S. and Sikdar, S.K., Modification of alpha subunit of RIIA sodium channels by aconitine, *Pflugers Arch.*, 439, 349–355, 2000.

Rennard, S.I., West, W.W., and Robbins, R.A., Epithelial cells in host defense, in *Pulmonary Defences*, Stockley, R.A., Ed., John Wiley and Sons Ltd., Chichester, UK, 1997, pp. 163–178.

Reynolds, H.Y., Integrated host defense against infections, in *The Lung: Scientific Foundations*, 2nd ed., Crystal, R.G., West, J.B., Weibel, E.R., and Barnes, P.J., Eds., Lippincott-Raven, Philadelphia, 2353–2365, 1997.

Robbins, A. and Rennard, S.I., Biology of airway epithelial cells, in *The Lung: Scientific Foundations*, 2nd ed., Crystal, R.G., West, J.B., Weibel, E.R., and Barnes, P.J., Eds., Lippincott-Raven, Philadelphia, 1997, 445–457.

Rogowski, R.S., Krueger, B.K., Collins, J.H., and Blaustein, M.P., Tityustoxin K alpha blocks voltage-gated noninactivating K+ channels and unblocks inactivating K+ channels blocked by alpha-dendrotoxin in synaptosomes, *Proc. Natl. Acad. Sci. USA*, 91, 1475–1479, 1994.

Roitt, I., Brostoff, J., and Male, D., *Immunology*, 3rd ed., Gower Medical Publishing, London, 1993a, p. 4.6.

Roitt, I., Brostoff, J., and Male, D., *Immunology*, 3rd ed., Gower Medical Publishing, London, 1993b, pp. 11.2–11.3.

Rosenstein, Y. and Lafarge-Frayssinet, C., Inhibitory effect of Fusarium T2-toxin on lymphoid DNA and protein synthesis, *Toxicol. Appl. Pharmacol.*, 70, 283–288, 1983.

Saint-Joanis, B., Garnier, T., and Cole, S.T., Gene cloning shows the alpha-toxin of Clostridium perfringens to contain both sphingomyelinase and lecithinase activities, *Mol. Gen. Genet.*, 219, 453–460, 1989.

Sakurai, J., Toxins of Clostridium perfringens, *Rev. Med. Microbiol.*, 6, 175–185, 1995.

Sallusto, F. and Lanzavecchia, A., Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha, *J. Exp. Med.*, 179, 1109–1118, 1994.

Sandvig, K., Olsnes, S., and Pihl, A., Binding, uptake and degradation of the toxic proteins abrin and ricin by toxin-resistant cell variants, *Eur. J. Biochem.*, 82, 13–23, 1978.

Sandvig, K. and van Deurs, B., Endocytosis, intracellular transport, and cytotoxic action of Shiga toxin and ricin, *Physiol. Rev.*, 76, 949–966, 1996.

Sarac, M.S., Peinado, J.R., Leppla, S.H., and Lindberg, I., Protection against anthrax toxaemia by hexa-D-arginine in vitro and in vivo, *Infect. Immun.*, 72, 602–605, 2004.

Schantz, E.J., Chemistry and biology of saxitoxin and related toxins, *Ann. N. Y. Acad. Sci.*, 479, 15–23, 1986.

Schengrund, C.L., Das Gupta, B.R., Thompson, J.P., and Ringler, N.J., Inhibition of the adherence of botulinum toxin serotype A to immobilized GT1b by multivalent oligosaccharide GT1b-derivatized dendrimers, *J. Neurochem.*, 69, S17A, 1997.

Schlesinger, R.B., Deposition and clearance of inhaled particles, in *Concepts in Inhalation Toxicology*, 2nd ed., McClellan, R.O. and Henderson, R.F., Eds., Taylor & Francis, Washington, DC, 1995, pp. 191–224.

Schnitzer, J.E., Liu, J., and Oh, P., Endothelial caveolae have the molecular transport machinery for vesicle budding, docking, and fusion including VAMP, NSF, SNAP, annexins, and GTPases, *J. Biol. Chem.*, 270, 14399–14404, 1995.

Schraufstatter, I.U. and Cochrane, C.G., Oxidants: Types, sources, and mechanisms, in *The Lung: Scientific Foundations*, 2nd ed., Crystal, R.G., West, J.B., Weibel, E.R., and Barnes, P.J., Eds., Lippincott-Raven, Philadelphia, 1997, pp. 2251–2258.

Shaw, D.M., Gaerthe, B., Leer, R.J., Van Der Stap, J.G., Smittenaar, C., Heijne Den Bak-Glashouwer, M., Thole, J E., Tielen, F.J., Pouwels, P.H., and Havenith, C.E., Engineering the microflora to vaccinate the mucosa: serum immunoglobulin G responses and activated draining cervical lymph nodes following mucosal application of tetanus toxin fragment C-expressing lactobacilli, *Immunology*, 100, 510–518, 2000.

Shortt, S.J., Titball, R.W., and Lindsay, C.D., An assessment of the in vitro toxicology of Clostridium perfringens type D epsilon-toxin in human and animal cells, *Hum. Exp. Toxicol.*, 19, 108–116, 2000.

Sibille, Y. and Reynolds, H.Y., Macrophages and polymorphonuclear neutrophils in lung defense and injury, *Am. Rev. Respir. Dis.*, 141, 471–501, 1990.

Simons, K. and Toomre, D., Lipid rafts and signal transduction, *Nat. Rev. Mol. Cell Biol.*, 1, 2000.

Simpson, L.L., Lautenslager, G.T., Kaiser, I.I., and Middlebrook, J.L., Identification of the site at which phospholipase A2 neurotoxins localize to produce their neuromuscular blocking effects, *Toxicon*, 31, 13–26, 1993.

Smallshaw, J.E., Firan, A., Fulmer, J.R., Ruback, S.L., Ghetie, V., and Vitetta, E.S., A novel recombinant vaccine which protects mice against ricin intoxication, *Vaccine*, 20, 3422–3427, 2002.

Smith, C.G. and Music, S.I., Pfiesteria in North Carolina. The medical inquiry continues, *N. C. Med. J.*, 59, 216–220, 1998.

Smith, L.A., Jensen, M.J., Montgomery, V.A., Brown, D.R., Ahmed, S.A., and Smith, T.J., Roads from vaccines to therapies, *Mov. Disord.*, 19, S48–S52, 2004.

Spiekermann, G.M., Finn, P.W., Ward, E.S., Dumont, J,.Dickinson, B.L., Blumberg, R.S., and Lencer, W.I., Receptor-mediated immunoglobulin G transport across mucosal barriers in adult life: Functional expression of FcRn in the mammalian lung, *J. Exp. Med.*, 196, 303–310, 2002.

Stedman's Medical Dictionary, 26th ed., Williams and Wilkins, Baltimore, 2000, p. 897.

Stockley, R.A., Ed., *Pulmonary Defences*, John Wiley & Sons Ltd., Chichester, England, 1997.

Stricklett, P.K., Hughes, A.K., Ergonul, Z., and Kohan, D.E., Molecular basis for up-regulation by inflammatory cytokines of shiga toxin 1 cytotoxicity and globotriaosylceramide expression, *J. Infect. Dis.*, 186, 976–982, 2002.

Su, M.J., Coulter, A.R., Sutherland, S.K., and Chang. C.C., The presynaptic neuromuscular blocking effect and phospholipase A2 activity of textilotoxin, a potent toxin isolated from the venom of the Australian brown snake, Pseudonaja textilis, *Toxicon*, 21, 143–151, 1983.

Swinker, M., Tester, P., Koltai Attix, D., Schmechel, D., Human health effects of exposure to Pfiesteria piscicida: a review, *Microbes Infect.* 4**,** 751–762, 2002.

Takx-Kohlen, B.C.M.J., Immunomodulators. Future prospects, *Pharm. Weekblad Scientific ed.*, 14, 245–252, 1992.

Tanaka, K.S., Chen, X.Y., Ichikawa, Y., Tyler, P.C., Furneaux, R.H., and Schramm, V.L., Ricin A-chain inhibitors resembling the oxycarbenium ion transition state, *Biochemistry*, **40,** 6845–6851, 2001.

Thompson, J.P. and Schengrund, C.L., Inhibition of the adherence of cholera toxin and the heat-labile enterotoxin of Escherichia coli to cell-surface GM1 by oligosaccharide-derivatized dendrimers, *Biochem. Pharmacol.*, 56, 591–597, 1998.

Titball, R.W., Bacterial phospholipases C, *Microbiol. Rev.*, 57, 347–366, 1993.

Todar, K.U., *Todar's Online Textbook of Bacteriology.* [Internet], Department of Bacteriology, University of Wisconsin-Madison, 2002. Available at http://www.textbookofbacteriology.net. [Accessed July 2002]

Toews, G.B., Pulmonary defense mechanisms, *Semin. Respir. Infect.*, 8, 160–167, 1993.

Tokuyama, T., Daly, J., and Witkop, B., The structure of batrachotoxin, a steroidal alkaloid from the Colombian arrow poison frog, Phyllobates aurotaenia, and partial synthesis of batrachotoxin and its analogs and homologs, *J. Am. Chem. Soc.*, 91, 3931–3938, 1969.

Tosteson, M.T., Halperin, J.A., Kishi, Y., and Tosteson, D.C., Palytoxin induces an increase in the cation conductance of red cells, *J. Gen. Physiol.*, 98, 969–985, 1991.

Tsuneoka, M., Nakayama, K., Hatsuzawa, K., Komada, M., Kitamura, N., and Mekada, E., Evidence for involvement of furin in cleavage and activation of diphtheria toxin, *J. Biol. Chem.*, 268, 26461–26465, 1993.

Tuerk, C. and Gold, L., Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase, *Science*, 249, 505–510, 1990.

Tzipori, S., Sheoran, A., Akiyoshi, D., Donohue-Rolfe, A., and Trachtman, H., Antibody therapy in the management of Shiga toxin-induced haemolytic uremic syndrome, *Clin. Microbiol. Rev.*, 17, 926–941, 2004.

Uchida, H., Kiyokawa, N., Taguchi, T., Horie, H., Fujimoto, J., and Takeda, T.. Shiga toxins induce apoptosis in pulmonary epithelium-derived cells, *J Infect. Dis.*, 180, 1902–1911, 1999.

Ueno, Y., Nakajima, M., Sakai, K., Ishii, K., and Sato, N., Comparative toxicology of trichothec mycotoxins: inhibition of protein synthesis in animal cells, *J. Biochem. (Tokyo)*, 74, 285–296, 1973.

Ueno, Y., Toxicological features of T-2 toxin and related trichothecenes, *Fundam. Appl. Toxicol.*, 4, S124–S132, 1984.

Ulrich, R.G., Sidell, S., Taylor, T.G., Wilhelmson, C.L., and Franz, D.R., Staphylococcal enterotoxin B and related pyrogenic toxins, in *Textbook of Military Medicine. Part 1. Medical Aspects of Chemical and Biological Warfare*, Zajtchuk, R. and Bellamy, R.F., Eds., Office of the Surgeon General, Department of the Army, USA, 1997, pp. 621–630.

Usami, M., Satake, M., Ishida, S., Inoue, A., Kan, Y., and Yasumoto, T., Palytoxin analogs from the dinoflagellate—Ostreopsis siamensis, *J. Am. Chem. Soc.*, 117, 5389–5390, 1995.

USAMRIID, Comparative lethality of selected toxins and chemical agents in laboratory mice, in *Medical Management of Biological Casualties. Handbook*, 3rd ed., Eitzen, E., Pavlin, J., Cieslak, T., Christopher, G., and Culpepper, R., Eds., U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD., Appendix C, 1998.

Van Dolah, F.M., Marine algal toxins: origins, health effects, and their increased occurrence, *Environ. Health Perspect.*, 108 (Suppl 1), 133–141, 2000.

Van Ness, B.G., Howard, J.B., and Bodley, J.W., ADP-ribosylation of elongation factor 2 by diphtheria toxin. Isolation and properties of the novel ribosyl-amino acid and its hydrolysis products, *J. Biol. Chem.*, 255, 10717–10720, 1980a.

Van Ness, B.G., Howard, J.B., and Bodley, J.W., ADP-ribosylation of elongation factor 2 by diphtheria toxin. NMR spectra and proposed structures of ribosyl-diphthamide and its hydrolysis products, *J. Biol. Chem.*, 255, 10710–10716, 1980b.

Van Vleet, T.R., Klein, P.J., and Coulombe, R.A., Metabolism and cytotoxicity of aflatoxin b1 in cytochrome P-450 expressing human lung cells, *J. Toxicol. Environ. Health A*, 65, 853–867, 2002.

Venant, A., Dazy, A.C., Diogene, G., Metezeau, P., and Marano, F., Effects of maitotoxin on calcium entry and phosphoinositide breakdown in the rabbit ciliated tracheal epithelium, *Biol. Cell*, 82, 195–202, 1994.

Vogel, P., Rivera, V.R., Pitt, M.L., and Poli, M.A., Comparison of the pulmonary distribution and efficacy of antibodies given to mice by intratracheal instillation or aerosol inhalation, *Lab. Anim. Sci.*, 46, 516–523, 1996**.**

Vogtle, F., Gestermann, S., Hesse, R., Schwierz, B., and Windisch, B., Functional dendrimers, *Prog. Polym. Sci.*, 25, 987–1041, 2000.

Wagner, S.D., Popov, A.V., Davies, S.L., Xian, J., Neuberger, M.S., and Bruggemann, M., The diversity of antigen-specific monoclonal antibodies from transgenic mice bearing human immunoglobulin gene miniloci, *Eur. J. Immunol.*, 24, 2672–2681, 1994.

Wang, S.Y. and Wang, G.K., Point mutations in segment I-S6 render voltage-gated Na^+ channels resistant to batrachotoxin, *Proc. Natl. Acad. Sci. USA*, 95, 2653–2658, 1998.

Ward, P.A., Role of complement, chemokines, and regulatory cytokines in acute lung injury, *Ann. N. Y. Acad. Sci.*, 796, 104–112, 1996.

Warren, J.R., Spero, L., Metzger, J.F., Stabilization of native structure by the closed disulfide loop of staphylococcal enterotoxin B, *Biochim. Biophys. Acta*, 359, 351–363, 1974.

Weibel, E.R., Design and morphometry of the pulmonary gas exchanger, in *The Lung: Scientific Foundations*, 2nd ed., Crystal, R.G. West, J.B., Weibel, E.R., and Barnes ,P.J., Eds., Lippincott-Raven, Philadelphia, 1997, pp.1147–1157.

Werkman, T.R., Gustafson, T.A., Rogowski, R.S., Blaustein, M.P., and Rogawski, M.A., Tityustoxin-K alpha, a structurally novel and highly potent K^+ channel peptide toxin, interacts with the alpha-dendrotoxin binding site on the cloned Kv1.2 K^+ channel, *Mol. Pharmacol.*, **44,** 430–436, 1993.

Wewers, M.D., Gadek, J.E., and Marsh, C.B., Pro-inflammatory cytokines, in *The Lung: Scientific Foundations*, 2nd ed., Crystal, R.G, West, J.B., Weibel, E.R., and Barnes, P.J., Eds., Lippincott-Raven, Philadelphia, 1997, pp. 117–132.

White, C.W. and Das, K.C., Role of cytokines in acute lung injury, in *The Lung: Scientific Foundations*, 2nd ed., Crystal, R.G., West, J.B., Weibel, E.R., and Barnes, P.J., Eds., Lippincott-Raven, Philadelphia, 1997, pp. 2451–2464.

WHO, Bases of quantitative estimates, in *Health Aspects of Chemical and Biological Weapons*, WHO, Geneva, 1970, pp. 84–100.

Wiberg, G.S. and Stephenson, N.R., Toxicologic studies on paralytic shellfish poison, *Toxicol. Appl. Pharmacol.*, 2**,** 607–615, 1960.

Wilhelmsen, C.L. and Pitt. M.L., Lesions of acute inhaled lethal ricin intoxication in rhesus monkeys, *Vet. Pathol.*,33, 296–302, 1996.

Woodward, R.B., Structure of tetrodotoxin, *Pure Appl*, *Chem.*, 9**,** 49–74, 1964.

Wu, A.M., Wu, J.H., Herp, A., Chow, L.P., and Lin, J,Y., Carbohydrate specificity of a toxic lectin, abrin A, from the seeds of Abrus precatorius (jequirity bean), *Life Sci***.,** 69, 2027–2038, 2001.

Xu, Q.H., Wei, C.H., Huang, K., and Rong, K.T., Toxin-neutralizing effect and activity-quality relationship for tetrodotoxin-specific polyclonal antibodies, *Toxicology*, 206, 439–448, 2005.

Yasumoto, T. and Satake, M., Chemistry, etiology and determination methods of ciguatera toxins, *J Toxicol. Toxin Rev.*,15, 91–107, 1996.

Yoshida, T., Sugiyama, T. Koide, N., Mori, I., and Yokochi, T., Human microvascular endothelial cells resist Shiga toxins by IFN-gamma treatment in vitro, *Microbiology*, 149, 2609–2614, 2003.

Yutsudo, T., Honda, T., Miwatani, T., and Takeda, Y., Characterization of purified Shiga toxin from Shigella dysenteriae 1, *Microbiol. Immunol.*, 30, 1115–1127, 1986.

Zambrano, F. and Canelo, E., Effects of microcystin-LR on the partial reactions of the Na^+—K^+ pump of the gill of carp (Cyprinus carpio Linneo), *Toxicon*, 34, 451–458, 1996.

Index