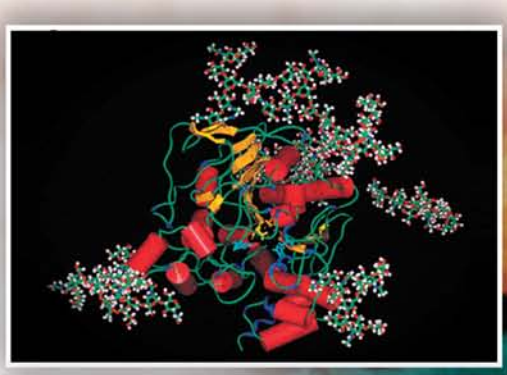
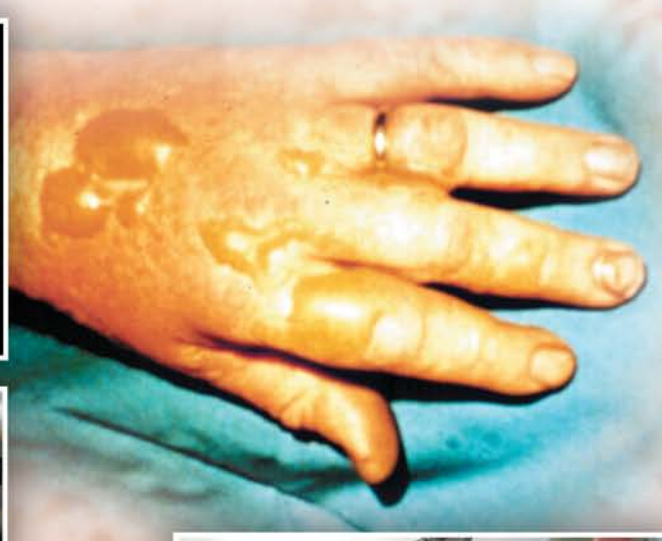
Second Edition

Chemical Warfare Agents

Chemistry, Pharmacology, Toxicology, and Therapeutics





Edited by

James A. Romano, Jr.
Brian J. Lukey
Harry Salem

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Dedication

The editors consider it a distinct honor to dedicate this book to the memory of our good friends and distinguished colleagues, Drs. Satu Somani and Brennie E. Hackley Jr.

Dr. Somani, professor of pharmacology and toxicology at Southern Illinois University (SIU) since 1974, was internationally recognized as a scholar and educator, as well as a pharmacologist and toxicologist. He was dedicated to research and teaching, as well as to his students and his native India. He was a driving force in the planning of the 35th Annual Conference of the Indian Pharmacology Society on ''Chemical and Biological Warfare'' (CBW). He edited two books in the area of CBW, including the first edition of this work with James Romano in 2001. He was particularly devoted to working with medical students in India in implementing problem-based learning. This philosophy, although new to India's medical schools, was a core philosophy at SIU. Conversely, he was eager to incorporate ayurvedic medicine into the medical pharmacology curriculum at SIU. Although Dr. Somani passed away on October 29, 2002, he remains a source of inspiration to the editors.

Dr. Brennie E. Hackley Jr. was chief scientist and scientific advisor to the commander of the United States Army Medical Research Institute of Chemical Defense. He authored or coauthored more than 75 publications and 15 U.S. patents. His publications and patents contributed significantly to the development of medical antidotes for chemical warfare agents. During his career, Dr. Hackley studied the relationship between chemical structures and chemotherapeutic activity with reference to efficacy against toxic agents. He contributed to the elucidation of mechanisms of reactions of nucleophiles with organophosphorus compounds. He synthesized a number of oximes, one of which was adopted as an antidote against chemical agents by the U.S. Air Force.

Dr. Hackley received numerous honors and commendations during 57 years of his continuous government service. He was an honorary life member of the American Chemical Society and fellow of the American Institute of Chemists. Dr. Hackley passed away on November 5, 2006.

The field of medical chemical defense will struggle to overcome the loss of Drs. Somani and Hackley, but in the end will prevail because of their legacy of scholarly effort and compassionate mentoring.

Contents

Preface

We previously published a book on chemical warfare agents (CWAs) in 2001. There have been many changes in this area in past years, driven by world events that have created a sense of urgency to this field. We believe it is time to update our previous work, citing the numerous developments in the field since 2001. We believe these to include epidemiological or clinical studies of exposed or potentially exposed populations, new treatment concepts and products, improved organization of the national response apparatus in the United States to address the potential for CWA terrorism, and improved diagnostic tests that enable rapid diagnosis and treatment. In the preface to our earlier work, we provided a rationale as to why an increased national investment had begun in the United States. That preface was written in January 2001.

As in our earlier work, we consider our chapter contributors to be experts who are recognized for their contributions to the science of toxic chemicals. Their contributions are summarized in the following paragraphs.

Salem, Ternay, and Smart tell us that chemicals have been used in warfare since almost the beginning of recorded history. Use of chemicals started out crudely using malodorous materials, irritants, poisonous plants and animals, as well as decaying bodies. Since the birth of chemistry, toxic chemicals have been created specifically for war. Lethal and disabling chemicals were developed, which incapacitated or killed the enemy without disfiguring or mutilating the body and without affecting or destroying the infrastructure. These chemicals appeared to offer distinct advantages. It is important to recognize that the advances in biotechnology, nanotechnology, genetic engineering, neurobiology, and computer sciences, among others, may assist not only in the proliferation of traditional CWAs, but also stimulate the emergence of nontraditional agents as well. Advances have also occurred in the delivery systems of these agents. The authors concluded that while the use of CWAs in terrorist activities appears to have been limited, this may not accurately reflect the potential of their future use.

Kikilo, Fedorenko, and Ternay provide an overview of the chemistry of selected substances that have been thought of as CWAs at one time or another. In general, this chapter is written from the perspective of an organic chemist. The authors begin with some general remarks regarding nomenclature and categories, followed by discussions of physical properties, synthesis, and chemical reactions. This chapter covers the chemistry of pulmonary (choking), asphyxiating (blood), nerve, and blister agents, as well as a brief discussion of incapacitating (one instance) and riot-control agents (one instance).

Salem, Whalley, Wick, Gargan, and Burrows point out that water supplies and their distribution systems are potential targets for terrorist activity in the United States. Even short-term disruption of water service can significantly impact a community and lead to serious medical, public health, societal, and economic consequences. In the United States, most of the water supply is treated and contains a disinfectant, such as free chlorine or chloramines, to destroy or control the growth of bacteria. Maintaining a residual free chlorine concentration of 0.5 mg/L for public water supplies, and 2.0 mg/L for field drinking water for deployed troops could provide adequate protection from most biological contamination. The authors considered chemical threats to the water supply and concluded that although it may be possible to contaminate a water supply system, a high degree of physical security, combined with maintaining a higher-than-usual residual chlorine level, should ensure its safety.

McDonough and Romano provide an update of their earlier contribution on ‘‘Health Effects of Low-Level Exposure to Nerve Agents.’’ They bring this area up-to-date by reviewing epidemiological or clinical studies of exposed or potentially exposed populations and new treatment

concepts and products to mitigate nerve agent toxicity. They point out the significant human epidemiological and clinical literature that has appeared since 2001. These studies are based on further follow-up of military volunteers from earlier research programs in the United States and the United Kingdom, and more intensive follow-up of the victims of Japanese terrorist attacks involving nerve agents in 1994 and 1995. They conclude by discussing four new potential ''product lines'' of improved treatment for these deadly nerve gases.

Van der Schans and Benschop present the rationale for toxicokinetic/toxicodynamic studies of nerve agents. They argue that toxicokinetic studies are of ultimate importance because the time period of the intoxication by nerve agents, perhaps contrary to earlier thinking, might span several hours. This suggests that the timing of the antidote administration has to be adapted to the toxicokinetic process. A section discussing the importance of distinguishing the stereoisomers of nerve agents precedes the discussion of the toxicokinetics of nerve agents. The distribution of sarin through tissues is discussed as part of a characterization of the elimination pathways of nerve agents. Finally, the toxicokinetics of soman in anesthetized, atropinized, and artificially ventilated, naive, and HuBuChE-pretreated guinea pigs were studied, demonstrating the utility of the toxicokinetic approach in evaluating the effectiveness of scavengers.

Sekowski and Dillman tell us that over the past several years, there has been a move away from a reductionist approach of studying one gene or protein at a time toward a more global approach of studying molecular and cellular networks and how these networks integrate information and respond to the environment. Recent technological developments allow researchers to study the function of a single gene or protein in the context of cellular and molecular networks, or to study the response of numerous genes or proteins to an environmental stimulus (e.g., CWA exposure). These new molecular techniques allow for global analysis of gene expression (genomics, transcriptomics), global analysis of protein expression, modification, function (proteomics), and global analysis of metabolism and metabolites (metabonomics, metabolomics). Within the Army life science research community, Sekowski and Dillman and many of their colleagues are following the lead of the pharmaceutical and biotechnology industries in applying these global approaches and associated technologies to the problem of CWA countermeasures. In this chapter, they provide an overview of each of these technologies and their current state-of-the-art, and provide examples of how these approaches are being applied to the development of CWA countermeasures.

Saxena, Luo, Chilukuri, Maxwell, and Doctor describe novel approaches to medical protection against CWAs. They begin by discussing enzyme-based pretreatments, to include both stoichiometric and catalytic scavenging enzymes, their isolation and purification, and the like. Interestingly, the authors speculate on the delivery of scavenging enzymes by gene therapy and carefully describe the benefits of that approach. Next, they consider advances in oxime-based, postexposure therapy, going beyond the treatment offered by McDonough and Romano in considering even more recently synthesized agents comparable to, or perhaps superior to, MMB-4. Finally, they consider centrally acting pretreatment drugs. They agree with the sentiment of Periera et al. in that a number of centrally acting pretreatments, many designed for treatment of Alzheimer's disease, and clearly superior to pyridostigmine bromide, are emerging from research in this area.

Lenz, Broomfield, Yeung, Masson, Maxwell, and Cerasoli describe the use of scavenger enzymes as alternatives to conventional approaches to the management of nerve agent casualties. This approach, described by them in our earlier volume, avoids side effects associated with current multidrug antidotal regimens. It also obviates the requirement, often difficult to achieve in a military setting, for rapid administration of pharmacologically sufficient drug to attain its therapeutic aim. Candidate bioscavenger proteins, which react quickly, specifically, and irreversibly with organophosphorus compounds, are presented and discussed. This bond may be stoichiometric and sequester substrate or may be catalytic, hydrolyzing substrate into biologically inert products. Promising examples of each approach are presented, and the advantages of the novel approach over conventional approaches are discussed.

Podoly, Diamant, Friedler, Livnah, and Soreq, at the Hebrew University of Jerusalem, demonstrated that BuChE, in addition to its endogenous scavenging of drugs, therapeutics, and CWAs, appears to attenuate the formation of amyloid fibrils in the human brain. Thus, BuChE may provide neuroprotection not only in the short term, but also within a longer time frame, increasing its potential for future therapeutic uses. These provocative findings suggest a common direction for both Alzheimer's research and research into the protection against nerve CWAs.

Pereira, Burt, Aracava, Kan, Hamilton, Romano, Adler, and Albuquerque describe the possible utility of Alzheimer's disease drugs in protecting against central as well as peripheral effects of nerve agents. One such drug, galantamine (also called galanthamine), has recently been found by the authors to be a superior antidote against intoxication by soman and other nerve agents, effective when administered both before and soon after exposure. It has been suggested that the neuroprotective effect of galantamine is a result of activation of a number of kinases, including the extracellular signal-regulated kinase, secondary to the nicotinic allosteric potentiating action of the alkaloid. It is likely that selective block of AChE versus BuChE, ability to penetrate the blood–brain barrier, and allosteric potentiation of nAChRs all contribute to the effectiveness of galantamine as a medical countermeasure against organophosphate (OP) poisoning. The data summarized here indicate that the introduction of galantamine into clinical practice for OP poisoning will provide a major advance.

Dabisch, Hulet, Kristovich, and Mioduszewski present an overview of the toxic effects associated with inhalation of a nerve agent vapor or aerosol. Many studies cited were conducted at the U.S. Army Chemical Biological Center from the 1950s up to the present day. The authors point out that the challenge for laboratory studies is to safely generate stable vapor or aerosol atmospheres and verify their atmospheric concentration, chemical characterization, and stability during the exposure period. The results of such well-controlled studies enhance human risk assessment modeling tools, support the operational risk management decision process, and help define physiologically relevant nerve agent detection thresholds.

Smith, Stone, Guo, Ward, Suntres, Mukherjee, and Das focus on the oxidative stress aspects of vesicant exposure, a subject that has received little attention previously. The authors discuss three key intermediate mechanisms in the pathogenesis of the mustard injury—activation of PARP, formation of toxic metabolites, and signaling pathways that invoke the action of a number of proinflammatory mediators, looking particularly at sphingomyelinase-produced ceramides as apoptotic triggers. The authors discuss approaches to antidotes or ameliorative measures for a family of vesicants and the pulmonary toxicants chlorine and phosgene. The results presented here provide a molecular and cellular basis for developing strategies for pharmacological intervention, with potential for clinical application.

Hurst and Smith discuss the health effects of exposure to vesicant agents. They consider the mustards (nitrogen and sulfur mustard) and lewisite. They describe the biochemical and physiological roots of the pathogenesis of these vesicating agents, the principal target organs, the clinical course of the pathology in each instance, residual long-term health effects, and medical management of casualties of vesicant exposure. The authors also provide a brief history of the circumstances of exposure of humans to vesicating agents, whether in warfare, volunteers in research, or, in the case of mustards, in medical treatment. This chapter considers current research and concludes, "Although much effort is being expended in developing therapeutic interventions that will limit the extent of tissue pathology, the best immediate approaches involve prevention of contact between mustard and tissues and medical procedures that ease patient trauma and discomfort."

Ballantyne and Salem discuss the experimental and human clinical toxicology of cyanides with particular reference to their potential for application as chemical warfare weapons and use by terrorists. They consider repeated exposure toxicity as well as specific organ, tissue, and functional end-point toxicity. Among the functional end-point toxicities, they review neurotoxicity, cardiotoxicity, vascular toxicity, developmental and reproductive toxicity, and genotoxicity. They conclude this review of the toxicology of cyanide by describing emergency first aid and poison-control

measures in current usage as well new approaches, still in the research stage, to the management of the problem of cyanide poisoning.

Salem, Ballantyne, and Katz present the argument that when chemicals are used to control civilian disturbances, it is necessary to use substances of low health hazard potential and employ delivery methods that carry the minimum potential for injury. This chapter reviews the nature and effects of chemicals used, and proposed for use in peacekeeping operations. Particular attention is given to their operational uses in various circumstances, pharmacology, toxicology, evaluation of safety-in-use, delivery, effects on humans, consequences and medical management of overexposure and injury, and the need for preparedness planning.

Adler, Oyler, Apland, Deshpande, Nicholson, Anderson, Millard, Keller, and Lebeda use the insights gained in their understanding of the mechanism of botulinum neurotoxin (BoNT) action to establish a conceptual framework within which to develop effective treatment strategies for intoxication. The authors also suggest that some vaccine approaches have proven effective, but generally require multiple inoculations and incubation times of up to a year from onset to generate adequate protection. In addition, vaccinated individuals may be precluded from the use of local BoNT administration for treatment of spasticity or movement disorders that may develop during their lifetime. These limitations argue strongly in favor of a supplementary pharmacological approach for the management of botulism. They point out that efforts to develop pharmacological inhibitors of BoNT have increased substantially during the last decade. The major focus of the current research is the design and synthesis of specific metalloprotease inhibitors. Most of the ongoing drug discovery efforts were initiated prior to the availability of the crystal structure for BoNT and will be aided enormously by the availability of precise structural information.

Millard and LeClaire reported that several aspects of ricin, including its significant human toxicity, past military interest, wide availability in ton quantities from castor seed meal, and increased attention from the world news media, have contributed to the international regulation of the toxin as a potential ''weapon of mass destruction.'' They provide an overview of this literature for scientists who are working toward practical medical solutions to prevent or mitigate the consequences of chemical warfare or bioterrorism. They summarize the biochemistry and pathophysiology of ricin and briefly review studies with experimental animal models to aid in preventing, diagnosing, and treating the poorly characterized human response to ricin exposure. Throughout the chapter, they compared ricin to several closely related proteins toxins of comparable potency of the same plant genus. This is done to clarify the gaps in our current understanding for this important class of plant toxins.

Ballantyne and Salem present the concept of screening smokes, for example, a fog-like atmosphere composed of light-scattering particles that limit visibility of troops or vehicles. These particles should not be of a biologically reactive nature, lest they be classified as CWAs under the Geneva Convention. In their chapter, they discuss the acute and chronic toxicity, as well as the environmental and ecotoxicological impact, of the most common screening smokes. They conclude by discussing the medical management of patients overexposed to screening smokes, which can in rare cases cause systemic toxicity.

Capacio, Smith, Gordon, Haigh, Barr, and Lukey describe some of the most recent approaches to improving nerve agent diagnostics. They describe efforts to develop portable, reliable, prompt (i.e., near real-time) assays capable of detecting exposure even when administered after some time. They remind us that these assays are compared to the delta pH method of Ellman, the historical standard for measurement of ChE as the biomarker of exposure. Lukey and his colleagues point to successful efforts to measure regenerated nerve agent in blood. Thus, given the potential increase in urban terrorism that may include the use of chemical warfare organophosphate agents, federal, state, and local authorities now have a variety of sensitive and accurate cholinesterase and OP detection assays for appropriate containment, decontamination, and treatment measures.

Jones provides an assessment of the importance of physical protection equipment in supporting effective prehospital interventions. He describes the requirements for, availability of, and

physiological burden associated with, various components of PPE. These include masks, respirators, protective suits, gloves, and the like. He discusses their ability to meet Occupational Safety and Health Administration (OSHA) standards. He considers alternatives to traditional PPE, such as Skin Exposure Reduction Paste Against Chemical Warfare Agents (SERPACWA), a barrier cream of demonstrated efficacy against a number of CWAs and skin irritants. He also considers how work–rest cycles and use of wet-bulb temperature information can be factored into response planning. Finally, he discusses how new Federal OSHA chemical, biological, radiological, and nuclear standards have ''taken the guesswork'' out of response planning for many local agencies.

Lukey, Slife, Clarkson, Hurst, and Braue describe the problems associated with the decontamination of CWAs from the skin. They begin by describing skin barrier and metabolizing properties. They move to describing historical approaches to protecting the skin, to include protective ensembles, skin barrier creams, both inert barriers and active (decontaminating or inactivating) creams, and to describing the properties of effective skin decontaminants. They review the effectiveness of a number of candidates or fielded skin-decontaminating kits, foams, solutions, and field-expedient measures. The authors conclude by stating their guiding principle; the best decontaminants are those that most rapidly remove threat agents from the skin.

Romano, King, Lumley, and Saviolakis discuss the concept of CWAs used in terrorism within the framework of the generalized catastrophe literature, that is, disasters, chemical spills, terrorism, and warfare. They suggest likely psychological, physiological, and neurobehavioral effects that may be encountered if CWAs are employed against U.S. forces, or even more troublesome, against U.S. citizens. Moreover, they discuss possible outcomes if CWAs are threatened or employed on a target population, drawing widely on historical disaster, warfare, and terrorism literature. In this chapter, they consider some of the latest physiological data and experimental results, which describe the underlying physiological basis of posttraumatic stress syndrome and relate those data to the nonlethal effects of nerve CWAs.

Moore and Saunders-Price describe the organization and capabilities of the ever-evolving national response apparatus to a domestic or international terrorist use of a ''weapon of mass destruction (WMD).'' This apparatus involves many Federal agencies, one new since 2001 (The United States Department of Homeland Security), which support and complement local and state response systems that respond to such incidents. Over the past 5 years, subsequent to the publication of the last edition of this text, enormous emphasis has been placed on domestic preparedness for possible use of WMD. Chemical warfare agents, along with nuclear weapons and biological warfare agents, are included in this category. The reader is referred to the previous edition, where much of the information on medical and public health considerations of CWAs remains accurate. Moreover, recognition of the possible terrorist use of toxic industrial chemicals and materials (TICs and TIMs) has presented additional challenges. This chapter expands on the previous work and puts this information into a more current context.

Pulley and Jones suggest that parts of this text are highly technical, discussing major chemical toxins, their physiologic and health consequences, and how to manage the toxins with antidotes and decontamination. The intent of their chapter is to create a framework where the emergency medical community can understand, and then employ, the basics of an organized response to a large-scale chemical event. The interested reader can then turn to the other chapters for more detailed technical information to increase the breadth of your plan and subsequent response. These major areas include command and control, communications, security, transportation and traffic, and planning and preparation.

The editors point to the passing of several contributors to our first volume, scientists of great accomplishment in the area of medical chemical defense. They include our great friend and mentor, Dr. Satu Somani of Southern Illinois University, Dr. Frederick Sidell, formerly of the United States Army Medical Research Institute of Chemical Defense, a dedicated physician and scholar, and Dr. Robert Sheridan, also of the United States Army Medical Research Institute of Chemical Defense, a gentle, soft-spoken scholar who contributed greatly to this field. The field of medical

chemical defense will struggle to overcome their loss, but in the end will prevail because of their leadership and efforts.

The editors of this text have been working in the area of chemical defense toxicology and medical response for many years. Colonel (Ret.) James Romano is currently a Program Manager at Science Applications International Corporation, Frederick, Maryland. Science Applications International Corporation is a leading provider of scientific, engineering, systems integration, and technical and biomedical services and solutions. He was formerly commander of the United States Army Medical Research Institute of Chemical Defense and deputy commander of the United States Army Medical Research and Materiel Command. Dr. Harry Salem is a senior biological scientist at the Edgewood (Maryland) Chemical and Biological Center, United States Army Research, Development, and Engineering Command. The Edgewood Chemical and Biological Center is the Army's lead research establishment for development of physical countermeasures to CWAs, such as protective ensembles, decontaminating solutions, sensors, and area survey devices. Its outstanding physical sciences capabilities have allowed it to be an effective inhalation toxicology laboratory and an excellent collaborator to the United States Army Medical Research Institute of Chemical Defense (USAMRICD), collocated at Aberdeen Proving Ground, Maryland. The USAMRICD is the Army's lead laboratory for the development of medical countermeasures to chemical warfare agents. Colonel Brian Lukey is currently commander of the USAMRICD. The USAMRICD has a long history of discovery and development of pretreatments, therapeutics, diagnostics, and skin barrier creams to protect against CWAs. The USAMRICD functions as a subordinate command of the United States Army Medical Research and Materiel Command, Fort Detrick, Maryland.

Finally, the editors wish to thank Candy Romano, the Salem family (Flo, Jerry, Amy, Joel, Marshall, and Abby Rose), and Marita Lukey and for their patience and encouragement. As with all previous efforts by the editors, they have been steadfast in their support and reassuring in their conversations. With their support, this task proved achievable and enriching.

Acknowledgments

The editors wish to thank Ms. Patricia Hurst whose persistence, attention to detail, and sense of purpose kept the editors and many of the contributors on track. Ms. Hurst skillfully polished the rough edges of nearly all the chapters of this book, while maintaining a positive and goal-directed demeanor. The editors also wish to thank Ms. Sandra Loukota for her excellent administrative contributions. In addition, the assistance of the Edgewood Chemical Biological Center Library Staff, Patsy D'Eramo, Edward Gier, Corky Smith, and Carolyn Sullivan, is greatly appreciated, as are the contributions of Judith Hermann, Mary Frances Tracey, Megan Lynch, and Susan Biggs of the Chemical and Biological Information Analysis Center.

1 Brief History and Use of Chemical Warfare Agents in Warfare and Terrorism

Harry Salem, Andrew L. Ternay Jr., and Jeffery K. Smart

CONTENTS

I. INTRODUCTION

Poisons and incendiary weapons first described in ancient myths included arrows dipped in serpent venom, water poisoned with drugs, plagues unleashed on armies, and secret formulas for combustible weapons. Exploiting lethal forces of nature was not just mythical fantasy, but was supported by numerous nonfiction authors in ancient times, to include Near Eastern records of 1770 BCE (before the Common Era), Greek myths recorded by Homer in about 750 BCE and Greek historians from 500 BCE through the second century of the Common Era (CE). From 500 BCE on, weapons of poison and combustible chemicals in China and Japan were described in military and medical treatises. The development of Greek fire and other incendiaries was described in Byzantine and Islamic sources of late antiquity from the seventh century through the fourteenth century CE. Archers in antiquity created toxic projectiles with snake venoms, poisonous plants, and bacteriological substances. Contamination of the enemy's water and food supplies was also accomplished (Mayor, 2003).

There were descriptions of the use of calmatives to tranquilize, disorient, or knockout enemies so they were unable to defend themselves. These were applied in warfare by ancient Greeks when they conquered Ionia which is modern Turkey. Intoxicants were used to gain victories by ancient armies in Gaul, North Africa, Asia Minor, and Mesopotamia. The calmatives and intoxicants of antiquity included toxic honey, drugged sacrificial bulls, barrels of alcohol, and mandrake-laced wine. Malodors also had their origins in antiquity when, over two millennia ago, armies in Asia and Germany employed noxious smells to overwhelm their foes.

The contents of this chapter are not to be construed as an official Department of the Army position, unless so designated by other authorizing documents.

II. CHEMICAL WARFARE AGENTS IN ANCIENT TIMES BCE

One of the earliest examples of chemical warfare was in the late Stone Age (10,000 BCE). Hunters known as the San, in southern Africa, used poison arrows. They dipped the wood, bone, and stone tips of their arrows in poisons obtained from scorpion and snake venoms, as well as poisonous plants (CBW Info, 2005; Tagate, 2006; Wikipedia, 2007a).

In about 2000 BCE, soldiers in India used toxic fumes on the battlefield. Chinese writings from as far back as the seventh century BCE contain hundreds of recipes for the production of poisonous and irritating smokes for use in war, along with numerous accounts of their use. These accounts describe the arsenic containing ''soul-hunting fog'' and the use of finely divided lime dispersed into the air (Geiling, 2003; DeNoon, 2004; Tagate, 2006).

The Assyrians in 600 BCE contaminated the water supply of their enemies by poisoning their wells with rye ergot (Mauroni, 2003). Solon of Athens used hellebore roots, a purgative that caused diarrhea, to poison the water in an aqueduct leading to the Pleistrus River, around 590 BCE, during the siege of Cirrha. The Cirrhaeans drank the water and developed violent and uncontrollable diarrhea, and were thus quickly defeated (Hemsley, 1987; United Kingdom Ministry of Defense, 1999; Noji, 2001; Robey, 2003; Tschanz, 2003; CBW Info, 2005; Tagate, 2006; Wikipedia, 2007a).

In the Peloponnesian War between Athens and Sparta in the fifth century BCE, the Spartan forces used noxious smokes and flame unsuccessfully against the Athenian city of Plataea. Later the Boeotians successfully used noxious smokes and flame during the siege of Delium by placing a lighted mixture of coal, brimstone, and pitch at the end of a hollow wooden tube. Bellows pushed the resulting smoke through the tube and up to the walls of the besieged city, driving the defenders away (Thucydides, 1989).

During the fourth century BCE, the Mohist sect in China used bellows to pump smoke from burning balls of mustard and other toxic vegetables into tunnels being dug by a besieging army (Tagate, 2006). In 200 BCE, the Carthaginians spiked wine with Mandrake root, a narcotic to sedate their enemies, feigned a retreat to allow the enemy to capture the wine, and then when the enemy was sleeping, returned to kill them (Batten, 1960; United Kingdom Ministry of Defense, 1999).

III. CHEMICAL WARFARE AGENTS IN THE CE TO WORLD WAR I

Around 50 CE, Nero eliminated his enemies with cherry laurel water that contained hydrocyanic acid (Hickman, 1999). Plutarch described irritating smokes in some of his writings around 46–120 CE. In 1000 CE, the Mongols used gas bombs made of sulfur, nitre, oil, aconite, powdered charcoal, wax, and resin. These bombs weighed about 5 pounds each. Aconite was a favorite poison which is derived from the perennial herb of the genus Aconitum. It is in the buttercup family and is also known as monkshood and wolfsbane. Aconite (*Aconitum napellus*) is an alkaloid acting on the central nervous system, heart, and skin. It first stimulates and then paralyzes the nerves and heart. The effects begin with a tingling of the mouth, fingers, and toes, and then spread over the entire body surface. Body temperature drops quickly and is followed by nausea, vomiting, and diarrhea. Fatal doses are marked by intense pain, irregular breathing, and a slowed and irregular heartbeat. Death results from heart failure or asphyxiation. Aconite has been used as a poison on arrowheads and to taint enemy water supplies, and it was also used as a poison by Indian courtesans when they applied it as a lipstick as the ''Kiss of Death'' (PDR Health, 2006).

In about 660 CE, Callinicus of Heliopolos invented a weapon called Greek fire, also referred to as Byzantine fire, wildfire, and liquid fire (Figure 1.1). Because its formulation was a carefully guarded military secret, the exact ingredients are unknown. It probably consisted of naphtha, niter, sulfur; petroleum, quicklime sulfur; or phosphorus and saltpeter. It may have been ignited by a flame, or ignited spontaneously when it came into contact with water. If it was the latter, the active ingredient could have been calcium phosphide, made by heating lime, bones, and charcoal. On contact with

FIGURE 1.1 Drawing of a fake dragon shooting Greek fire; U.S. Army (1918), The Gas Defender.

water, calcium phosphide releases phosphine, which ignites spontaneously. However, Greek fire was also used on land. The ingredients were apparently heated in a cauldron and then pumped out through a siphon or large syringe, known as a siphonarios, mounted on the bow of the ship. Greek fire could also be used in hand grenades made of earthenware vessels that may have contained chambers for fluids that when mixed, ignited when the vessel broke on impact with the target. It was used very effectively in naval battles as it continued burning even under water, and was known to the Byzantine enemies as a wet, dark, sticky fire because it stuck to the unfortunate objects hit and was impossible to extinguish. It was also very effective on land as a counter force suppression weapon used on besieging forces. Greek fire was first used on the battlefield to repel the Arab siege of Constantinople in 674–677 CE then at the Battle of Syllaeum, in 717–718 CE against the Moslems, and later against the Russian attacks in 941 and 1043 CE. The Byzantines also used Greek fire against the Vikings in 941 CE and against the Venetians during the fourth Crusade (Waitt, 1942; CBW Info, 2005; Wikipedia, 2007b).

Chinese soldiers during the period 960–1279 CE used arsenical smokes in battle (CNS, 2001), and the Germans used noxious smokes in 1155 CE. In the fifteenth and sixteenth centuries, the Venetians used poison filled mortar shells and poison chests to taint wells, crops, and animals (Geiling, 2003).

Leonardo da Vinci (1452–1519) described the preparation of Greek fire in his notebooks. His recipes included boiling charcoal of willow, saltpeter, sulfuric acid, sulfur, pitch, frankincense, camphor, and Ethiopian wool together. Liquid varnish, bituminous oil, turpentine, and strong vinegar were then mixed in and dried in the sun or oven. After forming balls, sharp spikes were added, leaving an opening for a fuse (Richter, 1970). Leonardo da Vinci also proposed throwing poison powder on enemy ships. For poison, he recommended chalk, fine sulfide of arsenic, and powdered verdigris (basic copper salts) (MacCurdy, 1938; Dogaroiu, 2003). To protect the friendly soldiers, da Vinci described a simple protective mask made of fine cloth dipped in water that covered the nose and mouth. This is the earliest known description of a protective mask (MacCurdy, 1938; Women in Military Service for America Memorial Foundation, Inc., 2007).

During the Thirty Years War (1618–1648), stink bomb grenades were used against fortifications. These were made from shredded hoofs, horns, and asafetida mixed with pitch. In 1672, during the siege of the city of Groningen, soldiers belonging to Christoph van Galen, the Bishop of Munster, used flaming projectiles and poisoned fireworks, but without much success (Beebe, 1923). Three years

later, in 1675, the French and Germans concluded the Strasbourg Agreement, the first international agreement that banned the use of poison bullets (Hemsley, 1987; Tagate, 2006).

During the Crimean War in 1854, British chemist Lyon Playfair proposed to fill a hollow shell with cacodyl cyanide [$(CH_3)_2AsCN$] for use against Russian ships (Miles, 1957b; Camerman and Trotter, 1963). The enclosed space of a ship would allow the chemical agent to be more deadly. The British military, however, considered chemical warfare inappropriate and thought it similar to poisoning water wells (Browne, 1922). In his letter to the Prince Consort, Playfair responded that ''why a poisonous vapor which could kill men without suffering is to be considered illegitimate is incomprehensible.'' He further added that ''no doubt, in time, chemistry will be used to lessen the suffering of combatants'' (Browne, 1922). Other British proposals for chemical weapons during the Crimean War included ammonia shells, sulfur dioxide smoke clouds, and cacodyl oxide shells (Miles, 1957a, 1958a; Hemsley, 1987).

The many suggestions, inventions, and concepts proposed during the American Civil War were among the forerunners of chemicals used on a much larger scale during World War I (Smart, 2004). In 1861, Confederate Private Isham Walker wrote a letter to the Secretary of War Lucius Walker proposing the use of poison gas balloons against Fort Pickens and the Federal ships guarding it near Pensacola, Florida. The plan was not accepted (Wiley, 1968). On April 5, 1862, the same day that the Union Army began the siege operations against the extensive fortifications at Yorktown, Virginia, John Doughty of New York City, a school teacher, sent a letter to the War Department suggesting that shells filled with chlorine be shot at the Confederates (Figure 1.2). He envisioned the shells exploding over the Confederate trenches and creating a chlorine cloud that would either disable or drive the defenders away. He also addressed the moral question of introducing chemical warfare and concluded that it ''would very much lessen the sanguinary character of the battlefield and at the same time render conflicts more decisive in their results'' (Haydon, 1938). Also in April 1862, shortly after the naval engagement between the USS *Monitor* and the CSS *Virginia* ended in a draw, Commodore L.M. Goldsborough reported a rumor that the Confederates were going to use chloroform as a knockout gas against the USS *Monitor* to produce insensibility of their crew. A similar suggestion was made by Joseph Lott from Hartford, Connecticut in 1862 to load hand pumped fire engines with chloroform and spray it on the enemy troops behind their earthworks defending Yorktown, Virginia, and Corinth, Mississippi (Thompson and Wainwright, 1918).

During the siege of Petersburg, Virginia, in 1864, Forrest Shepherd of New Haven sent a letter to President Abraham Lincoln, suggesting mixing sulfuric acid with hydrochloric or muriatic acid to form a dense toxic cloud. Being slightly heavier than the atmosphere and a visible white color,

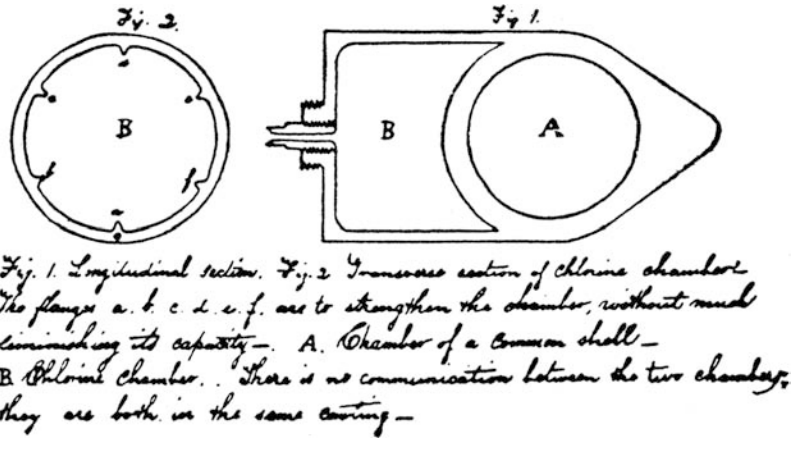

FIGURE 1.2 Drawing of John Doughty's proposed chlorine shell in 1862; National Archives.

the cloud would conceal the operator while the breeze blew it across enemy lines. Once it hits the enemy lines, it would cause coughing, sneezing, and tearing, which would prevent the enemy from aiming their guns, but would not kill them (Miles, 1958b). Also during the siege, Confederate Colonel William Blackford directed that tunnels be dug toward the Union lines to discover Union tunneling operations and, when the enemy tunnels were found, to use cartridges of smoke to suffocate or drive out enemy troops in them. The composition of the smoke was not clearly defined but could have been generated from gunpowder with a much higher proportion of sulfur to create a sulfur dioxide cloud when burned. Another possibility was that the material was similar to the mixture used in stink bombs, which would contain sulfur, rosin, pitch, asafetida, horse-hoof raspings, as well as other materials designed to produce nauseating smokes (Blackford, 1945; Miles, 1959). The use of stink shells was also suggested by Confederate Brigadier General William Pendleton in 1864 to break the siege of Petersburg. He wanted a shell that combined an explosive with an offensive gas that would ''render the vicinity of each falling shell intolerable'' (U.S. War Department, 1891). Also in 1864, Captain E.C. Boynton described a cacodyl glass grenade that combined an incendiary with a toxic gas. He envisioned this for use against wooden ships. The cacodyl ($C_4H_6As_3$), a heavy oily liquid, bursts into flame on contact with the air and also produces toxic fumes (Boynton, 1864).

During the 1899 Boer War, the British used picric acid in their shells. Although the shells were not particularly effective, the Boers protested their use (Hersh, 1968; Hemsley, 1987; Tintinalli, 2003).

At the First Hague Peace Conference in 1899, Article 23(a) banned the use of poisons or poisoned arms and was ratified by the United States. A separate declaration banning asphyxiating gasses in shells was rejected by the United States even though the major European powers all signed it (Taylor and Taylor, 1985).

The Russo–Japanese War also saw limited use of toxic chemical weapons. The Japanese used arsenical rag torches against Russian trenches. The torches were pushed toward the enemy by long bamboo poles to create a choking cloud (Chemical Warfare Service, 1939).

IV. CHEMICAL WARFARE AGENTS USED IN WORLD WAR I (1914–1918)

World War I has been called the ''Chemist's War'' because it ushered in the beginning of the modern era of chemical warfare. Most of the key chemical warfare agents used during the war, however, were eighteenth- and nineteenth-century discoveries. These included: chlorine (1774); hydrogen cyanide (1782); cyanogen chloride (1802); phosgene (1812); mustard agent (1822); and chloropicrin (1848) (Sartori, 1939).

Chlorine (Cl_2), designated Cl by the United States and Betholite by the French, is the only substance that has been used in its elementary state as a war gas. It is a greenish-yellow gas with an irritating and disagreeable odor. Chlorine causes spasm of the larynx muscles, burning of the eyes, nose, and throat, bronchitis, and asphyxiation. Its asphyxiating properties were first recognized by the Swedish chemist Karl Wilhelm Scheele in 1774. Chlorine was the first chemical agent used on a large scale by the Germans in April 1915 (Sartori, 1943; Field Manual 3–11.9, 2005).

Although substances containing cyanide have been used for centuries as poisons, it was not until 1782 that hydrogen cyanide or hydrocyanic acid (HCN) was also isolated and identified by Karl Scheele, who later may have died from cyanide poisoning in a laboratory accident. The agent is a colorless liquid with a faint odor of bitter almonds. It causes faintness, pain in the chest, difficulty in breathing, and ultimately death. Hydrogen cyanide, also referred to as prussic acid, was designated AC by the United States and called Vincennite and Manganite by the French. It was reportedly first used by the Austrians in September 1915 (Foulkes, 1934; Baskin and Brewer, 1997; Field Manual 3–11.9, 2005).

Cyanogen chloride (CNCl) was discovered by Wurtz and first prepared by Berthollet in 1802. It is a colorless gas with an irritating odor that immediately attacks the oral–nasal passages.

Its symptoms are similar to hydrogen cyanide and in high concentrations, it eventually causes death. The U.S. designation was CC which was later changed to CK. The French called it Mauguinite and Vitrite. It was first used by the French in October 1916 (Sartori, 1943; Field Manual 3–11.9, 2005).

Phosgene ($COCl_2$) or carbonyl chloride, designated CG by the United States, Collongite by the French, and D-Stoff by the Germans, was obtained in 1812 by Humphrey Davy when he exposed a mixture of chlorine and carbon monoxide to sunlight. Phosgene is a colorless gas with an odor like musty hay that attacks the lungs causing pulmonary edema and eventually death. It was first used by the Germans as a war gas in December 1915 (Sartori, 1943; Field Manual 3–11.9, 2005).

Mustard agent or dichloroethyl sulfide ($S(CH_2CH_2)_2Cl_2$) was discovered by Despretz who obtained it by the reaction of ethylene on sulfur chloride in 1822. It is normally a pale yellow to dark brown oily liquid with odor like garlic (although the German mustard agent had an odor similar to mustard). The agent normally attacks the eyes and blisters the skin. The U.S. designated it HS, and then later HD after a purified version was developed in 1944. The French called it Yperite and the Germans Lost. The German name was derived by taking the first two letters of the two Germans Lommel and Steinkopf, who proposed and studied the use of this agent in warfare. The first use of mustard agent by the Germans near Ypres, Belgium, in July 1917, marked the beginning of a new phase of chemical warfare, and inflicted about 15,000 British casualties in three weeks (Prentiss, 1937; Sartori, 1943; Field Manual 3–11.9, 2005).

Chloropicrin or trichloronitromethane (CCl_3NO_2) was prepared in 1848 by Stenhouse and was extensively used in World War I. It is a pungent, colorless, oily liquid that caused oral–nasal irritation, coughing, and vomiting. In high dosages, it causes lung damage and pulmonary edema. It was first employed dissolved in sulfuryl chloride by the Russians in 1916 in hand grenades. In Germany it was known as Klop, in France as Aquinite, and PS in the United States. It has also been used as an insecticide and fungicide, as well as for eradicating rats from ships (Sartori, 1943).

At the beginning of World War I, both sides used munitions filled with irritants such as ethylbromoacetate ($CH_2BrCOOC_2H_5$), chloroacetone (CH_3COCH_2Cl, or French Tonite, German A-Stoff), *o*-dianisidine chlorosulfonate, xylyl bromide ($C_6H_4CH_3CH_2BR$, German T-Stoff), or benzyl bromide ($C_6H_5CH_2Br$) (Dogaroiu, 2003). Other irritants used in World War I included acrolein (CH_2CHCHO, French Papite), bromoacetone (CH_3COCH_2Br, U.S. BA, German B-Stoff, French Martonite), and bromobenzyl cyanide ($C_6H_5CHBrCN$, U.S. CA, British BBC, French Camite) (Salem et al., 2006). Thus, the first use of chemicals in World War I involved nonlethal tear gases, which were used by both the French and the Germans in late 1914 and early 1915 (Figure 1.3).

Germany was the leader in first using chemical weapons on the battlefield and then introducing or developing new chemical agents to counter new developments in protective equipment. Fritz Haber was the designer behind many of Germany's chemical weapons. Although he was not a toxicologist, he profoundly influenced the science of chemical toxicology. Haber and colleagues conducted acute inhalation studies in animals with numerous chemical agents thought to be useful in chemical warfare. He also developed Haber's Law which is usually interpreted to mean that identical products of the concentration of an airborne agent and duration of exposure will yield similar biological responses (Witschi, 2000). Actually, the product of the concentration (C) of the gas in air in parts per million (ppm) and the duration of the exposure (t) in minutes was referred to as Haber's Constant (Haber, 1986). It was also referred to as the mortality-product, the Haber Product W ($C \times t = W$), or the lethal index, the lower the product or the index number, the greater the toxic power (Sartori, 1939). Although Haber's Law has been used by toxicologists to define acute inhalation toxicity for toxic chemicals, it can also be useful for quantitative risk or safety assessment (Rozman and Doull, 2001).

Germany's use of chemical weapons on the battlefield began on October 27, 1914 when they fired shells loaded with dianisidine chlorosulfonate, a tear gas, at the British near Neuve Chapelle. This tear gas normally produces violent sneezing. In this case, however, the chemical dispersed so rapidly in the air that the British never knew they were attacked by gas (Charles, 2005). Following this experiment, the Germans continued to test other potential chemical weapons.

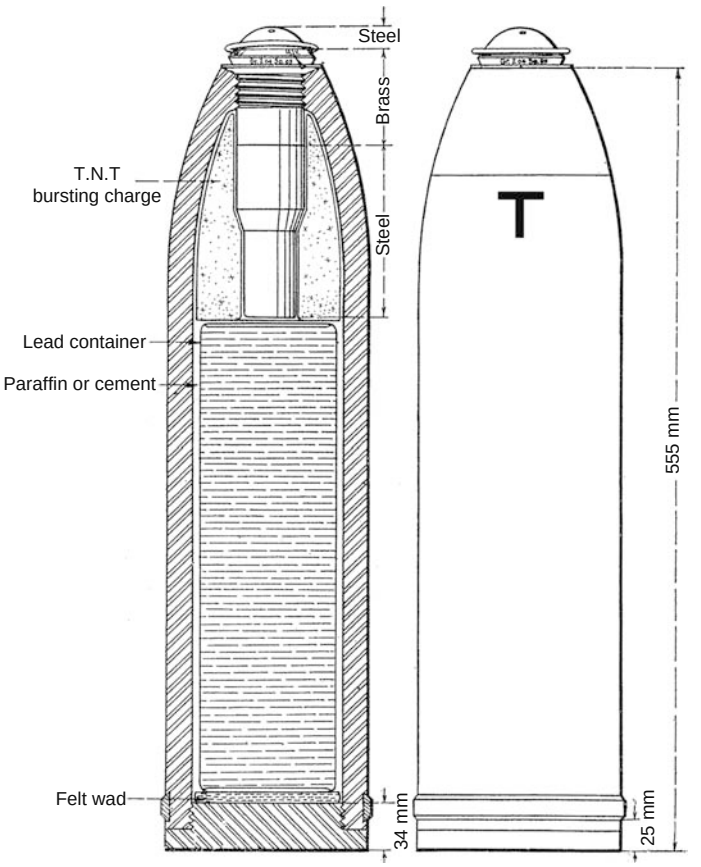

FIGURE 1.3 The German 150 mm xylyl bromide (T-Stoff) shell; Army War College (1918), Gas Warfare, Part I, German Methods of Offense.

In mid December 1914, Haber's assistant was killed while working on an arsenic containing weapon (cacodyl: $(CH_3)_2As–As(CH_3)_2$). In January of 1915, the Germans used xylyl bromide (T-stoff) against the Allies, but it was so cold that the gas froze and settled in the snow.

By the spring of 1915, Haber convinced the German High Command to use chlorine gas, and to create a special gas unit, the 35th Pioneer Regiment. This unit included Otto Hahn, Wilhelm Westphal, Erwin Madelung, James Franck, and Gustav Hartz. Three of these were future Nobel Laureates, Hahn, Franck, and Hertz (Charles, 2005). In preparation for the release of chlorine gas, Haber arranged for over 5000 chlorine cylinders to be placed near Ypres, Belgium (Figure 1.4). Only some of the ordinary German soldiers had protective masks made of cotton gauze, while Haber had provided Draeger masks for the Pioneers. While waiting for the wind to blow in the right direction, enemy fire hit some of the chlorine cylinders, and released their gas. Three German soldiers were reportedly killed and 50 were injured. On April 22, 1915, a strong wind from the northeast arrived, and at 5 PM, Haber's gas troops opened the valves on the 5730 high-pressure steel tanks containing about 168 tons of chlorine along a four mile frontline. The chlorine drifted southward toward the French and Canadian lines. It formed a yellow–green smoke wall about 50 feet high and 4 miles long. The wind shifted and the cloud moved to the east toward the trenches occupied by the 45th Algerian Division (French). Those who tried to stay were quickly overcome, retching and gasping for air as they died. The rest fled in panic, stumbling and falling, and throwing away their rifles. The cloud moved on at about 100 ft/min and opened up a four mile wide hole

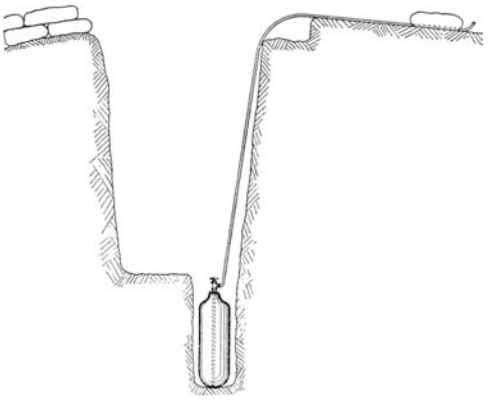

FIGURE 1.4 German trench with a gas cylinder ready for discharge; Army War College (1918), Gas Warfare, Part I, German Methods of Offense.

in the Allied front. After 15 minutes, the German troops emerged from their trenches and advanced cautiously. Had the German High Command provided enough reserves to sustain the offensive, they might have been able to break through the Allied defensives and capture Ypres. Instead, the German forces gained only about two miles of territory. The French soldiers used rudimentary defensive equipment including cans of water, along with wads of cotton that they were supposed to soak and hold to their faces. Estimated casualties for the battle ranged from 3,000 to 15,000 killed and wounded (McWilliams and Steel, 1985; Charles, 2005; MSN, 2005; Landersman, 2003).

Following this attack, the Germans led repeated chlorine gas attacks on the Allies and drove them back almost to Ypres, but were unable to capture the objective. The initial attacks caught the Allies completely unprepared. However, shortly after the first attack, the British troops were told to urinate on their handkerchiefs, and tie them over their faces for emergency protection. This caused problems for some soldiers when there were multiple gas attack alerts within a short time. Within a week of the first attack, Emergency Pad Respirators made of cotton waste soaked in sodium carbonate and sodium thiosulfate (hypo) were available for British soldiers. By early 1916, protective masks included goggles, exhaust valves, and provided adequate protective against most of the chemical agents being used on the battlefield. This was the beginning of a competition between the developers of chemical warfare agents that could penetrate the masks and the developers of protective equipment. The developers of protective equipment were ahead in the competition until the summer of 1917.

Mustard agent (HS) was first used by the Germans against the French on July 12, 1917, also near Ypres (Harris and Paxman, 1982; Mitretek, 2005a). The attack led to about 15,000 Allied casualties. Unlike phosgene, which was disseminated as a gas, mustard agent is relatively non-volatile and looks much like motor oil. It is persistent and will remain on objects for long periods of time. The introduction of mustard agent on the battlefield created a dilemma for the protective equipment developers. No longer were the oral–nasal passages and the eyes the only areas that needed protection. Mustard agent required full body protection for both soldiers and all animals used on the battlefield for transportation and communication (Figure 1.5). Unprotected soldiers suffered blisters on all exposed skin that appeared hours after the initial exposure. Less than 5% of the mustard casualties who reached medical aid stations died, but the average convalescent period was greater than six weeks. Mustard agent damages eyes, lungs, and skin, and ties up large amounts of medical resources (Figure 1.6).

On March 9, 1918, a German chemical bombardment began between St. Quentin and Ypres firing half a million shells containing mustard agent and phosgene at the rate of about 700 shells/min. On that day, a total of 1000 tons of chemicals were used by Germany. The Germans

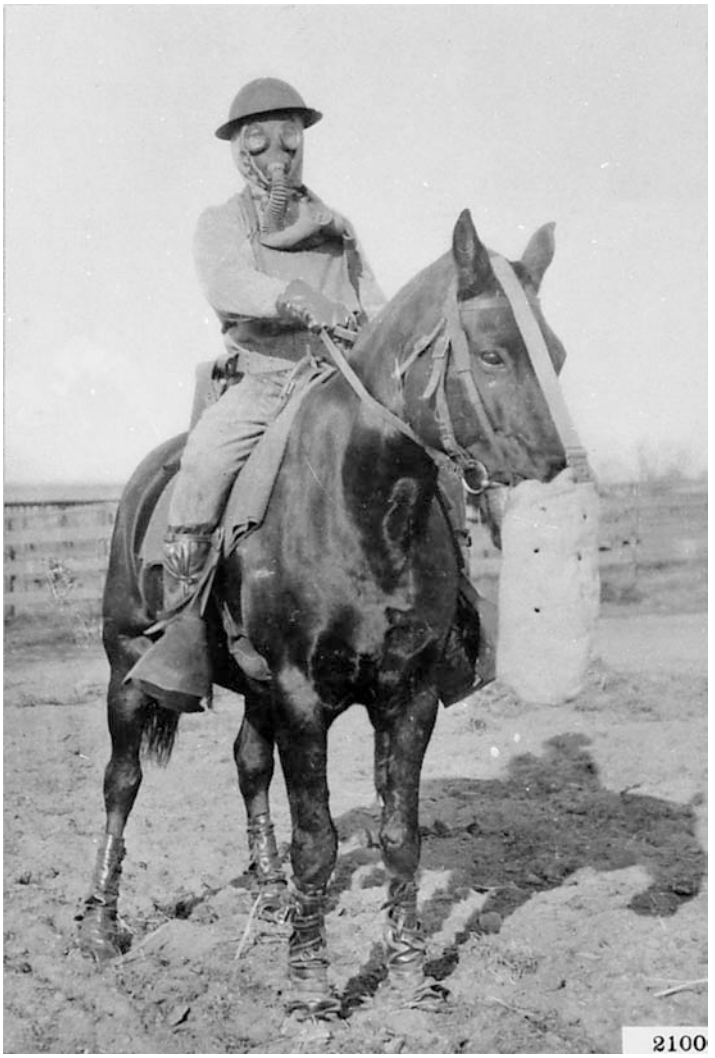

FIGURE 1.5 Rider and horse protected against mustard agent during World War I; U.S. Army.

also attacked Salient du Fey on March 9, 1918, where Colonel Douglas MacArthur led the capture of a German machine gun nest and was awarded the Distinguished Service Cross. Two days later, MacArthur was among those gassed by the Germans, and for this he received the Purple Heart. On March 19, 1918, the British launched a pre-emptive retaliatory strike against the German positions near St. Quentin. They used nearly 85 tons of phosgene and killed 250 German troops.

There were attempts late in the war to develop more potent vesicants than mustard agent. In 1918, Associate Professor Winford Lee Lewis left Northwestern University to become director of the Offensive Branch of the Chemical Warfare Service Unit at Catholic University. This Unit was called Organic Unit No. 3 and was tasked to develop and produce novel gases containing arsenic. Lewisite (dichloro-(2-chlorovinyl) arsine) ($C_2H_2AsCl_3$) was developed based on early research conducted in 1904 at the university by J.A. Nieuwlands. It is a brown liquid that smells like geraniums. The new agent was also designated G-34 or M-1. For the popular press, it was referred to as Methyl or the ''Dew of Death.'' Like mustard agent, it is also a vesicating agent that attacks the eyes and blisters the skin, but its effects occur much quicker than mustard agent. Developed late

FIGURE 1.6 Sample of mustard agent; U.S. Army.

in the war, it was not used on the battlefield (Anonymous, 1921; Vilensky and Sinish, 2005; Field Manual 3–9.11, 2005).

By the end of the war, it was estimated that approximately 1.2 million soldiers were wounded by the use of gas and over 91,000 were killed. Reportedly, the Russians alone suffered approximately half a million chemical casualties. Over 113,000 tons of chemicals were released and over 66 million gas shells were fired during World War I. What started as innocent, peaceful, and even humanitarian commercial use of chemical products led to the horrors of chemical warfare on the battlefield. Haber, the scientist who synthesized ammonia from nitrogen and hydrogen, and for which he was awarded the Nobel Prize in 1918, shifted his interests from fertilizer to gunpowder production in collaboration with I.G. Farben. The Germans used chlorine as a weapon, rather than the more deadly phosgene, and finally mustard agent. Mustard was the most effective casualty causing agent used in World War I. It produced about eight times the casualties of all other German chemicals and no effective defense was developed against it during the war (Landersman, 2003). It was also Haber's group that developed the use of hydrogen cyanide as an insecticide for flour mills and granaries. Known as Zyklon A, the gas contained an odorous marker as a warning system to prevent poisoning. One of his insecticides, Zyklon B, later became a standard means for killing detainees in Nazi concentration camps during the World War II Holocaust. Among the victims reportedly were some of Haber's relatives (Wilson, 2006).

V. CHEMICAL WARFARE AGENTS BETWEEN WORLD WAR I AND WORLD WAR II

Following the end of World War I, many nations attempted to ban chemical warfare. In 1925, 16 of the world's major nations signed the Geneva protocol pledging never to use gas in warfare again. The United States signed the agreement, but the U.S. Senate refused to ratify it due to a growing

isolationism sweeping the country and also concerns that the nation needed to continue preparing in case chemical weapons were used again. Not until 1975 did the United States ratify the protocol. Despite the Geneva protocol, there were continued incidents of chemical weapon usage.

During the Rif War (1921–1926) in Spanish occupied Morocco, Spanish forces reportedly fired gas shells and dropped mustard agent bombs on the Riffians (Anonymous, 1923; Waitt, 1942; SIPRI, 1971). In 1935, Italy used chemical weapons during their invasion of Ethiopia. The Italian military primarily dropped mustard agent in bombs, and experimentally sprayed it from airplanes and spread it in powdered form on the ground. In addition, there were reports that the Italians used chlorine and tear gas. Some sources estimated chemical casualties were 15,000, mostly from mustard (Clark, 1959; SIPRI, 1971). Japan used chemical weapons against Chinese forces during their war starting in 1937. Reports indicated that the Japanese used mustard agent, lewisite, phosgene, hydrogen cyanide, and tear gases filled in bombs, shells, and smoke pots (SIPRI, 1971). The Soviet Union also used chemical weapons on its own people during this period reportedly using them to suppress a massive peasant uprising around Tambov (Wikipedia, 2007a).

Until the mid 1930s, the World War I chemical agents, phosgene and mustard agent, were considered the most dangerous chemical weapons. They could readily be identified by their smell. That changed when Germany discovered nerve agents. The history of nerve agents began on December 23, 1936 when Dr. Gerhard Schrader of I.G. Farben in Germany accidentally isolated ethyl *N, N*-dimethylphosphoramidocyanidate ($C_5H_{11}N_2O_2P$) while engaged in his program to develop new insecticides since 1934. It was a colorless to brown liquid with a faintly fruity odor. Controlled animal laboratory studies revealed that death could occur within 20 min of exposure. In January 1937, Schrader and his assistant were the first to experience the effects on humans. A small drop spilled on a laboratory bench caused both of them to experience miosis and difficulty in breathing. Schrader reported the discovery to the Ministry of War which was required by the Nazi decree passed in 1935 that required all inventions of military significance be reported. The chemical was quickly recognized as a new, more deadly, chemical warfare agent. It was initially designated as Le-100, and later as Trilon-83. It would eventually become known as Tabun. The United States, when it became aware of the agent, designated it GA for German Agent A. Tabun was designated a chemical warfare agent by the military. The first Tabun production was at Elberfeld. In 1940, production was relocated to Dyhernfurth. Tabun was apparently tested on German death camp inmates (SIPRI, 1971; Harris and Paxman, 1982; MTS, 2005a, 2005b; CBW; Field Manual 3–9.11, 2005).

In 1938, Schrader discovered a second potent nerve agent, isopropyl methylphosphonofluoridate ($C_4H_{10}FO_2P$), whose name Sarin is an acronym for the names of the members of the development team: Schrader, Ambrose, Rudriger, and van der Linde. The Germans designated it T-144 or Trilon-46. The United States eventually designated it GB. It is a colorless liquid with no known odor. Animal tests indicated that it was 10 times more effective than Tabun (Harris and Paxman, 1982; MTS, 2005a; Field Manual 3–11.9, 2005).

VI. CHEMICAL WARFARE AGENTS IN WORLD WAR II

With the start of World War II, Germany filled bombs, shells, and rockets with Tabun nerve agent. The Germans, however, never used them on the battlefield. They remained in storage until the end of the war when the Allies captured them and discovered their existence (Figure 1.7). The reasons Germany did not use nerve agents or any other chemical weapons are still debated. One possible explanation was that Adolph Hitler had been exposed to mustard agent as a young soldier and did not want to use chemical agents again. Another possible reason was that by the time nerve agents could have made a difference on the battlefield, Germany had already lost air superiority and risked massive attack against their cities. President Franklin Roosevelt's pledge in 1943 to not use chemical weapons unless the United States was attacked first probably also helped convince the Germans not to initiate chemical warfare (Landersman, 2003).

FIGURE 1.7 German Tabun bombs discovered after the defeat of Germany in 1945; Office of the Chief of Chemical Corps (1947), The History of Captured Enemy Toxic Munitions in the American Zone European Theater, May 1945 to June 1947.

The U.S. preparation for chemical warfare, however, came at a cost. As part of their preparations in case Germany or Japan did use chemical weapons, the U.S. forward positioned chemical agent stockpiles around the world. The only U.S. casualties from mustard agent in hostile action during World War II, were those injured or killed following a German air raid on the harbor of Bari, Italy, that was heavily congested with merchant ships off-loading war supplies on December 2, 1943. Among the ships was the SS *John Harvey* whose cargo included 100 tons of mustard agent bombs. German bombers attacked the harbor sinking 16 ships and damaging 8 others. The *John Harvey* was one of the first hit, and all those on board knowledgeable of the chemical weapons were killed. The destroyed ship spread a mixture of oil and mustard agent across the harbor. Hospitals were unaware of the contamination and were unprepared to treat the patients. There were over 600 mustard agent casualties of whom 83 died within a month (Harris and Paxman, 1982; Landersman, 2003).

During the war, research continued on both sides to find new chemical warfare agents. Soman (pinacolyl methyl phosphonofluoridate) ($C_7H_{16}FO_2P$), eventually designated GD by the United States, was developed by the Germans in 1944. Its name might have been either derived from the Greek verb ''to sleep'' or the Latin stem ''to bludgeon.'' It is a colorless liquid with a fruity or camphor odor. Soman was discovered by Dr. Richard Kuhn, a Nobel Laureate while he was working for the German Army on the pharmacology of Tabun and Sarin (SIPRI, 1971; MTS, 2005a; Field Manual 3–9.11, 2005). Soman combines features of both Sarin and Tabun (CBW). Initial tests showed that Soman was even more toxic than Tabun and Sarin (Harris and Paxman, 1982). The Germans also apparently researched two other nerve agents, later designated GE (ethyl Sarin, $C_5H_{12}FO_2P$) and GF

(cycloSarin, $C_7H_{14}FO_2P$) which were not discovered by the Allies until they overran Germany in 1945 (Harris and Paxman, 1982).

The United States developed a process to purify mustard agent by distillation in 1944 and designated it as HD ($C_4H_8Cl_2S$). The World War I mustard agent, referred to as Levinstein mustard, had a higher percentage of sulfur which made it less effective and less stable in storage. Distilled mustard (HD) had less smell, had a greater blistering capability, and was more stable in storage. The United States and the British also researched mixing different chemicals with mustard agent. This work identified HL (a mixture of mustard agent and lewisite), HQ (sesqui mustard), HT (a mixture that was more persistent and had a lower freezing point), and HV (a thickened mustard agent).

Both sides also researched the nitrogen mustard agents. First identified in the 1930s by Kyle Ward Jr., eventually three nitrogen mustard agents were identified during the war. HN-1 ($C_6H_{13}Cl_2N$), HN-2 ($C_5H_{11}Cl_2N$), and HN-3 ($C_6H_{12}Cl_3N$) were all similar to mustard agent but had quicker reaction in the eyes. The United States focused on HN-1, while the British concentrated on HN-2 and HN-3. The Germans focused on HN-3 and filled it in shells and rockets (Brophy et al., 1959).

VII. CHEMICAL WARFARE USE AFTER WORLD WAR II

As the Allies overran Germany in 1945, they discovered the German nerve agent program for the first time. Both the United States and the Soviet Union took the German technology and made it their primary focus for chemical warfare agents. In the United States, Sarin (GB) was the nerve agent produced during the 1950s at Rocky Mountain Arsenal in Colorado (Figure 1.8). The agent was filled in bombs, shells, and rockets.

FIGURE 1.8 The U.S. Sarin (GB) nerve agent plant at Rocky Mountain Arsenal, CO; U.S. Army.

Within less than a decade, however, a new nerve agent was discovered by the British. The new agent, eventually designated VX (V for venomous), was developed at the ICI Protection Laboratory in 1952. VX ($C_{11}H_{26}NO_2PS$), both an organophosphate and an organosulfate compound which was immediately toxic to mammals as well as to insects was discovered by the British chemist Dr. Ranajit Ghosh. Its chemical name is *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothiolate. VX is a colorless and odorless liquid. The research was originally intended to find a replacement for the insecticide DDT, but as it was too lethal to employ as a pesticide, it was passed to the Chemical and Biological Weapons Facility at Porton Down. Because the British were already committed to the production of Tabun and Sarin, they passed the compound on to the United States and Canada. Knowledge of the VX project somehow leaked to the Soviets who developed their own version of VX which they designated as VR-55. It was later discovered that VR-55 was only a thickened version of Soman. In 1960, the United States completed a VX plant at Newport, Indiana (Figure 1.9). VX was filled in shells, rockets, and a newly designed land mine.

The nerve agents are the most toxic of the known chemical warfare agents. They are hazards in their liquid and vapor states and can cause death within minutes after exposure. Nerve agents inhibit acetylcholinesterase in tissue, and their effects are caused by the resulting excess of acetylcholine. Nerve agents are liquids under temperate condition. When dispersed, the more volatile ones constitute both a vapor and a liquid hazard. Others are less volatile and represent primarily a liquid hazard. The G-agents are more volatile than VX. GB (Sarin) is the most volatile, but evaporates less readily than water. GF is the least volatile of the G-agents (FAS). VX is persistent, that is, it does not degrade or wash away easily. The consistency of VX is similar to motor oil, so it is primarily a contact hazard.

The United States never used nerve agents on the battlefield. However, continued testing and long-term storage created dangers that eventually impacted the entire U.S. chemical weapons

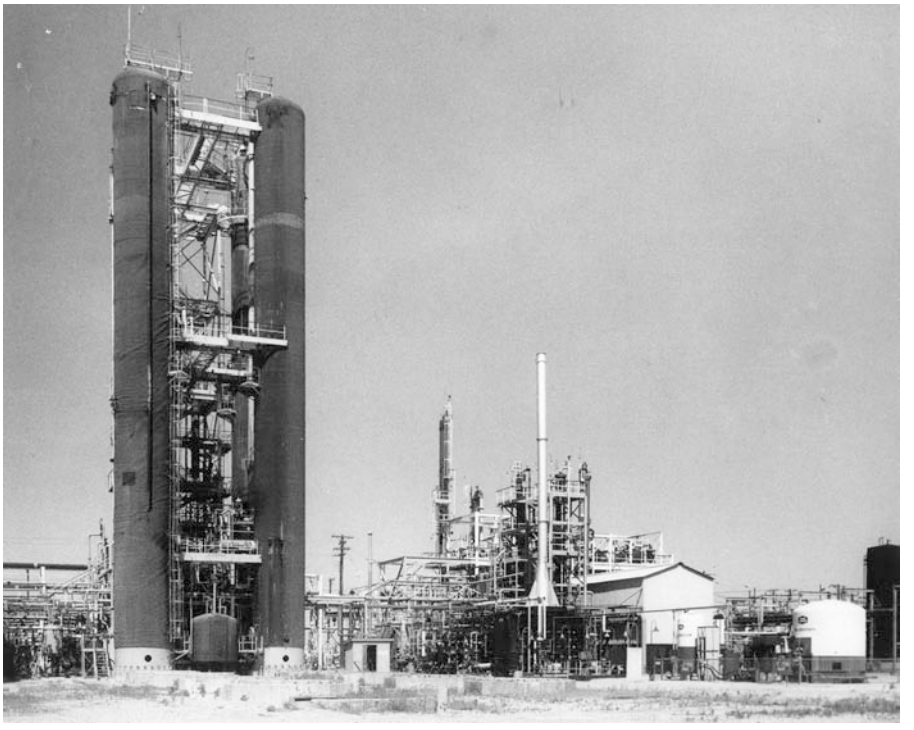

FIGURE 1.9 The U.S. VX nerve agent plant at Newport, IN; U.S. Army.

program. In 1968, VX apparently leaked from an aerial spray tank which was believed to be empty, and drifted across Skull Valley over the borders of Dugway Proving Ground, Utah, and sickened or killed 6000 sheep. In 1969, GB leaked from a Navy bomb, part of a secret chemical weapons stockpile stored on the island of Okinawa, injuring 23 soldiers and one civilian. As a direct result of these accidents, President Richard Nixon issued an executive order in 1969 to halt U.S. production of chemical warfare agents (Newby, 1969; CBW; Mitretek, 2005b; Wikipedia, 2007c).

Following World War II, there were incidents of chemical weapon usage around the world. During the Yemen Civil War (1963–1967), Egypt reportedly used tear gas, mustard agent, and possibly nerve agents against the Yemeni Royalists (SIPRI, 1971; Taylor and Taylor, 1985). The Soviets allegedly used chemical weapons during their invasion of Afghanistan (1979–1989). Intelligence reports indicated the Soviets used nerve agents, phosgene oxime ($CHCl_2NO$), and tear gas (Haig, 1982). The most significant use of chemical weapons occurred when Iraq used them against Iran during the Iran–Iraq War (1980–1988). The reports indicated extensive mustard agent and probable nerve agent usage. There was no consideration of international law and little deterrence, although Iran may have retaliated with chemical weapons of their own near the end of the war. Iraq had ratified the 1925 Geneva Protocol against chemical warfare agent use in 1931 (United Nations, 1986; Pringle, 1993; Landersman, 2003). Toward the end of the war, in 1988, Iraq's military conducted a massive chemical agent attack by aircraft against their own people in Halabja, an unprotected city of 45,000 Iraqi Kurds, knowing they could not retaliate. There were 5000 total casualties with 200 fatalities (Landersman, 2006). Libya reportedly used mustard agent against Chad in 1987 (Pringle, 1993). In all these incidents, there was little outcry or objection from the rest of the world, although the United Nations investigated some of the incidents. There was no deterrence because only one side had chemical weapons in most cases. In addition, the chemical weapons were only marginally effective in their use and did not win the war.

Following these incidents, the United States decided to again produce GB nerve agent in 1987 for a retaliatory capability. However, instead of the 1950s version, they produced binary nerve agent (Figure 1.10). The nerve agent GB was broken down into two less-than-lethal precursor chemicals

FIGURE 1.10 The binary nerve agent plant at Pine Bluff Arsenal, AR; U.S. Army.

that were stored in separate canisters for loading into artillery shells. The production of binary chemical agents continued until 1990 when the Soviets agreed to end chemical weapons production. Eventually in 1993, many countries joined to sign the Chemical Weapons Convention that banned all chemical weapons development, production, acquisition, stockpiling, transfer, or use and also required the destruction of all existing stockpiles (Field Manual 3–11.9, 2005; Smart, 1997).

The Russians apparently continued to research new nerve agents after World War II. According to public disclosures, the Russians developed a highly toxic binary nerve agent series designated Novichuk during the 1980s. In Russian, Novichok means newcomer. Other than tidbits disclosed by defectors and disgruntled scientists, very little else about the Novichuk series is publicly known. For the Russians, the advantage of having new chemical agents is that they have never been previously used on the battlefield. Thus the agents are not banned by treaty, there is no existing detection and warning devices, and the current protective equipment is not effective (Englund, 1992, 1993; Khripunov and Averre, 1999; Wikipedia, 2007c).

VIII. CHEMICAL WARFARE AGENTS USED IN TERRORISM

The FBI has defined terrorism as the unlawful use of force or violence against persons or property to intimidate or coerce a government, civilian population, or any segment thereof, to furtherance of political or social objectives (FBI, 2003).

The first large-scale chemical terrorism incident occurred in the United States in 1982 (Cooke, 2002). Seven relatively young people in the Chicago, Illinois area collapsed suddenly and died after taking Tylenol capsules that had been laced with 65–100 mg cyanide per capsule. The lethal dose of cyanide is approximately 0.5–1.0 mg/kg or about 70 mg for an adult person (Cai, 1998). These were the first victims to die from product tampering. The police believed that the murderer bought or stole the products from the stores, tampered with them, and returned them to the stores. The police speculated that the terrorist could have had a grudge against the producers of Tylenol, society in general, or even the stores where the tainted products were found, and he or she may have even lived in the area of the stores. The perpetrator was never apprehended even though Johnson and Johnson, maker of the capsules offered a $100,000 reward (Kowalski, 1997). As a result of these events in 1983, congress passed the *Federal Anti-Tampering Act*. This legislation made it a federal crime to tamper with food, drugs, cosmetics, and other consumer products. In addition, many manufacturers have made their products tamper resistant (Fearful, 2003). Copycat tampering followed in 1986 with Lipton Cup a Soup, Excedrin, and Tylenol again, and Sudafed in 1991 and Goodies Headache Powder in 1992, resulting in deaths. Sporadic tamperings have continued in the Chicago vicinity, Detroit, and Tennessee (Kowalski, 1997).

After the first World Trade Center bombing in 1993, sodium cyanide was reported to have been in the bomb, but had burned in the heat of the explosion. It was speculated that had it vaporized, the cyanide would have been dispersed into the North Tower and all in the Tower would have been killed, whereas only six people died in the explosion (Mylroie, 1994; 1995/96; Phillips, 1994). Not all chemical terrorism incidents involved lethal chemicals. For example, a Palestinian homicide bomber sprinkled an anticoagulant rat poison among the nuts and bolts of his bomb that he exploded in a bus in Jerusalem, Israel. Among the survivors, a 14 year old girl was bleeding uncontrollably from every one of her puncture wounds. Use of a coagulant drug eventually stopped the young victim's bleeding (Hammer, 2002).

Perhaps the most publicized incident involving chemical terrorism occurred in Japan in 1995. The Aum Shinrikyo, a Japanese religious doomsday sect used Sarin against civilians in Japan on June 27, 1994. The sect targeted a dormitory in Matsumoto where three judges who ruled against them in a land deal trial lived. The sect attempted to spread Sarin in the open, however, the chemical reaction did not work right and the wind changed direction. The judges survived, but became ill. Seven victims in the neighborhood died that evening and over 144 civilians were injured. Aum Shinrikyo's next attempt was to spread Sarin in a closed area. They chose the subway system in

Tokyo, Japan on March 20, 1995, where five trains would meet at 8:15 AM. Members of the sect on the trains pierced plastic bags containing 30% Sarin with sharp tipped umbrellas and let the Sarin evaporate into a lethal gas cloud. Twelve passengers on the train died and about 5500 sought medical treatment (Biema, 1995; Ohbu and Yamashina, 1997).

IX. CONCLUSIONS

Chemicals have been used in warfare since almost the beginning of recorded history. It started out crudely using malodorous materials, irritants, poisonous plants and animals, as well as decaying bodies. Since the birth of chemistry, toxic chemicals have been created specifically for war. Less than lethal and lethal chemicals were developed that incapacitated or killed the enemy without disfiguring or mutilating the body, and without affecting or destroying the infrastructure. These appeared to be distinct advantages offered by the use of these chemicals.

In asymmetric warfare and terrorism, it is sometimes difficult to recognize or identify the enemy. Because terrorists may avail themselves of toxic industrial chemicals and materials that are transported and already stockpiled, a working knowledge of the chemistry of chemical warfare agents is no longer a necessity.

It is important to recognize that the advances in biotechnology, nanotechnology, genetic engineering, neurobiology, computer sciences among others, may assist not only in the proliferation of traditional chemical warfare agents, but also stimulate the emergence of nontraditional agents as well. Advances have also occurred in the delivery systems of these agents.

Although the use of chemical warfare agents in terrorist activities appears to have been limited, this may not accurately reflect the potential of their future use.

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2 Chemistry of Chemical Warfare Agents

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CONTENTS

I. INTRODUCTION

The purpose of this chapter is to provide an overview of the chemistry of selected substances that have been thought of as chemical warfare agents (CWAs) at one time or another. Although ''toxins'' are chemicals, they have not been included since, in some circles, they are thought of as biological threats even though they are perhaps better considered as toxic chemicals arising from various organisms. In general, this chapter is written from the perspective of an organic chemist.

Given size and other restrictions, it was possible to mention only a fraction of the work that we might otherwise have included. The chemicals described in this chapter are identified using what appear to be their most common names. Alternative names and abbreviations are presented in each specific discussion, along with CAS numbers and molecular formulas. In this chapter, we focus on reactions that are representative or of special value. Each section includes, as appropriate, some general remarks, followed by discussions of physical properties and chemical reactions. With a few exceptions, discussions of analytical chemistry are not included. The core chemistry related to the decontamination of threat agents has been summarized elsewhere (Yang et al., 1992). Issues of biological reactions and syntheses are intentionally excluded.

II. SPECIFIC AGENTS

A. Chlorine—Cl_2

General Remarks: Chlorine (molecular chlorine; dichlorine; CAS# 7782-50-5) is among the top 10 chemicals produced in the United States, and was the first chemical used as a weapon (Red Star) on a large scale during World War I (WWI). Mixed with 20% sulfur chloride (SCl_2 or S_2Cl_2), it was used for a relatively short period under the name ''Blue Star.'' ''White Star'' was a mixture of chlorine (50%) and phosgene (50%) (Richter, 1992). Because it is very reactive, chlorine does not occur in nature as Cl_2. It normally is produced from compounds that are relatively rich in chlorine, for example, sodium chloride. Manufactured in large quantities for commercial applications, chlorine now is considered to be a toxic industrial chemical (TIC).

Physical Properties: Chlorine (atomic chlorine, Cl) has an atomic number of 17, an electronegativity of 3.2 (Pauling scale), and an atomic mass of 35.46. It is the third most electronegative element, behind fluorine and oxygen. Throughout this chapter, the term ''chlorine'' refers to Cl_2, unless otherwise noted. Chlorine is a highly reactive nonmetal (halogen). It is a greenish-yellow gas with a density of 3.2 g/L at Standard Temperature and Pressure (STP). Solid chlorine melts at $-101°C$, and the gas liquefies at room temperature at 7.9 atm or at $-34°C$ at 1 atm. Chlorine has a water solubility of 0.64 g of Cl_2/100 g of water at room temperature and a pungent odor.

Synthesis and Reactivity: Chlorine was first prepared by the Swedish chemist K.W. Scheele in 1774 using the reaction of manganese dioxide with hydrochloric acid. Erroneously, he thought that it was a compound of oxygen, and ultimately it was named and identified as an element by Sir Humphry Davy in 1810. The reaction of hydrochloric acid with potassium permanganate provides a second convenient route to small quantities of chlorine.

Commercial syntheses employ several techniques including those listed below. The first two of these are collectively called the chlor-alkali process and are responsible for the consumption of approximately 50% of the sodium chloride produced in the United States.

1. Electrolysis of brine (two types of cells used) to form chlorine at the anode
2. Electrolysis of fused NaCl
3. Electrolysis of hydrochloric acid

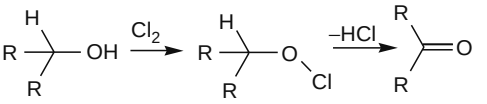
When chlorine dissolves in water, it forms ''chlorine water.'' Only a portion of the chlorine remains intact in this solution, and the remainder converts to hypochlorous acid (aqueous HOCl) and hydrochloric acid (aqueous HCl). It is this formation of acid that enhances the corrosiveness of ''moist'' chlorine gas. By way of contrast, fluorine reacts with water to form HF and O_2.

Most organic functional groups, including those in biomolecules, react with chlorine. Some reactions yield products containing only one chlorine, whereas others yield products with two. Some slow reactions may be accelerated by altering conditions (e.g., solvent, temperature, catalyst). Chlorine reacts by either radical or ionic processes, depending on both substrate and reaction conditions. Radical reactions require some initiator, for example, heat, light, or radicals, and may involve the conversion of molecular to atomic chlorine. Reactions with alkanes most often are radical processes (Ternay, 1979). In a nonreactive solvent, alkenes can readily add chlorine to form *vic*-dichlorides by either radical or ionic mechanisms. Anti-addition is frequently observed, especially in ionic additions. For example, cyclohexene affords *trans*-1,2-dichlorocyclohexane. At high temperatures, chlorine can replace an allylic hydrogen by a radical process. The double bond is preserved in these allylic chlorinations. For example, propene yields allyl chloride and hydrogen chloride (Carey, 2000a).

Alkenes, $R_2C=CR_2$, can react with chlorine and water to form halohydrins, $R_2C(OH)C(Cl)R_2$. With alcohol ($R'OH$) solvents, the product is a haloether, $R_2C(OR')C(Cl)R_2$. In the presence of a carboxylic acid ($R'CO_2H$), the products include the corresponding halohydrin esters, $R_2C(O_2CR')CClR_2$ (Wagner and Zook, 1965b).

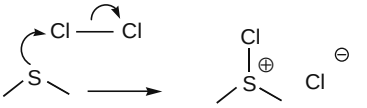
In general, aromatic hydrocarbons are less nucleophilic than are simple alkenes. Chlorination of less nucleophilic aromatics, for example, C_6H_6, requires the use of a Lewis acid catalyst (e.g., anhydrous aluminum chloride) and either excess of the liquid aromatic or an inert solvent. The product, C_6H_5Cl, results from a net substitution process. Chlorination (ortho or para) of activated aromatics such as anisole, $C_6H_5OCH_3$, may not require a catalyst.

Alcohols (1° and 2°) are oxidized to carbonyl-containing substances, the creation of a ketone from a secondary alcohol being typical. The oxidation, accelerated in alkaline solutions, involves a hypochlorite intermediate.



In the presence of base and excess chlorine, both α-chlorination and oxidation may occur. For example, a cyclohexanol-water-calcium carbonate mixture reacts to form 2-chlorocyclohexanone (Wagner and Zook, 1965a).

Chlorine also reacts with the sulfur in sulfides to form reactive sulfonium salts.



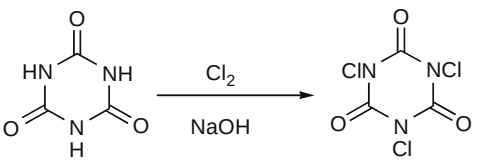
A reactive sulfonium salt

The reaction of chlorine with aqueous ammonia and ammonium salts is noteworthy since it leads to various chloramines, including the unstable (explosive) nitrogen trichloride, NCl_3 (Chloramines, 1999). Both Faraday and Dulong were injured working with this substance (Cardillo, 2001). The reaction of chlorine with phosphorus represents a commercial route to PCl_3 and PCl_5. In turn, this becomes an important process in the synthesis of nerve agent precursors (Greenwood and Earnshaw, 1986).

Chlorine reacts with many metals, often violently if the metal is finely divided, to form binary compounds such as iron (III) chloride, magnesium chloride, and so forth. Traces of water may accelerate the reaction. The product of the reaction of chlorine with calcium hydroxide is "bleaching powder." Although sometimes shown as a mixed salt, CaCl(OCl), bleaching powder also has been represented as a mixture of $Ca(OCl)_2$, $CaCl_2$, and $Ca(OH)_2$.

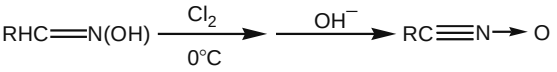
Chlorine is more potent an oxidant than is bromine but less so than fluorine (Clifford, 1961a). Thus, chlorine is able to oxidize both bromide and iodide ions to Br_2 and I_2, respectively, whereas fluorine oxidizes chloride, bromide, and iodide to their corresponding halogens. The oxidizing ability of chlorine is the basis for the starch-iodide test for chlorine. Here, a mixture of potassium iodide, soluble starch, and zinc chloride produces a blue-violet color in the presence of chlorine. Unfortunately, and like many "spot tests," this is a nonspecific test and gives a positive result with many oxidants, for example, bromine.

Trichloroisocyanuric acid (TCCA) (Tilstam and Weinmann, 2002), itself an interesting oxidant, can be prepared in approximately 75% yield by passing a stream of chlorine into an alkaline solution of cyanuric acid (Monson, 1971).



Cyanuric acid TCCA

In contrast to this imide-based synthesis, amides of the type $RC(O)NH_2$ are decarbonylated to primary amines (RNH_2) with chlorine in the presence of base. This process, often called the Hofmann reaction, involves an intermediate isocyanate (R–N = C = O) (Fieser and Fieser, 1961; Sandler and Karo, 1983). Aromatic oximes, lacking an α-hydrogen, react with chlorine to form intermediates that are converted to nitrile *N*-oxides with base. (Nitrile *N*-oxides are highly reactive species.)



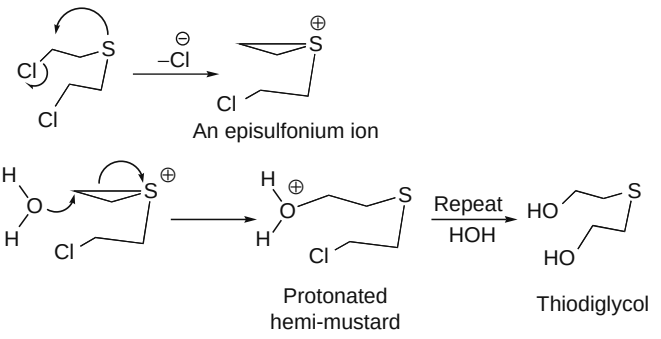
B. Sulfur Mustard—$C_4H_8Cl_2S$

General Remarks: Sulfur mustard (2,2′-dichloroethyl sulfide; bis-(2-chloroethyl) sulfide; CAS# 505-60-2) was introduced during WWI as a blister agent (vesicant). Although often called "HD" (distilled sulfur mustard) today, it also has been known as "Lost," "S-Lost," "Yellow Cross," "H" (a cruder form of sulfur mustard), "HS," and "Yperite." It is one member of a class of compounds known as mustards that generally bear a heteroatom (Z) separated from a leaving group (L) by two atoms, that is, contain the Z-$(CH_2)_2$-L fragment. Sulfur mustard has the structural formula $ClCH_2CH_2SCH_2CH_2Cl$. Nitrogen mustards, where Z = N, have been considered as anticancer drugs and CWAs but are not now considered as likely chemical warfare or terrorist threats. They are not discussed in this chapter.

Physical Properties: Sulfur mustard (mustard gas) is a colorless oil with bp of 227°C, mp of 14°C, molecular dipole moment 1.78 D (hexane), and molecular mass of 159. It normally is encountered as an impure, pale yellow–brown, odoriferous liquid. The color generally deepens with increasing amounts of impurity. HD has a vapor density of 5.4 relative to air and a vapor pressure of 0.072 mm Hg at 20°C. As a liquid, it is slightly denser than water (1.27 g/mL at 20°C). It is miscible in typical organic solvents (e.g., carbon tetrachloride, acetone or chloroform) but has a lower solubility in water (0.092 g/100 g at 22°C) (Sidell et al., 1998; Somani, 1992).

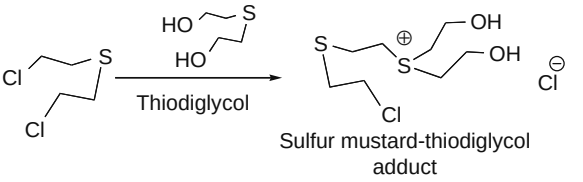
Chemistry: There are several routes to sulfur mustard. The first production of sulfur mustard in reasonable yield is often credited to Meyer (1886). A second route (Levinstein process) produces elemental sulfur as a contaminant in the mustard. The Steinkopf synthesis of sulfur mustard involves $S(CH_2CH_2OH)_2$. (Steinkopf et al., 1920).

The reactions of sulfur mustard are dominated by its heteroatoms. First, divalent sulfur is a good nucleophile and encourages S_N reactions (Whitman, 1995a). Sulfur's location β to a good leaving group (chlorine) leads to neighboring group participation (Eliel and Wilen, 1994) and, hence, to episulfonium ion formation. This internal nucleophilic displacement is favored by entropic factors. The resulting episulfonium ion possesses substantial small-ring strain and is equally prone to ring opening at either ring carbon. Using water as an external nucleophile, repetition eventually affords a diol mustard analog, thiodiglycol. The intermediate hemi-mustard possesses vesicant properties.

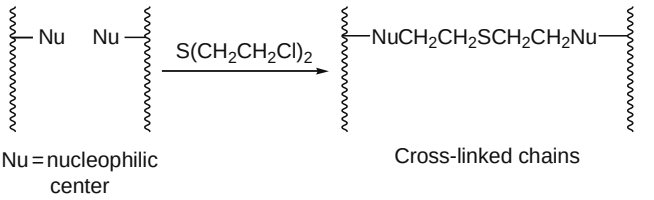


Structural variations that hinder episulfonium ion formation or sulfur nucleophilicity reduce activity. Thus, $S(C(O)CH_2Cl)_2$ is reported to show little vesicant behavior (Sartori, 1939).

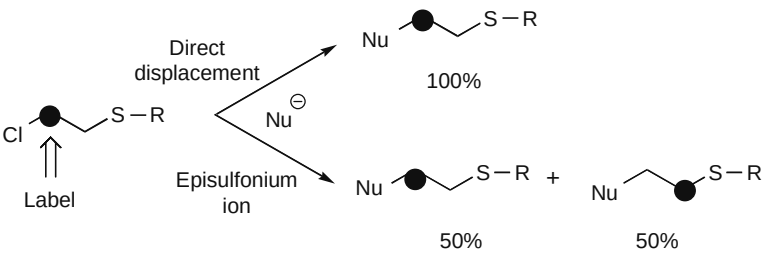
Mustard is denser than water, not very water soluble, and hydrolyzes rather slowly in cold water. Thus, it can remain a threat for sometime in bodies of water even though it ultimately hydrolyzes to the relatively safe thiodiglycol. Depending on specific conditions, the hydrolysis also can produce 1,4-thioxane ($O(CH_2CH_2)_2S$), 2-(vinylthio)ethanol ($CH_2{=}CHSCH_2CH_2OH$), and a variety of other compounds, some in quite minor amounts (D'Agostino and Provost, 1985). For example, mustard can react with thiodiglycol to form, depending on concentrations, mono- or disulfonium ions. One example in which the product is hazardous follows (Yang et al., 1992; Munro et al., 1999).



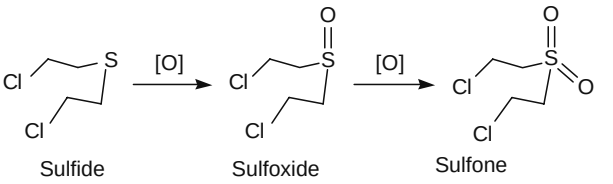
If the external nucleophile is attached to a chain, then two consecutive attacks on HD lead to cross-linking of two chains.



Molecules with more extensive separation between sulfur and leaving groups, such as chlorine (e.g., $Cl(CH_2)_6S(CH_2)_6Cl$), behave like simple aliphatic halides (or sulfides) since three-membered ring (episulfonium ion) formation is no longer possible. One convenient method for verifying the formation of an episulfonium ion intermediate involves isotopic carbon labeling. Since this ion is symmetric, it would ultimately lead to a nearly 1:1 distribution of an appropriately placed label, something not observed in a direct displacement.

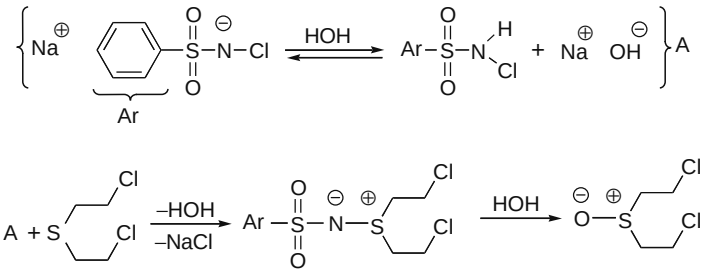


Sulfides, including HD, undergo oxidation at sulfur. Initial oxidation produces a sulfoxide, whereas further oxidation produces a sulfone (below). Oxidants include hydrogen peroxide, peroxyacids, nitric acid, permanganate ion, ozone, dinitrogen tetroxide, and dichromate ion. Oxides of mustard are not as volatile as mustard. This may help to explain why British air raid shelters were sometimes ''painted'' using the oxidant calcium hypochlorite ($Ca(OCl)_2$). ''Episulfonium'' ion formation is less likely in the sulfoxide of HD than in HD itself.

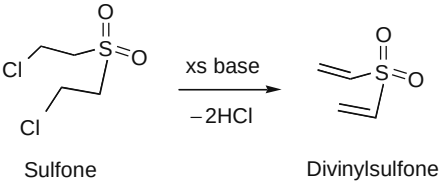


Titanium dioxide and iron oxide particles (anatase TiO_2 and ferrihydrite) have been explored as "detoxification" materials for threat agents including HD and nerve agents (soman and VX). (Kleinhammes et al., 2005; Ohtani et al., 1987). TiO_2 was found to be the more rapidly acting against HD and was able to convert HD into nontoxic materials with 99% effectiveness. Kinetic data for reactions involving both oxides and three threat agents at 25°C are provided. Analyses focused on the disappearance of threat agents, and identification of products was not provided. The authors claim that these oxides are more reactive than AP-MgO (Stengl et al., 2005; Kopeer et al., 1999).

Chloramine B oxidizes HD to its sulfoxide via an intermediate containing an S–N bond (Whitman, 1995b).



Strong bases (e.g., alkoxide) can dehydrochlorinate mustard and its sulfoxide and sulfone, converting one or both of their 2-chloroethyl fragments to vinyl groups.



Didehydrohalogenation of HD is reported to be complete within 1 min at room temperature using $CH_3OCH_2CH_2O^-$ as the base (Beaudry et al., 1992). This is a "component" of the decontaminating solution known as DS2 (a mixture of diethylene triamine, ethylene glycol monomethyl ether, and sodium hydroxide). Nanosize particles of calcium oxide have been shown to dehydrohalogenate HD (Wagner et al., 2000b).

Although anhydrous mustard is not a substantial corrosion threat to most metals, hydrolysis forms hydrochloric acid and does contribute to mustard's corrosive behavior. An interesting pmr and gas chromatograph–mass spectrometry (GC–MS) study of the hydrolysis of HD has been reported (Logan and Sartori, 2003). In this work, it was shown that hydrolysis (D_2O at 22°C) had a half-life of approximately 7 min, but that in the presence of sodium chloride, the half-life increased to approximately 24 min. These results are consistent with those reported by Bartlett and Swain (1949).

Sulfur mustard has been shown to react with nucleic acid components and the N7-guanine adduct studied by several techniques (Rao et al., 2002).

C. Cyanogen Chloride—ClCN

General Remarks: This substance CAS# 506-60-2 has several additional names, including (1) chlorine cyanide, (2) chlorocyanogen, (3) chlorcyan, and (4) CK (previously CC). It has been described as a ''blood agent,'' a term used to identify chemical threats first thought to act in the blood. They also have been thought of as ''systemic agents'' or ''systemic poisons.'' Generally, these contain the –CN fragment, form ''cyanide'' in the body, and include both hydrogen cyanide and cyanogen chloride.

Although ''cyanogen'' is a distinct molecule (below), that term sometimes is used to describe any substance that forms cyanide in the body. By this extended definition, cyanogen chloride is a cyanogen.

Cl–Cl	N≡C–Cl	N≡C–C≡N
Molecular chlorine	Cyanogen chloride	Cyanogen

The correct formula for CK was first established by Gay-Lussac in 1815. Cyanogen chloride can be viewed either as the monocyanide of molecular chlorine or as the chloride of cyanogen. (Note: Like CK, cyanogen is poisonous.)

Physical Properties: CK has a low bp (13°C), a mp of −6°C, and a molecular mass of 61.5. Under standard conditions (STP), CK has a vapor density of about 2.1, whereas that of HCN is 0.93. Its density at 0°C is 1.2. A colorless gas at room temperature, cyanogen chloride is often handled as a cylinder of liquefied gas. The volatility of cyanogen chloride renders it a ''nonpersistent'' threat agent. Cyanogen chloride shows solubility in water (69 g/L) and in most organic solvents (e.g., ethanol, chloroform, or benzene).

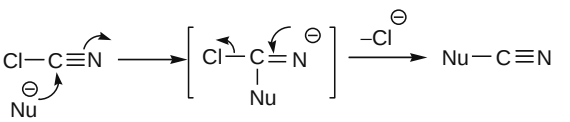
Chemistry: Cyanogen chloride's first reported preparation was by French chemist Claude Louis Berthollet (1789). Jennings and Scott observed that various conditions can influence the stability of impure ClCN (Jennings and Scott, 1919). A larger-scale synthesis of CK appeared shortly after the end of WWI (Price and Green, 1920). A typical synthesis involves the reaction of chlorine with an aqueous solution of sodium cyanide. A related anhydrous procedure yields the desired product with a density of 1.2 at 0°C. After purification, it can be stored (refrigerated) for months (Coleman et al., 1946).

$$Cl_2 + NaCN \rightarrow ClCN + NaCl.$$

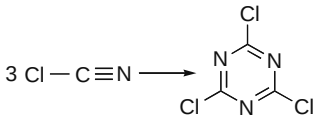
Commercially, ClCN has been produced, in the main, by the chlorination of aqueous HCN. An unintended synthesis of ClCN may occur during the treatment of cyanide-containing waste water with chlorine or hypochlorite. Aspects of this issue have been considered by Bailey and Bishop (1973a).

Trimethylsilylcyanide has been used to produce cyanogen chloride (Nachbaur et al., 1978). The reaction of active nitrogen and chloromethanes (e.g., carbon tetrachloride) has been reported to involve formation of cyanogen chloride (Sobering and Winkler, 1958).

Some of the reactions of cyanogen chloride are rationalized by the ''addition–elimination'' process shown below (''Nu'' represents a nucleophile). For example, diethyl malonate, $CH_2(CO_2C_2H_5)_2$, reacts with cyanogen chloride in the presence of sodium ethoxide to produce diethyl cyanomalonate, $CH(CN)(CO_2C_2H_5)_2$. This helps to explain why nucleophilic solvents, such as ethanol or water, eventually lead to the decomposition of CK.



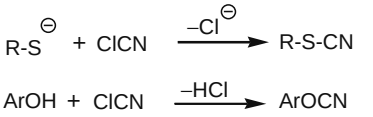
During storage, CK tends to form the cyclic trimer cyanuric chloride (cyanuryl chloride $(ClCN)_3$).



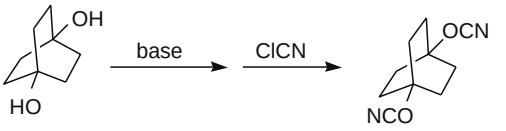
Cyanuric chloride

Cyanuric chloride, being less toxic than CK, is of reduced value as a CWA. To minimize this, the French produced an agent for use in WWI that they called Vitrite (also identified as Vivrite), a mixture containing 70% cyanogen chloride and 30% arsenic trichloride. CK also can be stabilized using sodium pyrophosphate ($Na_4P_2O_7$) (Kleber and Birdsell, 1959).

Cyanogen chloride reacts with sodium sulfide, Na_2S, to form sodium thiocyanate, NaSCN. An extension of this involves the reaction of thiolate anions, RS^-, with ClCN to form thiocyanates, RSCN. Similarly, the reaction of phenols produces aromatic cyanates (Kaupp et al., 1998). In such reactions, 3° amines are used to trap hydrogen chloride.



Some alkyl cyanates can be prepared by the reaction of alkoxide ions with cyanogen chloride. Their stability is enhanced by bulk around the OCN fragment since that inhibits subsequent trimerization (Kauer and Henderson, 1964).



The application of gas–solid reactions permits the synthesis of a variety of functionalities from ClCN (Kaupp et al., 1998). An example is shown below:

$$ArNH_2 + ClCN + N(Me)_3 \rightarrow ArNHCN + N(Me)_3H^+Cl^-.$$

CK hydrolyzes to form HCl and HOCl only slowly. However, the hydrolysis rate (Bailey and Bishop, 1973) increases in the presence of bases, such as sodium hydroxide. Although cyanogen chloride can be prepared by reacting cyanide with chlorine water, ClCN reacts with hypochlorite at pH 7–8 (Price et al., 1947). Complete hydrolysis (aqueous sodium hydroxide/heat) of cyanogen chloride produces ammonia, and the detection of this ammonia was the basis for an early, nonspecific test for ClCN. Ammonolysis of ClCN using ethanolic ammonia produces cyanamide, NH_2CN (Cloez and Cannizzaro, 1851):

$$ClCN + 2NH_3 \rightarrow NH_2CN + NH_4Cl.$$

Amines can be formed by the addition of cyanogen chloride to substituted alkenes (Smolin, 1955). The reaction occurs in the presence of sulfuric acid:

$$CH_3CH_2C(CH_3) = CH_2 + ClCN + H_2SO_4 \rightarrow CH_3CH_2C(CH_3)_2NH_2(23\%).$$

Cyanogen chloride reacts with sulfite ion as well as sulfur dioxide (Bailey and Bishop, 1973). With sulfur trioxide, ClCN adds to alkenes to produce sulfonyl chlorides (Arlt, 1970).

The unstable (explosive when dry) cyanogen azide is formed when sodium azide reacts with cyanogen chloride (Noller, 1966b):

$$NaN_3 + ClCN \xrightarrow{MeCN} NaCl + N_3 - CN.$$

A kinetic study of the shock tube decomposition of ClCN led to the conclusion that a set of six reactions are involved in this thermal decomposition (Schofield et al., 1965). Details of the valence shell photoionization of cyanogen halides have been published (Holland and Shaw, 2004), as have the UV absorption spectra of cyanogen halides (King and Richardson, 1966).

D. Hydrogen Cyanide—HCN

General Remarks: Other names for hydrogen cyanide, HCN, include (1) prussic acid, (2) formonitrile, (3) methan(e)nitrile, (4) Blausaure, (5) hydrocyanic acid, and (6) carbon hydride nitride. It has CAS# 74-90-8. The terms ''hydrocyanic acid'' and ''prussic acid'' are best limited to aqueous solutions of hydrogen cyanide. Liquid HCN mixed with substances designed to reduce polymerization (e.g., cyanogen chloride) was dubbed ''Zyklon B'' in Germany. Hydrogen cyanide is currently identified by the military as ''AC.'' Like CK, it is described as a ''blood agent'' and owes its toxicity to the liberation of cyanide ion (CN^-) under physiological conditions.

The importance of cyanides in commerce is indicated by the annual U.S. production capacity for HCN which, in 2004, was estimated to be over 1 billion pounds. Approximately one-half of the U.S. production is used to prepare adiponitrile for nylon 66. Its other uses include the preparation of acrylonitrile, methyl methacrylate (via acetone cyanohydrin), methionine, and cyanide salts.

Physical Properties (http://webbook.nist.gov): HCN is a highly toxic gas with a density of 0.94 relative to air. Its molecular dipole moment (gas) is 2.95 D, whereas that of cyanogen chloride (gas) is 2.80 D (McClellan, 1963). In these linear molecules, the nitrogen atom resides at the more negative end of the molecular dipole (Orville-Thomas, 1966). The liquid (density 0.687 g/mL at 10°C) has a low bp (26°C), a freezing point of −13°C, and a molecular mass of 27.0. It has a closed-up flash point of −18°C. AC is colorless as both a gas and liquid and has a bitter, almond-like odor. The odor threshold is estimated to be 2–5 ppm, although some cannot detect it by odor, even at lethal concentrations. Estimates suggest that 40%–60% of the population may not be able to detect HCN by its odor (http://web.princeton.edu). Its rather high vapor pressure (620 mm/Hg at 22°C) ensures that this is a nonpersistent CWA.

HCN is soluble in water, ethanol, chloroform, and benzene. Unlike sulfuric acid, hydrochloric acid, and so forth, hydrocyanic acid (i.e., aqueous HCN) is a rather weak acid with $pK_A = 9.32$ (Clifford, 1961b). An aqueous solution of HCN barely turns litmus red. While forming salts with strong bases, it does not with carbonates.

Chemistry: Due to its high toxicity, when needed for laboratory use, it is common for HCN to be used in situ. A typical procedure employing potassium cyanide and dilute sulfuric acid is outlined by Streitwieser and Heathcock (1976). A synthesis using sodium cyanide and sulfuric acid is available (Ziegler, 1941). HCN can be prepared commercially by several routes, including the reaction of ammonia/air with methane.

HCN is not completely stable and is marketed as a stabilized (often with H_3PO_4), flammable, anhydrous material. It reacts when heated, or in the presence of base or water, and may polymerize violently in contact with strong acids (e.g., sulfuric acid). Polymerization, once initiated, can be autocatalytic and, under confined conditions, lead to an explosion. It can be removed from waste streams by conversion to ammonium thiocyanate, a process involving scrubbing the waste stream with elemental sulfur in water (http://www.chemalliance.org).

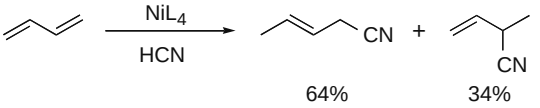
HCN undergoes many well-studied reactions (*Cyanides in Organic Reactions; A Literature Review*, 1962), the most famous of which may be addition to a carbonyl group (aldehyde or ketone). The products of these additions are called ''cyanohydrins'' or ''hydroxynitriles'' (Ziegler et al., 1990). This reaction, shown below, demonstrates the formation of a pair of enantiomers from an aldehyde. Rates of these addition reactions are pH dependent, maximum rates generally occurring at about pH 4–5. Substituents around the carbonyl group determine the position of such equilibria, steric hindrance destabilizing cyanohydrins.

Cyanohydrins derived from aldehydes are generally more stable than those from ketones (Ternay, 1976). Cyanohydrin formation is the first step in the well-known chain-lengthening sequence, the Kiliani–Fischer synthesis. For example, D-arabinose, an aldopentose, ultimately affords both D-glucose and D-mannose by this set of reactions (Carey, 2000b).

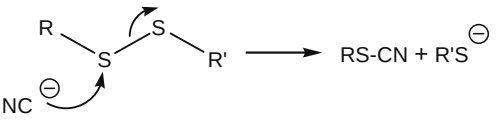
Cyanohydrin formation, being reversible and occurring in nature, is relevant to the potential hazards from ingesting cyanohydrins. Thus, *Prunus amygdalus dulcis* is a source of an oil used in confectionery. However, when crushed, the kernels of *Prunus amygdalus amara* yield an oil commonly known as ''oil of bitter almonds'' (*oleum amygdalae amarae*). On hydrolysis, the latter produces as much as 6% of hydrogen cyanide (http://en.wikipedia.org).

Conjugated aldehydes or ketones can add HCN by a 1,4-addition pathway (''conjugate addition'' or ''Michael addition'') and produce β-cyano carbonyl compounds. For example, 1-phenylpropenone ($C_6H_5C(O)CH{=}CH_2$) adds HCN to afford 4-oxo-4-phenylbutanenitrile ($C_6H_5C(O)CH_2CH_2CN$) in approximately 65% yield.

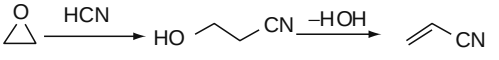
HCN also adds to alkenes in the presence of an appropriate catalyst (Arthur et al., 1954; Jackson and Lovel, 1982). Thus, cobalt carbonyl leads to Markownikov addition, for example, 1-propene yields isopropyl cyanide in approximately 75% yield. HCN adds to alkynes in the presence of metal complexes, and the use of a nickel complex may lead to syn addition (Jackson and Lovel, 1983; Jackson et al., 1988). Hydrogen cyanide reacts with conjugated dienes, the mechanism involving a π-allyl intermediate. The course of addition is complex and may lead to more than one product (Keim et al., 1982).



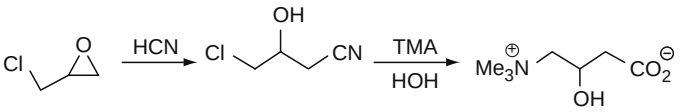
Cyanide ion is a good nucleophile and is able to cleave various bonds including the disulfide linkage (Gould, 1959).



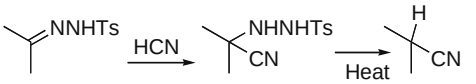
Epoxides can be ring opened by HCN. Thus, ethylene oxide reacts with HCN to form ethylene cyanohydrin (Mowry, 1948). A critical step in one commercial route to acrylonitrile involves the ring opening of ethylene oxide using HCN.



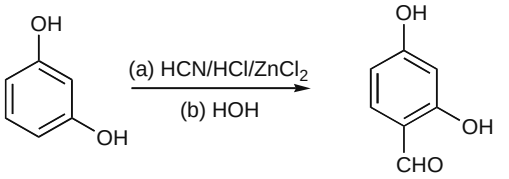
The ring opening of epichlorohydrin with HCN begins a sequence that leads to carnitine (Yamagutchi, 1989).



Ketones are converted to the corresponding nitrile by a sequence that begins with conversion of the ketone to a tosylhydrazone, followed by HCN addition across the C = N bond. Thermolysis of the intermediate 1-cyanohydrazine affords the cyanide in acceptable yields (Buehler and Pearson, 1976).



The Gatterman aldehyde synthesis involves a Friedel–Crafts-type reaction on activated aromatic rings to afford aryl aldehydes (Noller, 1966a).



The Ritter reaction is a method for creating amides beginning with alkenes or alcohols (2° or 3°) and cyanide (Reddy, 2003). Preparation of a formamide is shown below:

$$R_3COH + HCN \xrightarrow{H_3O^+} H - C(O)NHCR_3.$$

The interaction of methylphosphonocyanidate with HCN or CN^- has been described (Morozik et al., 2003).

The ability of cyanide to form strong complexes with various metals explains its use in extracting gold from gold-containing rock, a process called ''heap leaching'' (http://www.newmont.com). Cyanide ion reacts with iron salts to form complexes, such as is found in ferric ferrocyanide (also known as Prussian Blue, Hamburg Blue, Paris Blue, and mineral blue). The stronger affinity of cyanide for Fe(III) (ferric) than for Fe(II) (ferrous) ion provides the foundation for one therapy for cyanide poisoning (Baskin and Brewer, 1997). The binding of cyanide to carbonic anhydrase has been studied (^{13}C nuclear magnetic resonance [NMR]), and it was concluded that the unprotonated cyanide binds to zinc (Feeney and Burgen, 1973).

The color and bonding of cyanide complex ions has been summarized (Clifford, 1961). The infrared spectra of these complexes have been studied extensively (Rao, 1963; Bowser, 1993). The photoionization of hydrogen cyanide has been reported by Dibeler and Liston (1968), and the absorption spectrum of HCN has been compared with that of DCN (Nagate et al., 1981).

HCN adds chlorine to form cyanogen chloride that, in turn, trimerizes to cyanuric chloride (see ''Cyanogen chloride'' section). Catalytic (platinum catalyst) hydrogenation of hydrogen cyanide affords methylamine (Barratt and Titley, 1919). A catalyst (Ag or Au) can be used in the high-temperature oxidation of HCN using oxygen. Products of this include HOCN (cyanic acid) and lesser amounts of cyanogen, $(CN)_2$ (Zima, 1959).

The reaction of HCN with dilute aqueous sodium hydroxide can be used to produce sodium formate, and this reaction has served as the basis for an analytical method for measuring cyanide (Doizine et al., 1982).

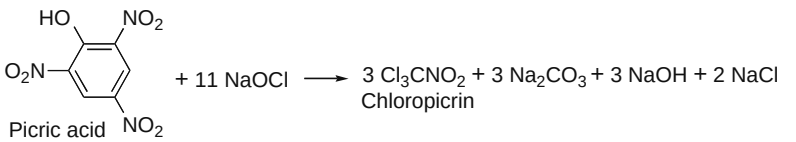
E. Chloropicrin—CCl_3NO_2

General Remarks: Alternate names for chloropicrin include (1) nitrochloroform, (2) trichloronitromethane, and (3) nitrotrichloromethane. During WWI, it was called ''Aquinite'' (French), ''vomiting gas'' (British), and ''Klop'' (Germans). Its CAS# is 76-06-2, and its military abbreviation is ''PS'' (or ''PK'' in older parlance). A mixture of 30% chloropicrin and 70% chlorine was called ''Yellow Star,'' which should not be confused with ''Yellow Cross,'' a term for sulfur mustard. ''Green Star'' was a mixture of chloropicrin (65%) and hydrogen sulfide (35%). PS was used during WWI as a tear gas and was found to be a useful fumigant by the end of the war. Now its use as a fumigant (Jackson, 1934) is almost phased out.

Physical Properties: Chloropicrin is a colorless oil, bp of 112°C, and mp of −69°C, with a pungent, stinging odor that has been described as ''anise-like.'' Its vapor pressure is approximately 20 mm/Hg at 20°C, and its molecular mass is 164 (Redeman et al., 1948). This liquid has a density of 1.66 g/mL and a vapor density of 5.6 relative to that of air. PS has low solubility in water (approximately 2 g/L), but is quite soluble in typical organic solvents (chloroform, acetone, ethyl acetate, etc.). Chloropicrin dissolves a variety of organic compounds including, for example, benzoic acid and various resins.

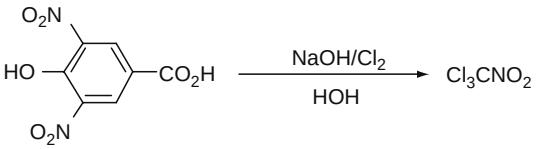
Although chloropicrin is nonflammable, contact with oxidizing agents may lead to fires or explosions. High temperatures can produce toxic gases, including phosgene and carbon monoxide, from PS.

Chemistry: Chloropicrin's first reported preparation, by the Scottish scientist John Stenhouse, was in 1848 and involved the reaction of a chlorinating agent (bleaching powder) with picric acid (*sym*-trinitrophenol). It is this synthesis that led to the name ''chloropicrin,'' even though the ring present in picric acid is absent in the product chloropicrin.



Another route to this compound involves the chlorination of nitromethane with chlorine in the presence of calcium carbonate (Orvik, 1980). The nitration of chloroform with nitric acid also has served as a route to chloropicrin on a large scale (Szmant, 1957).

The reaction of chlorine gas and aqueous sodium hydroxide with 4-hydroxy-3,5-dinitrobenzoic acid can be used to prepare chloropicrin (Simandi and Soos, 1986).



Chloropicrin does not decompose rapidly in either cold water or cold mineral acids (e.g., hydrochloric acid), is stable in cold, dilute aqueous sodium hydroxide, but slowly decomposes in ethanolic potassium hydroxide. It undergoes more rapid decomposition with sodium ethoxide or ethanolic sodium cyanide. As with many other highly chlorinated organics, PS's stability is decreased in the presence of light as evidenced by color formation in solutions in benzene. Solutions of PS in acetone decompose slowly and deposit ammonium chloride.

Slow thermolysis under reflux produces phosgene ($COCl_2$) and nitrosyl chloride (NOCl). Photolysis acts similarly (Ashmore and Norrish, 1951; Castro and Belser, 1981). Heating with 20% fuming sulfuric acid rapidly decomposes PS to form NOCl and phosgene. Chloropicrin is dehalogenated with aqueous sulfite (SO_3^{-2}) to form dichloronitromethane ($CHCl_2NO_2$) (Croue and Reckhow, 1989). In the absence of sulfite, hydrolysis of PS is slow and rather independent of pH.

Studies of the mass spectrum of PS exist (Murty et al., 2005). The vibrational spectra of bromo- and chloropicrin have been analyzed (Mason et al., 1959), and electron diffraction studies of chloropicrin have been reported (Knudsen et al., 1966).

Chloropicrin can be used to prepare orthocarbonates, $C(OR)_4$, the process involving four replacements by alkoxide ions (Hennig, 1937):

$$\underset{\text{chloropicrin}}{Cl_3CNO_2} + 4RONa \rightarrow \underset{\text{an orthocarbonate}}{C(OR)_4} + NaNO_2 + 3NaCl.$$

Similarly, chloropicrin reacts with iodide to form carbon tetraiodide (Kretov and Mehnikov, 1932).

Chloropicrin has been shown to react with iron-bearing clay minerals to produce both chloronitromethane and dichloronitromethane (Cervini-Silva et al., 2000).

An interesting discussion of the microdetermination of chloropicrin in air using a colorimetric procedure has been presented, and the authors demonstrated that analyses based on either chloride or nitrite afforded similar results (Feinsilver and Oberst, 1953).

F. Phosgene—$COCl_2$

General Remarks: Phosgene, perhaps the most effective CWA of WWI, also has been called (1) carbonyl chloride (or dichloride), (2) ''Collongite,'' (3) ''D-Stoff,'' (4) chloroformyl chloride, and (5) CG (Ryan et al., 1996). Its CAS# is 75-44-5. It is estimated that recent annual U.S. production demand is approximately 5400 million pounds. Its most important industrial use is in the production of isocyanates (R–N=C=O) (http://www.the-innovation-group.com).

Physical Properties: Phosgene is a colorless gas at room temperature with a density, relative to air, of 3.4. It has mp of −118°C, bp of 8°C, and vapor pressure of approximately 1200 mm at 20°C. It is slightly soluble in water, in which it hydrolyzes, and rather soluble in most hydrocarbons (e.g., hexane and benzene) and in glacial acetic acid. Phosgene has an odor of newly mown or ''musty'' hay. It is estimated that the least detectable odor occurs at approximately 1 ppm, but that the lowest concentration affecting the eyes is about 4 ppm.

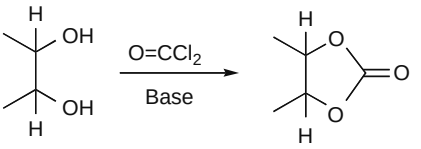
Chemistry: Phosgene can be prepared in the laboratory using a reaction similar to its commercial synthesis. In this gas-phase process, equimolar amounts of chlorine and carbon monoxide are passed over a bed of activated charcoal granules.

Phosgene is the acid dichloride of carbonic acid, HO–C(O)–OH, and, like all acid chlorides, reacts rapidly with water to produce the corresponding acid and hydrogen chloride. Since carbonic acid is unstable, the ultimate products of reaction with water are hydrogen chloride and carbon dioxide. The hydrogen chloride produced dissolves in excess water to form hydrochloric acid (corrosive).

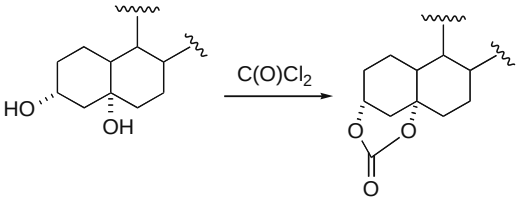
Accompanied by the loss of HCl, phosgene reacts with alcohols to produce either a chloroformate or a carbonate, depending on stoichiometry:

$$C(O)Cl_2 + ROH \rightarrow \underset{\text{a choloroformate}}{ClC(O)OR} \xrightarrow{ROH} \underset{\text{a carbonate}}{O=C(OR)_2}.$$

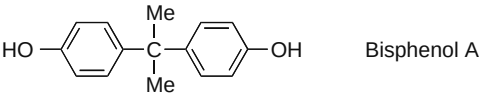
The reaction of phosgene with *vic*-diols (including sugars) leads to cyclic carbonates (Kawabata et al., 1986).



A similar reaction may occur with proximal diols (Le Boulch et al., 1967).

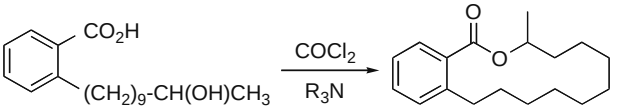


A number of important polymers, for example, polycarbonates, are synthesized using phosgene. For example, Lexan is made by reacting bisphenol A with phosgene in the presence of base.



Bisphenol A

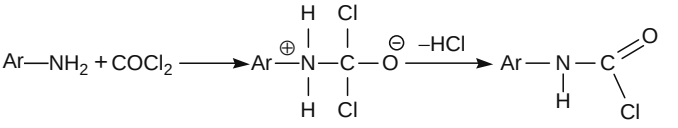
Phosgene also has been used to lactonize an hydroxyacid (Wehrmeister and Robertson, 1968) and to convert a carboxylic acid to its anhydride (nicotinic acid to its anhydride) (Rinderknecht and Gutenstein, 1967).



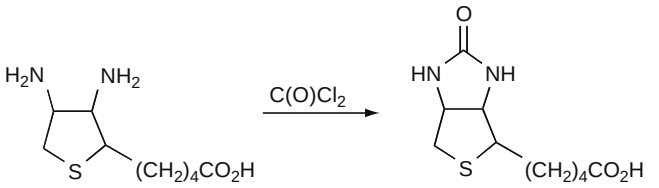
Chloroform produces phosgene when exposed to light and oxygen or air. For this reason, chloroform has sometimes been stabilized with traces of ethanol since it reacts with any phosgene impurity to form ethyl carbonate.

The reaction of phosgene with benzyl alcohol yields benzyl chloroformate (carbobenzoxy chloride), $ClCO_2CH_2C_6H_5$, useful in protecting (Ege, 1999) amino groups of amino acids. (Deprotection is accomplished with $H_2/Pd/C$.)

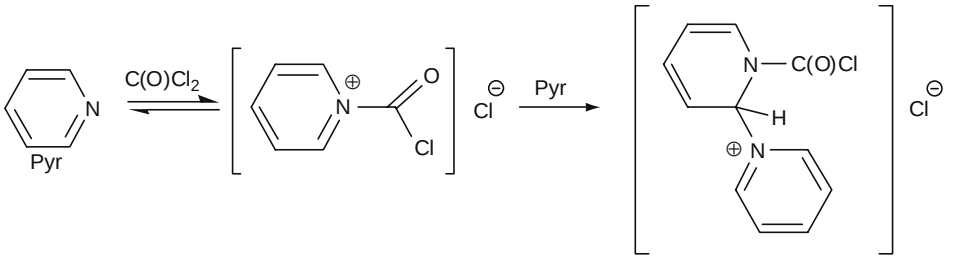
Reactions with nucleophiles other than alcohols can produce other carbonic acid derivatives. For example, reaction with anhydrous ammonia yields urea, $O=C(NH_2)_2$. Aromatic primary amines can, depending on reaction conditions, react with phosgene to form carbamoyl chlorides (Csuros et al., 1969).



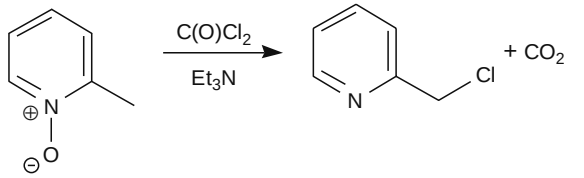
Isocyanate formation from diamines is commercially quite important since they are polymer precursors (Khardin and Pershin, 1979). Under appropriate conditions, proximal diamines can be converted to cyclic structures, the preparation of the biotin skeleton being representative (Marx et al., 1977).



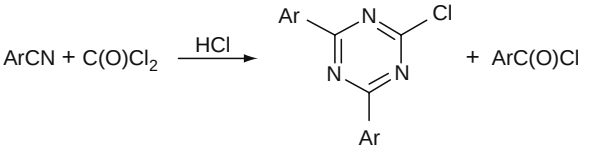
Pyridine reacts with phosgene to form a complex containing two pyridine fragments per carbonyl chloride.



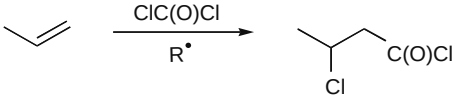
Some amine *N*-oxides are reduced by phosgene. Thus, in the presence of base, 2-methylpyridine *N*-oxide is deoxygenated with phosgene (Ash and Pews, 1981).



Aryl nitriles, ArCN, react with phosgene in the presence of HCl to yield triazines (Yanagida et al., 1969).

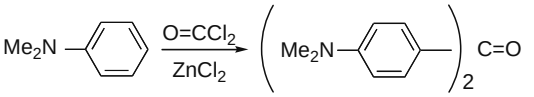


Like chlorine, phosgene can add to alkenes by a free radical process. The addition can be initiated using $CH_3CO_2^{\bullet}$.



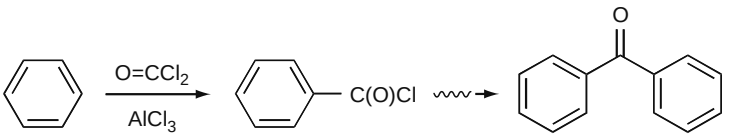
The photolytic cleavage of phosgene is believed to begin with a homolysis to form [COCl] and Cl. In turn, [COCl] rapidly decomposes to form carbon monoxide and a second chlorine atom (Wijnen, 1961).

Like other acyl chlorides, phosgene can participate in Friedel–Crafts reactions. A classic example is the synthesis of Michler's ketone, which is used to make some dyes (Robertson, 1937).



Michler's ketone

With less-reactive aromatics, a stronger Lewis acid may be required, but the monosubstituted acyl chloride can be isolated. Under appropriate conditions, a symmetric, disubstituted ketone is formed. Thus, benzophenone can be isolated by the reaction with excess benzene (Wilson and Fuller, 1922).



Benzophenone

III. NERVE AGENTS—GENERAL REMARKS

The ''nerve gas'' (Black and Harrison, 1996b; Carlsen, 2005) era in chemical warfare is rooted in attempts to develop improved pesticides (Ecobichon, 1991b). These organophosphates (OPs) have a central phosphorus atom bonded to three heteroatoms. In each of these, the tetrahedral phosphorus is surrounded by four different groups. Organophosphates can be divided into two general types, V and G agents. The common G agents (GA, GB, and GD) were developed shortly before or during World War II (WWII) and possess two oxygen atoms bonded to phosphorus. Development in Germany led to their being dubbed as ''G'' agents. The V agent, VX, is an alkylphosphonothiolate. It was developed in the early 1950s and contains sulfur, as well as two oxygens bonded to phosphorus. VX is less volatile, hence more persistent, than are the G agents, for example, sarin. Among the nerve agents under discussion, the general order of volatility is sarin (GB) > soman (GD) > tabun (GA) > VX. Chemically, GA has a cyano group bonded to phosphorus, whereas GB and GD have fluorine bonded to phosphorus. GA, GB, and VX exist as a pair of enantiomers, whereas GD, with two stereogenic (chiral) centers, exists as four stereoisomers. As would be expected, these sets of stereoisomers have different biological activities. Rate constants for anticholinesterase activity of soman, sarin, tabun, and VX have been summarized (Benschop and De Jong, 1988).

Much of the recent study of the chemistry (as opposed to biochemistry) of nerve agents has focused on routes to decomposition, decontamination, and destruction (Yang, 1995; Amitai et al., 2006). This also has led to studies of the analysis of various agents (Carrick et al., 2001; Cody et al., 2005). A description of enzyme inhibition by a variety of materials, including nerve agents, can be found in many modern biochemistry books (Mathews and Van Holde, 1996).

A. Nerve Agents—VX ($C_{11}H_{26}NO_2PS$)

General Remarks: Normally called VX, this nerve agent can be identified as (1) (*O*-ethyl *S*-[2-(diisopropylamino)ethyl)methylphosphonothioate; (2) *O*-ethyl *S*-[2-(diisopropylamino)ethyl) methylphosphonothiolate); or (3) methylphosphonothioic acid, *S*-[2-[bis(1-methylethyl)amino) ethyl *O*-ethyl ester. It bears CAS# 50782-69-9.

Physical Properties: With a molecular mass of 267.4, VX has mp $\leq$51°C, bp of 298°C, vapor pressure of 7×10^{-4} mm Hg at 20°C, and a density similar to that of water (1.008 g/mL at 20°C). It has a vapor density of 9.2 relative to air and is slightly soluble in water (approximately 30 g/L at room temperature). VX is both odorless and colorless.

Chemistry: The most extensively studied of VX's reactions are related to its decomposition (oxidation and hydrolysis) and this, in turn, may be related to its persistence. Hydrolysis occurs rather slowly (at pH 7; 25°C; $_{1/2} \approx 30$ days) and its products, of which there are several, are pH dependent. Thus P–S bond cleavage predominates at pH < 6 or > 10, but P–O cleavage is substantial in between. The former leads to 2-mercapto (*N,N*-diisopropyl)ethylamine, whereas the latter leads to *S*-(2-diisopropylaminoethyl) methyl phosphonothioate (Munro et al., 1999). Because nerve agents may hydrolyze over time (Yang et al., 1992), they have been stabilized for storage by moisture removal (Henderson, 2002):

$$\underset{\text{a diimide}}{\text{RN}=\text{C}=\text{NR}} \xrightarrow[\text{rapid}]{\text{HOH}} \underset{\text{a urea}}{\text{RNHC(O)NHR}}.$$

Oxidation is a potentially important route to destroying nerve agents. The oxidation reaction between VX and gaseous ozone has been shown to lead to a wide variety of products. In general, the most reasonable sites for initial oxidation of VX are sulfur or nitrogen, the former yielding a ''sulfoxide,'' with the latter affording an amine oxide. Oxidation in a polar medium enhances *S*-oxide formation, perhaps (at least in part) by stabilizing the adjacent dipolar (S–O and P–O) bonds. It has been reported that VX was oxidized to ''VX *N*-oxide'' before the subsequent oxidation or hydrolysis to *O*-ethyl methylphosphonate using a variety of oxidants (Cassagne et al., 2001).

The photochemical oxidation of both VX and sulfur mustard using TiO_2 or acetonitrile has been examined. Although effective, the reported quantum yields are somewhat low ($<0.3\%$) (Fox, 1983).

Highly reactive, nanosize magnesium oxide offers potential for the destruction of a VX, as well as GD and sulfur mustard. Half-lives for these processes were determined, as were decomposition products (Wagner et al., 1999).

It has been suggested that Cu(II) catalyzes the hydrolysis of both thiophosphoric esters and methylphosphonofluoridates (Ketelaar et al., 1956), although it does not significantly catalyze VX hydrolysis (Albizo and Ward, 1988). VX hydrolysis products may include a substance, EA2192, that is nearly as toxic as VX itself. The reactions of VX with a variety of reagents that should be capable of destroying it have been reviewed (Yang, 1999). The fate of VX in the environment, including the influence of solids on hydrolysis, has been studied (Davisson et al., 2005).

Supercritical water oxidation is of interest in the destruction of CW agents, including VX (Yesodharan, 2002).

B. Nerve Agents—GA (Tabun) ($C_5H_{11}N_2O_2P$)

General Remarks: Tabun and GA are the two most often used names for this nerve agent, although others include (1) *O*-ethyl *N,N*-dimethylphosphoramidocyanidate; (2) ethyl *N,N*-dimethylphosphoramidocyanidate; (3) Le-100; (4) *N*-Stoff; and (5) Trilon-83. It bears CAS# 77-81-6.

Physical Properties: Tabun is a colorless compound, which is said to have a somewhat fruity odor. The odor changes with decomposition so that with lesser amounts of decomposition the odor of cyanide (like bitter almonds) is apparent, whereas with greater amounts the odor of dimethylamine (like fish) is apparent. Tabun's vapor pressure is the lowest of all the G agents (approximately 0.04 mm Hg at 20°C). This suggests that tabun could be a relatively persistent threat at lower temperatures. Tabun has a vapor density of 5.6 relative to air and a liquid density of 1.08 g/mL (25°C). Its mp is -50°C, and its bp is approximately 240°C. It is three times as water soluble (approximately 10% at 20°C) as is VX, and also soluble in typical organic solvents (e.g., ethanol, diethyl ether, and chloroform). As with other nerve agents, dissolution in inert solvents (e.g., diethyl ether) enhances tabun's stability.

Chemistry: After its discovery in 1936, tabun was produced in Germany on a large scale in 1942 (Borkin, 1997). Although others exist, one synthesis begins with phosphorus trichloride and employs the Arbuzov reaction (Kosolapoff, 1950).

GA's acidic hydrolysis initially produces $C_2H_5OP(OH)(O)CN$ and dimethylamine (as salt) (Benes, 1963). The CN group is lost next, followed by the loss of the EtO fragment. The ultimate product of hydrolysis is phosphoric acid.

Base-induced hydrolysis (and direct water hydrolysis) of tabun has been studied (Sanchez et al., 1993; McNaughton and Brewer, 1994). Analyzed by GC–MS, a number of previously unrecognized materials have been observed (D'Agostino and Provost, 1992). Larsson (1952) has reported a spectrophotometric study of tabun hydrolysis.

Ultimately, depending on conditions (pH, reaction times, and so forth), tabun hydrolysis products may include: cyanide ion; hydrocyanic acid, the monoethyl ester of dimethylphosphoramidic acid, ethanol, dimethylamine, and phosphoric acid. Under acidic conditions the P–N bond is cleaved early, whereas under alkaline conditions the P–C bond cleaves much more readily than does P–N. Tabun has a half-life of about 7 h at pH 4–5, but this increases to about 8.5 h at pH 7. Tabun hydrolysis is less than 5% complete after 20 h at pH 8.5.

Tabun has a stereogenic (chiral) phosphorus atom and exists as a pair of enantiomers. A gas chromatograph study of the enantiomers of tabun has been reported (Degenhardt et al., 1986). Separation was achieved through the use of bis[(1*R*)-3-(heptafluorobutyryl-camphorate)nickel(II). This approach also separated stereoisomers of both sarin and soman. These authors also reported the stereospecific hydrolysis of racemic tabun using phosphorylphosphatases. They noted the species (mouse, rat, horse) dependence of the hydrolysis. Dilute solutions of tabun in inert solvents (e.g., carbon tetrachloride) exhibit optical stability for months at −25°C.

Shift reagents have aided in the study of pmr spectra of tabun and other nerve agents (Van Den Berg et al., 1984). Two-dimensional NMR (^{1}H-^{31}P) has been applied to samples containing tabun (Albaret et al., 1997).

C. Nerve Agents—GB (Sarin) ($C_4H_{10}FO_2P$)

General Remarks: GB carries several names including (1) isopropoxymethylphosphoryl fluoride, (2) isopropylmethylphosphonofluoridate, (3) methylphosphonofluoridic acid, 1-methylethyl ester, (4) Trilon-46, (5) N-stoff, and (6) T-144. It has CAS# 107-44-8.

Physical Properties: This colorless and odorless liquid has a molecular mass of 140.1 and a density of approximately 1.1 g/mL (20°C). Its mp is −57°C and its bp is approximately 147°C. Sarin is the most volatile of the G agents (vapor pressure 2.10 mm Hg at 20°C). It has been reported that sarin will evaporate from a sandy surface in about 2 h at 10°C (Sidell et al., 1998).

Chemistry: Like tabun, sarin was first prepared (1938) by Schrader's group at I.G. Farben. An early synthesis involved the use of methylphosphonic dichloride ($CH_3P(O)Cl_2$) as a starting material. An interesting route involves the reaction of a tetraalkoxysilane, $(RO)_4Si$, as a source of the alkoxy fragment in GB (Black and Harrison, 1996a).

The preparation of both nonradioactive (Bryant et al., 1960) and radioactive (at P) sarin has been reported (Reesor et al., 1960). Optically active (o.a.) sarin has been prepared using the enantiomeric sodium salts of *O*-isopropyl methylphosphonothioic acid. It has been found that o.a. sarin racemizes in less than 1 day when stored at 25°C in a polarimeter tube. On the other hand, dilute solutions in some solvents exhibit greater optical stability (Boter et al., 1966).

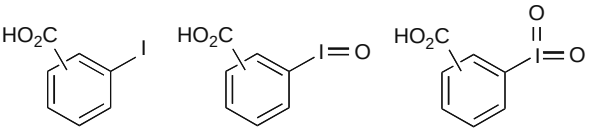
GB is both miscible with water and hygroscopic, and like GA, is subject to both basic and acidic hydrolysis. The initial step in these hydrolyses involves loss of fluoride. Hydrolysis of the C–F bond in both GB and GD (soman) is accelerated by the presence of bleach (hypochlorite ion), although the process is complex and both pH and concentration dependent (Epstein et al., 1956).

The effect of micelle formation on the hypochlorite-induced decomposition of toxic esters of phosphorus has been examined (Dubey et al., 2002). The presence of the calcium and magnesium cations in sea water appears to accelerate the hydrolysis (Demek et al., 1970).

The reaction of sarin with hydrogen chloride has been reported and kinetics determined by H^1 NMR imaging (Bard et al., 1970). With rate constants determined at 25°C, 81.5°C, and 100°C, Arrhenius analysis led to a calculated activation energy of 17.8 kcal/mole. The base-induced hydrolysis of sarin analogs and tabun was studied by Larsson (1958b) and the half-life of GA has been estimated to be 1.5 min at pH = 11 at 25°C. Ultimately, and depending on conditions (pH, reaction times, and so forth), hydrolysis products may include fluoride ion (or hydrogen fluoride), the 1-methylethyl ester of methylphosphonic acid, methylphosphonic acid, and 2-propanol.

(+)-Sarin is reported to be a weaker inhibitor of acetylcholinesterase (AChE) than is the enantiomer (Boter et al., 1966). Racemic sarin is reported to have an LD_{50} that is twice that of the (−) enantiomer. The kinetics of the fluoride-induced racemization of sarin is available (Christen and Van Den Muysenberg, 1965).

Both sarin and soman react with aqueous $KHSO_5$ to produce the corresponding phosphonic acids (Yang et al., 1992). Valuable studies of the use of hypervalent iodine derivatives (below) to hydrolyze phosphorus esters have been reported (Moss et al., 1983, 1984, 1986; Katritzky et al., 1988).



Iodobenzoic acid Iodosobenzoic acid Iodoxybenzoic acid

Sarin undergoes thermolysis–dealkylation on reflux. After approximately 80 min at elevated temperatures, the decomposition is essentially complete (Hoffman and Reeves, 1961).

At substantially higher temperatures (300°C–400°C), pyrolysis of sarin in the air leads almost exclusively to propylene and methyl phosphonofluoridic acid. Over platinized alumina, there are formed stoichiometric amounts of phosphoric acid, water, hydrogen fluoride, and carbon dioxide (Baier and Weller, 1967).

Several reports exist on the effect of metal cations on sarin hydrolysis (Wagner-Jauregg et al., 1955; Gustafson and Martell, 1962; Epstein and Mosher, 1968). Some of the metal ions that appear to be effective include Cu(II), Au(II), Ag(I), Ni(II), and Zn(II). The effect of hypochlorite on sarin decomposition has been studied (Epstein et al., 1956), including the effect of surfactant micelles on the process (Dubey et al., 2002). It was shown that the use of cetyltrimethylammonium bromide substantially increased the speed with which sarin was destroyed by hypochlorite. The effect of surfactant on the oxidation of sarin (and other OPs) with *o*-iodosobenzoic acid has been examined (Hammond et al., 1989). As part of an extensive study of the reactivity of nerve agents, Larsson (1958a) conducted a study of hydrogen peroxide's reaction with sarin.

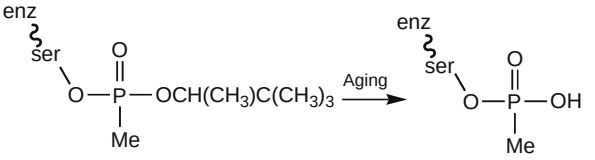
There is an interest in sorbents that can destroy chemical threats. A study at 23°C of the reactivity of DFP (diisopropyl fluorophosphate, a nerve agent simulant) with (1) γ-alumina, (2) Ambergard XE-555, and (3) base-impregnated divinylbenzene has been reported. Analyses were accomplished using ^{31}P MAS NMR spectra. Divinylbenzene-based systems were the most reactive against DFP (Wagner and Bartram, 1999). Study of the use of enzymes to hydrolyze G agents is an active area of research (Cheng et al., 1999). The toxic solution used in the Tokyo subway attack was analyzed for sarin quantitatively using ^{31}P-NMR (Miyata and Ando, 2001).

D. Nerve Agents—GD (Soman) ($C_7H_{16}FO_2P$)

Other names for this agent include (1) *O*-pinacolyl methylphosphonofluoridate, (2) pinacolyl methylphosphonofluoridate, (3) EA1210, (4) methyl pinacolyl phosphonofluoridate, (5) methyl pinacolyloxy phosphoryl fluoride, (6) T-300, and (7) PMFP. It carries CAS# 96-64-0. Thickened soman (TGD) (aka VR-55) is created by adding thickener to GD.

Physical Properties: Pure soman is a colorless liquid with a somewhat fruity odor. It has density 1.01 g/mL (20°C), vapor pressure of 0.27 mm/Hg (20°C), mp of −80°C, and bp of 190°C (85°C at 15 mm/Hg). Distillation is accompanied by decomposition that begins near 130°C. Soman's solubility in water is about 20% at 25°C. It is only about 20% as soluble in water as is tabun. With two different chiral centers, it exists as four stereoisomers (Benschop et al., 1985), each with a different toxicity. The lethal concentration (inhalation) in humans is estimated at approximately 25–50 mg min/m^3 (Somani et al., 1992).

Chemistry: The synthesis of soman is similar to that of sarin. The major alteration is that pinacolyl alcohol replaces isopropanol. Soman hydrolyzes over a range of pHs, with pinacolyl methylphosphonate and fluoride ions being the initial products. Subsequent hydrolysis of pinacolyl methylphosphonate is similar to the aging process associated with GD poisoning (below) (Michel et al., 1967).



In water (pH = 7; 20°C), the half-life for hydrolysis is approximately 80 h. Hydrolysis of this nerve agent is catalyzed by several reagents. The pH dependence of the hydrolysis of soman has been examined (Ward et al., 1988). Hydrolysis has a half-life of 45 h at pH 6.65 (25°C) but is complete within minutes in 5% aqueous sodium hydroxide (lye). The effect of metal ions (Cu(II), Ce (II), and Mn(II)) on the hydrolysis (25°C) of soman has been noted (Epstein and Rosenblatt, 1958).

It has been reported (Wagner et al., 2000a) that GD (neat) does not react with gaseous ozone even after 1 h exposure. This is in contrast to the report of oxidation that was conducted using ozone-containing fluorocarbons (Snelson et al., 1986). Photochemical reactions in sunlight do not appear to be important for soman.

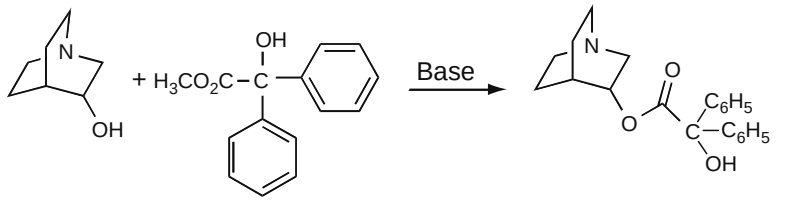
Aging: Nerve agents inhibit AChE by forming an adduct with the enzyme via a serine residue on that enzyme. These adducts may be decomposed hydrolytically or, for example, by the action of some oximes and thereby regenerate the enzyme. A second reaction type, one in which the enzyme–OP complex undergoes a subsequent reaction, is usually described as ''aging'' (Bencsura et al., 1995). Once the enzyme–OP complex has aged it is no longer regenerated by the common, oxime reactivators. The rate of aging depends on the specific OP, with soman aging extremely rapidly (a few minutes) (Berry and Davies, 1966). Due to the implications in treating nerve agent exposure, there are numerous studies of the process (Coult et al., 1966; Bucht and Puu, 1984; Curtil and Masson, 1993; Segall et al., 1993; Shafferman et al., 1997; Kovach, 2004; Worek et al., 2004).

E. BZ ($C_{21}H_{23}NO_3$)

General Remarks: BZ, also known as (1) QNB, (2) 3-quinuclidyl benzilate, and (3) benzilic acid, 3-quinuclidinyl ester, is a synthetic glycolic ester. Its medical action is as a competitive muscarinic cholinergic receptor antagonist, that is, an incapacitating agent. Like other such agents, its purpose is to produce temporary disability, not death (Wiener and Nelson, 2004). Indeed, the onset of symptoms may not appear until several hours after exposure.

Physical Properties: With one chiral center, BZ exists as a pair of enantiomers. The racemic mixture, a white solid, has mp of 164°C–166°C (Whitaker, 1966). It has a low vapor pressure, and in the environment, has an estimated half-life of approximately 1 month (25°C). It has low solubility in water but is soluble in typical, polar organic solvents. Protonation of nitrogen enhances its solubility in dilute, aqueous acids. With a flash point of 246°C, it has significant thermal stability but is slowly hydrolyzed in refluxing aqueous solutions. The single crystal x-ray analysis of the hydrobromide salt has been reported (Meyerhoeffer and Carlstroem, 1969).

Chemistry: The transesterification of methyl benzilate with 3-quinuclidinol provides a convenient route to this ester.



3-Quinuclidinol Methyl benzilate BZ

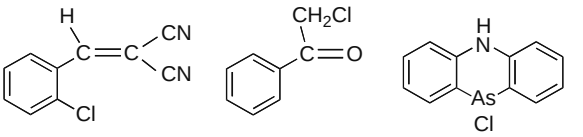
Like most bioactive substances that exist as enantiomeric pairs, the BZ enantiomers have different magnitudes of biological activity. The individual enantiomers have been prepared by beginning with the enantiomers of 3-quinuclidinol (Rzeszotarski et al., 1988). The *R* enantiomer exhibits a higher affinity for the muscarinic receptor than does the *S* enantiomer (guinea pig isolated ileum) (Lambrecht, 1979).

Both ^{18}O- and ^{11}C-labeled analogs of BZ have been synthesized (Calvin et al., 1949). The source of the carbon label was labeled carbon dioxide (Prenant et al., 1989). The oxygen label was introduced as a result of reacting enriched water with quinuclidinone and then reducing the labeled ketone with borohydride (Sniegoski et al., 1989). The synthesis of a number of tritium labeled "neurochemicals," including BZ, has been reported (Bloxsidge et al., 1981).

The (ester) hydrolysis of BZ over a range of conditions of pH and temperature has been investigated (Hull et al., 1979). Bromocresol Green forms a colored complex with 3-quinuclidinyl esters of hydroxyacetic acids, which has been used in the spectrophotometric analysis of mixtures containing these esters and 3-quinuclidinol (Stan'kopv et al., 1997). Analysis of the mass spectra of a number of quinuclidine derivatives is available (Vincze et al., 1980). BZ has been safely destroyed pyrolytically (Jensen, 1991).

F. CS ($C_{10}H_5ClN_2$)

General Remarks: CS is a lacrimator and is used as a riot-control agent. It is one member of a group of compounds that includes, for example, 1-chloroacetophenone (CN), and 10-chloro-5,10-dihydrophenarszine (DM). These agents rapidly (seconds to minutes) induce irritation of the eyes and upper respiratory tract.



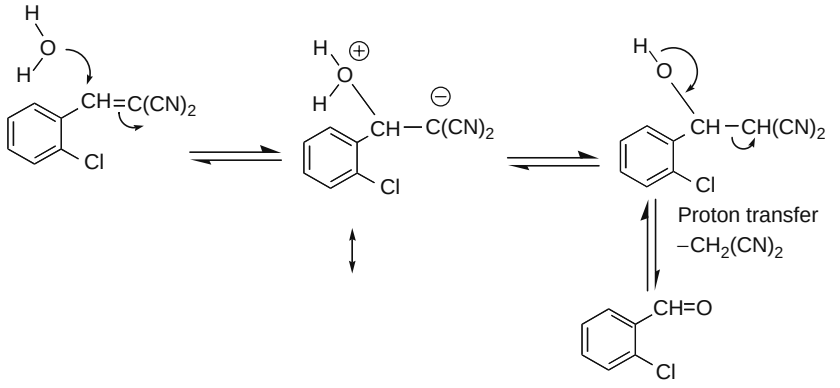
CS CN DM

Alternate names for CS include: (1) 2-chlorobenzylidene malononitrile; (2) *o*-chlorobenzylidenemalonitrile, (3) [(2-chlorophenyl)methylene]propanedinitrile, and (4) β,β-dicyano-*o*-chlorostyrene. Its CAS# is 2698-41-1.

Physical Properties: This white solid has molecular mass of 188.6, mp of 95°C, bp of 313°C, and vapor pressure at 20°C of approximately 3.5×10^{-5} mm/Hg. It has a relatively low water solubility (52 mg/L at 25°C), but is soluble in typical polar organic solvents.

Chemistry: A reliable synthesis of CS was reported in 1928 by Corson and Stoughton. The procedure involved condensation of a starting aldehyde with malononitrile and was catalyzed with base. Even uncatalyzed reactions eventually were successful, although significantly slower.

The sequence below is written to illustrate hydrolysis but the reverse represents the (uncatalyzed) synthesis of CS and similar α,β-unsaturated dinitriles (Corson and Stoughton, 1928).



Unlike simple alkenes, CS has a rather rapid rate of hydrolysis ($t_{1/2} = 15$ min at 25°C), and this is accelerated by the use of dilute, aqueous sodium hydroxide (Brooks et al., 1976). Potassium permanganate (aqueous) oxidizes CS and similar α,β-unsaturated dinitriles to the corresponding benzoic acids.

It has been reported that aerosol exposure to CS leads to an increased excretion of thiocyanate. This, along with other data, suggested that cyanide is released after CS intraperitoneal administration (Frankenberg and Sorbo, 1973).

The thermal (300°C, 500°C, 700°C, and 900°C) decomposition of CS has been shown to produce 14 products, including quinoline, *o*-dicyanobenzene, 2-chlorobenzaldehyde, 2,2-dicyano 3-(2-chlorophenyl)oxirane, and 2-chlorocinnamonitrile. The higher temperatures led to a greater diversity of products. Analysis was done by GC–MS (Wils and Hulst, 1985; Kluchinsky et al., 2002).

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3 Chemical Warfare Agent Threat to Drinking Water

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CONTENTS

I. INTRODUCTION

The use of chemical warfare agents (CWAs) and other weapons of mass destruction (WMD) by terrorists to contaminate military drinking-water supplies and our domestic water systems is a reality. In addition to the WMD (which include Chemical, Biological, Radiological, Nuclear, and Explosive—CBRNE), toxic industrial chemicals (TICs) and toxic industrial materials (TIMs) are also of concern. TIMs, unlike TICs, which have a defined chemical structure, do not consist of an exact or constant composition of a particular chemical, but a variety or mixture of many chemicals. Examples of TIMs include asbestos (variety of fibers and minerals) and gasoline (composition varies with octane level, manufacturer, and season of manufacture) (Salem, 2006). On February 13, 2001, President George W. Bush, in an address at the Norfolk Naval Air Station in Norfolk, Virginia, stated "... the grave threat from nuclear, biological, and chemical weapons has not gone away with the Cold War. It has evolved into many separate threats, some of them harder to see, and harder to answer. And the adversaries seeking these tools of terror are less predictable and more diverse" (Bush, 2001). Water infrastructure systems are highly linked with other infrastructures, especially electrical power, transportation, and the chemical industry, making security of all of them an issue of concern (Copeland and Cody, 2006). The spectrum of Chemical and Biological warfare agents that need to be considered as threats to both military and civilian populations is

The contents of this chapter are not to be construed as an official Department of the Army position, unless so designated by other authorizing documents.

TABLE 3.1
Chemical and Biological Warfare Agents' Threat Spectrum

Clearly Chemical Warfare Agents	Midspectrum Warfare Agents			Clearly Biological Warfare Agents
Classic Chemicals	**Emerging Chemicals**	**Bioregulators**	**Toxins**	**Pathogens**
Blood	Protection defeating	Pain	Plant	Bacteria
Vesicant	Physical incapacitant	Sleep	Bacterial	Viruses
Nerve	Nontraditional agents (NTAs)	Blood pressure	Venom	Rickettsiae
Incapacitants		Mood enhancers	Marine	Genetic engineered
Psychological			Fungal	microorganisms
Riot control agents			Algal	
Choking				
Toxic industrial chemicals				

presented in Table 3.1 (Salem, 2003). This spectrum is from the clearly chemical to the clearly biological, with midspectrum agents such as emerging chemicals, including those that are protection defeating, physical incapacitants, and nontraditional agents, as well as bioregulators producing pain, sleep, mood enhancers, and blood pressure effects, to toxins from plants, bacteria, or animals (Salem et al., 2004).

II. BACKGROUND

The U.S. Army has had a long-standing concern about deliberate contamination of military drinking water, which has previously been addressed in collaboration with Lawrence Livermore National Laboratories (LLNL) and reported in 1990 (Daniels, 1990). Military drinking water includes source, treated, stored, and distributed water. In addition to drinking, these waters may also be used for cooking, washing, bathing, and showering. Thus, the routes of exposure could be oral, cutaneous, and inhalation. Contamination of the water supply could also occur by military action, deliberately directed to, or falling into the water supply, or by vandals or terrorists directly contaminating the water. In collaboration with LLNL, Tri-Service Standards (TSS) were developed by the Army in the 1980s and reported in 1990 (Daniels, 1990). These standards were developed as a defensive measure to protect military personnel in the event of a chemical attack with the nerve agent values based on a 50% reduction of red blood cell (RBC) cholinesterase activity (Dacre and Burrows, 1988; Daniels, 1990; Palmer, 1990). The National Research Council (NRC) Committee on Toxicology (COT) reviewed the adequacy and toxicity of the CWAs, and concluded that the TSS should be considered as guidelines for levels that should neither cause acute adverse health effects, nor cause degradation of military performance following ingestion for up to 7 days (NRC, 1995). The levels of contamination were based on 5 and 15 L of water ingested per day, representing consumption during normal and stressful activity, respectively, by our military personnel. The COT accepted the LLNL proposed standards, except for the nerve agents tabun (GA), sarin (GB), soman (GD), and VX, which they recommended be cut in half, corresponding to a 25% reduction in RBCs cholinesterase activity. The Army Medical Department did not accept the COT recommendation for nerve agents and a document supporting this position was prepared in late 1996 and signed by all of the service Surgeons General, justifying the looser standards. Among the supporting data was a study by Bauer et al. (1949). In their study of the effects of the oral ingestion of GB, rats that drank water contaminated with as much as 16 ppm GB for 9 days, showed relatively few signs of GB poisoning. Although the cholinesterase levels in their blood had dropped almost to zero, the animals were apparently healthy, lost no weight, and showed no gross or microscopic pathological

changes after sacrifice (Bauer et al., 1949). The North Atlantic Treaty Organization (NATO) has also accepted these U.S. military standards (STANAG 2136, 2002). These TSS values (TB MED 577, 2005) are presented in Table 3.2. These recommended standards were neither considered applicable to civilian populations, nor to water-quality standards for drinking water treated at properly functioning fixed installations (Daniels, 1990).

The literature reports that deliberate chemical and biological contamination of water supplies has been common in history. These have ranged from crude dumping of human and animal cadavers into water supplies to well-orchestrated contamination with chemicals and biologicals such as rye ergot, hellebore root as well as anthrax and cholera. The crude contamination of water sources, including wells and reservoirs, meant for armies and civilian populations, with filth, cadavers, animal carcasses, and contagious materials, dates back to antiquity and continues even today (Christopher et al., 1997). It has been reported that as far back as 600 BCE the Assyrians poisoned wells with rye ergot, and Solon of Athens poisoned drinking water with hellebore roots during the siege of Krissa. The Cirrhaens drank the water and developed violent and uncontrollable diarrhea and were quickly defeated (Smart, 1997; U.K. MOD, 1999; Noji, 2001; Robey, 2003; Tschanz, 2003). During the Peloponnesian War in 430 BCE, when plague broke out in Athens, the Spartans were accused of poisoning most of the water sources in Athens (Tschanz, 2003). Persian, Greek, and Roman literature from around 300 BCE quote examples of dead animals used to contaminate wells and other sources of water (Dire, 2003). About 200 BCE, the Carthaginians spiked wine with mandrake root to sedate the enemy (Smart, 1997; U.K. MOD, 1999). For thousands of years, cyanide has been used as a waterborne poison. In ancient Rome around 50 CE, Nero eliminated his enemies with cherry laurel water, which contains hydrocyanic acid (Hickman, 1999). During the battle of Tortona in 1155 CE, Barbarossa used bodies of dead and decomposing soldiers to poison the enemy's water supply (Smart, 1997; U.K. MOD, 1999; Dire, 2003). During the American Civil War in 1863, the confederate troops, under General Johnston, contaminated wells and ponds with animal carcasses during their retreat from Vicksburg. General Sherman and his troops, in pursuit of the Confederates, found the water en route unfit to drink (Harris, 1994; Smart, 1997; U.K. MOD, 1999; Robey, 2003). In World War II, the Japanese attacked at least 11 Chinese cities, intending to contaminate food and water supplies with anthrax, cholera, and various other bacteria. Hitler's forces released sewage into a Bohemian reservoir, deliberately sickening the population. In deliberate violation of the Geneva Convention, Yugoslav federal forces or those allied with them appeared to have poisoned wells throughout Kosovo in October and November of 1998. They dumped animal carcasses and hazardous materials such as paints, oil, and gasoline in most of the wells in the area, deliberately sickening the populace and denying them the use of the wells (Smith, 1998; Hickman, 1999). Earlier, in 1939 and 1940, over 1000 Manchurian wells were laced with typhoid bacteria that were effective killers. Outbreaks of typhoid fever devastated many villages. About forty 13–15 year old youngsters who drank lemonade made from the contaminated well water contracted typhoid fever and subsequently died (Harris, 1994). In 1970, the Weather Underground, a revolutionary movement, planned to put incapacitating chemical and biological warfare agents into urban water supplies to demonstrate the impotence of the federal government (Tucker, 1999). A group of college students, influenced by ecoterrorist ideology and the 1990s drug culture, wanted to kill most of humanity to prevent the destruction of nature and restart the human race all over again with a select few. Their plan to use aerosol attacks to contaminate urban water supplies with eight microbial pathogens, including agents of typhoid fever, diphtheria, dysentery, and meningitis, was aborted upon discovery of these agents (Tucker, 1999). In 1980, the Marxist revolutionary group called the Red Army Faction, had cultivated botulinum toxins in a safe house in Paris, which was discovered along with plans to poison water supplies in Germany (Tucker, 1999). In spite of this historical background, various governmental agencies have conducted discordant assessments and have failed to evaluate the risk of sabotage contaminating water supplies with chemical and biological warfare agents. The President's Commission on Critical Infrastructure Protection (PCCIP) indicated that most water-supply systems in the United States are vulnerable to

TABLE 3.2
Tri-Service Standards (TSS) in Drinking Water (Chemical and Biological Agents)

Nerve Agents	TSS (mg/L) 2 L/day	5 L/day	15 L/day	Target Dose for Detection (mg/L)	Daily Human Oral Dose Ingested (mg/kg/day)	Estimated Human Oral Acute LD_{50} (mg/kg)	Average % of Estimated Human Oral Acute LD_{50}
GA (tabun)	0.350	0.140	0.046	0.046	0.01	0.357–0.714[1,2]	2.1
GB (sarin)	0.070	0.028	0.009	0.009	0.002	0.071–0.286[1,2]	1.8
GD (soman)	0.030	0.012	0.004	0.004	0.0009	0.071–0.286[1,2]	0.8
GF (cyclosarin)[a]	0.030	0.012	0.004	0.004	0.0009	0.14[2]	0.6
VX	0.037	0.015	0.005	0.005	0.001	0.043–0.143[1–3]	1.5
Blood Agent							
HCN (hydrogen cyanide)	15	6	2	2	0.43	0.71–1.43[4] 2.9[22]	45
Vesicants							
HD (distilled mustard)	0.35	0.14	0.047	0.047	0.01	100[5]	0.01
HL (mustard/Lewisite)[a]	0.20	0.08	0.027	0.027	0.006	30[5]	0.02
HN_3 (nitrogen mustard)[a]	0.35	0.14	0.047	0.047	0.01	10[5]	0.1
HT (60% HD and 40% T)[a]	0.35	0.14	0.047	0.047	0.01	100[5]	0.01
L (Lewisite)	0.20	0.08	0.027	0.027	0.006	30[5]	0.02
Incapacitating Agent							
BZ (3-quinuclidinyl benzilate)	0.0175	0.007	0.0023	0.0023	0.0005	100[6]	0.0005
Biotoxins							
T-2 mycotoxin	0.0653	0.026	0.0087	0.0087	0.0019	0.23–1.05[32,42]	0.51
Ricin[a]	2.273	0.909	0.3033	0.3033	0.0649	5–20[33]	0.50
Botulinum toxins[a]	0.00045	0.00018	0.00006	0.00006	0.000013	0.001–0.006[34,35,42,49]	0.47
Staphylococcal enterotoxin B (SEB)[a]	0.0227	0.0091	0.00303	0.00303	0.00065	0.05–0.36[36]	0.45
Aflatoxin[a]	0.0129	0.0052	0.00173	0.0173	0.0037	0.02–4.5[37]	0.40

[a] Estimated TSS (none established by Lawrence Livermore National Laboratory).

terrorist attacks through the distribution networks (CIAO, 1997). Senator Bill Frist, the Senate Majority Leader and the Senate's only physician in 2002, stated that our water supply is generally considered safe, but that there is anxiety over the threat of bioterrorism on our water supply (Frist, 2002). The President's Commission concluded that the water supplied to U.S. communities is potentially vulnerable to terrorist attack by insertion of chemical agents, biological agents, or toxins. The possibility of attack is of considerable concern, and the agents could be a threat if they were inserted at critical points in the system, theoretically causing a large number of casualties (CIAO, 1997). Bill Bracken of the Alabama Department of the Environmental Management Laboratory stated that after September 11, 2001, concern about safety of the nation's drinking water has increased. He described a variety of chemical agents that could be placed on a community's water supply. These included nerve agents, pesticides, cyanide, and radioactive materials (Corey, 2002). Nonfissionable radioactive materials, such as cesium-137, strontium-90, and cobalt-60, which cannot be used in nuclear weapons, can be used to contaminate water supplies, business centers, government facilities, or transportation networks. Although these radioactive attacks are unlikely to cause significant casualties, they could still cause damage to property and the environment, and produce societal and political disruption (Oehler, 1996). In April 1985, a threat was made to poison the water supply of New York City with plutonium. To evaluate the credibility of the threat, the New York City water supply was monitored for radioactivity. A sample taken on April 17, 1985, indicated a radioactive level of 21 femtocuries/L, which was a factor 100 times greater than that previously observed. This level, however, was substantially lower than the maximum of 5000 femtocuries considered safe for drinking water under federal guidelines (Purnick, 1985; Bogen et al., 1988).

More recently, evidence has surfaced that terrorists trained in Afghanistan were planning to poison water supplies with cyanide, which would be easy to do and very hard to prevent (Brennan, 2001). In January 2002, a bulletin from the Federal Bureau of Investigation's (FBI) National Infrastructure Protection Center, pointed out that members of al-Qaeda were trying to gain control of U.S. water supplies and waste-water treatment plants. Later, in July 2002, the FBI issued a series of warnings regarding possible terrorist attacks against American targets. The nation's water utilities were told to prepare to defend against attacks on pumping stations and pipes that serve the cities and suburbs. This action was a result of documents discovered in Afghanistan, which indicated that al-Qaeda terrorists were investigating ways to disrupt the U.S. water supply on a massive scale (Isenberg, 2002). Additionally, President Bush noted in his State of the Union address, that captured al-Qaeda documents included detailed maps of several U.S. municipal public drinking-water systems (Bush, 2002). Also in 2002, the Italian police reported that they had foiled a major terrorist attack by a gang of Moroccans linked to al-Qaeda. They were plotting a chemical attack of the United States Embassy in Rome and the city's water network. These sites were highlighted on a map found along with nine pounds of powdered potassium ferrocyanide, which is far less deadly than potassium cyanide (McGrory, 2002). As part of their 2002 Congressional Report, the NRC of the National Academy of Sciences (NAS) concluded that water-supply contamination and disruption should be considered a possible terrorist threat in the United States (NRC, 2003). Again, in September 2003, the FBI issued a warning of the potential use of nicotine and solanine by terrorists to poison the food and water supplies (Anderson, 2003).

It has also been recognized that a greater threat to our water supply might exist from more commonly available chemicals and materials such as TICs and TIMs as well as naturally occurring pathogens (Salem et al., 2004). The nation's largest outbreak of waterborne disease occurred in Milwaukee, Wisconsin, in the spring of 1993. *Cryptsporidium*, a protozoan, passed undetected through two water-treatment plants. It caused over 403,000 illnesses (mostly diarrhea), 4,000 hospitalizations, and 54 deaths in the 800,000 customers who drank the water. Investigators estimated that 725,000 productive days were lost as a result of the water-contamination event, at a cost in excess of $54 million in lost work time or additional expenses to residents and local authorities in Milwaukee. Although the cause was not clear, it confirmed that pathogens introduced

into the water supplies could cause death (Isenberg, 2002). In the year 2000, the municipal water supply of Walkerton, Ontario, Canada was contaminated with *Escherichia coli* 0157:H7, resulting in 2300 symptomatic residents and seven deaths attributed to the waterborne-disease outbreak. More than $11 million were required to reconstruct the community municipal water system and install temporary filtration after the contamination event. Estimates of the total cost of the Walkerton waterborne-disease outbreak and municipal-water contamination event reached $155 million (Meinhardt, 2005).

The Marine Corps has confirmed and suggested that terrorists might employ TICs because they are easily available and accessible. In addition, recent attacks by terrorists have shown their preference to use assets of the host country, rather than smuggling in raw materials or weapon systems (Jakucs, 2003). Although aerosol dispersion of an agent may be more effective in contaminating a larger number of people than through contamination of our water supplies, the PCCIP considered most water-supply systems vulnerable to terrorist attacks primarily through the distribution network (CIAO, 1997). Ideal poisoning agents that can be used to contaminate water should be reasonably or highly toxic. They should be highly soluble or miscible in water, have no taste or odor, be chemically and physically stable, and be resistant to aqueous hydrolysis and water treatment. Additionally, ideal contaminants should be difficult to recognize and detect, as well as having delayed activity, and no known antidote (Deininger, 2000).

Because of the critical need for water in every sector of our industrialized society, the water supplies and their distribution systems are potential targets for terrorist activity in the United States. Even short-term disruption of water service can significantly impact a community and lead to serious medical, public health, societal, and economic consequences (Meinhardt, 2005).

In the United States and in areas where our troops are deployed, most of the water supply is treated and contains a disinfectant such as free chlorine or chloramines to destroy or control the growth of bacteria. Maintaining a residual free chlorine concentration of 0.5 mg/L for public water supplies and 2.0 mg/L for field drinking water for deployed troops could provide adequate protection from most biological contamination (Burrows and Renner, 1999). However, to effectively contaminate the water supplies, large amounts of sodium thiosulfate could be added to deactivate the chlorine. Deininger et al. (1999) stated that although it may be possible to contaminate a water-supply system, a high degree of physical security combined with maintaining a higher than usual residual chlorine level, should ensure the safety of our water supply.

Deininger (2000) also described potential attack points to our water-supply system: upstream of the water intake or treatment plant (which would require large amounts of contaminant); the water intake or treatment plant; a service reservoir; and a point in the distribution system such as a fire hydrant. Individual houses and buildings, not isolated by backflow prevention devices, could also become attack points to the distribution system. He also concluded that there would be greater chances of success if contamination occurred after the treatment plant. There are many and varied agents that could be introduced into water-supply systems, and any one of the contaminants, or even the threat of contamination of the water supply, could cause panic, societal and political disruption.

III. APPROACH AND METHODOLOGY

The intent of the authors (subject matter experts) was to review the published literature and to determine the impact of contamination of our drinking water with CWAs, whether in warfare or by terrorist activity.

The CWAs that could effectively contaminate our water supplies as a viable threat were targeted. Literature searches were conducted, and the publications were retrieved and reviewed. Areas of interest included TSS (USACHPPM, 2003), oral toxicity doses, water solubilities or miscibilities, stabilities, and half-lives. Attempts were also made to identify toxic hydrolysis products. The data identified are presented in Tables 3.2 and 3.3, and their references are presented in Table 3.4.

TABLE 3.3
Agent Physical Characteristics

Nerve Agents	Solubility in Water (gm/100 gm)	Half-Life	Hydrolysis Products	Chlorine Resistance
GA (tabun)	7.2 at 20°C 9.8 at 100°C[7,8]	8.5 h at 20°C, pH 7[7,23]	Hydrogen cyanide (AC), dimethyl-aminocyanophosphonic acid[7,23] (may react with chlorine to form cyanogen chloride [CK])[7]	No[4]
GB (sarin)	Completely miscible[7,11,12] Completely soluble[9]	27 min at 20°C, pH 1 3.5 h at 20°C, pH 2 80 h at 20°C, pH 7 5.4 min at 20°C, pH 10 0.6 min at 20°C, pH 11[7,24]	Under acidic conditions, hydrogen fluoride (HF) and isopropyl methylphosphonic acid (IMPA) are formed which further hydrolyze to produce methylphosphonic acid (MPA) and isopropanol. Under alkaline condition, methylfluorophosphonic acid (MFPA) and isopropyl alcohol are initially formed which further hydrolyze to produce MPA and HF[7,16]	No[4]
GD (soman)	2.1 at 20°C[7,13] 3.4 at 0°C[7]	3 h at 25°C, pH 2 45 h at 25°C, pH 6.65 60 h at 25°C, pH 10[7,39]	Pinacolyl methylphosphonic acid (PMPA) and HF[39]	No[4]
GF (cyclosarin)	3.7 at 20°C 5.1 at 0°C[7,8] Insoluble[10]	42 h at 25°C[39]	HF and cyclohexyl methylphosphonic acid (CMPA)[7]	No[4]
VX	5.0 at 21.5°C[14]	1.8 min at 22°C in 1.25 M NaOH 10.8 min at 22°C in 0.25 M NaOH 31 min at 22°C in 0.10 M NaOH 3.3 h at 22°C in 0.01 M NaOH 20.8 h at 22°C in 0.001 M NaOH 60 h at 22°C in pure water[7,27] 350 days at 25°C[1,38] 110–120 h in well water[45] May survive up to 3 years[47]	VX hydrolyzes via three different pathways (P–S, P–O, and C–S), which vary significantly with temperature and pH. At pH below 12, the P–O bond cleavage path produces ethyl methyl-phosphonate (EMPA) and the toxic *S*-[2-diisopropylaminoethyl] methylphosphonothiolate ion (EA 2192). At room temperature, EA 2192 reacts very slowly with OH^- (EA 2192, $t_{1/2} = 7.4$ days [1.0 M NaOH] eventually producing less toxic products) Using an equimolar ratio of VX and water at elevated temperatures appears to reduce the persistency of EA 2192[6,7,27]	No[4] (half-time for destruction by hypochlorite is 1.5 min at pH 10)

(*continued*)

TABLE 3.3 (Continued)
Agent Physical Characteristics

Agent	Solubility in Water (gm/100 gm)	Half-Life	Hydrolysis Products	Chlorine Resistance
Blood Agent				
HCN (hydrogen cyanide)	Miscible with water and common organic solvents, completely soluble in water[7,15–17]	Stable but volatizes[7,40]	Ammonia, formic acid (HCOOH), and amorphous brown solids[7,16] (may react with chlorine to form cyanogen chloride [CK])[7]	No[48]
Vesicants				
HD (distilled mustard)	Practically insoluble in distilled water 0.92/100 g at 22°C[7,18] Mustard can survive in sea water up to 5 years[47]	5 min at 25°C[7,25,26] 60 min at 25°C[7,26]	Hydrogen chloride, thiodiglycol, and sulfonium ion aggregates—one of which is also highly toxic[6,7]	No[4]
HL (mustard/ Lewisite)	Slightly soluble in water[7,16,17]	Minutes[7,19,20,25,26]	Hydrogen chloride, thiodigylcol, sulfonium aggregates, 2-chlorovinylarsonous acid (CVAA), and Lewisite oxide based on HD and L[6,7,28]	No[4]
HN_3 (nitrogen mustard)	4 g/L at 25°C[7,19]	1.3 min at 25°C[7,19]	Hydrochloric acid, ethyl diethylnolamine, cyclic ammonium salts, dimer[7,19,29]	
HT (60% HD and 40% T)	Slightly soluble in water[7]	Minutes[7,25,26]	Hydrogen chloride, thiodiglycol, sulfonium aggregates; based on HD[6,7]	No[4]

L (Lewisite)	Hydrolyzes to form Lewisite oxide (solid), which dissolves very slowly in water[7,19,20]	Minutes[7,19,20]	CVAA, 2-chlorovinylarsenious oxide (Lewisite oxide), and HCl (hydrochloric acid)[7,28,43,44]	No[4]
Incapacitating Agent				
BZ (3-quinuclidinyl benzilate)	Approximately 1.18 g/L, slightly soluble in water[7,13,21]	6.7 h at 25°C, pH 9.8[7,30] 1.8 min at 25°C, pH 13[7,30] 3–4 weeks at 25°C, moist air pH 7[7,30] 12 min at 34°C, pH 12[7,21] 1.4 h at 50°C, pH 8.5[7,21] 9.5 h at 100°C, pH 0[7,21]	3-Quinuclidinol and benzylic acid[7,30]	Yes[4]
Biotoxins				
Botulinum toxins	Soluble[42]	Days (spores may survive years)[41,45]		Inactivated, 6 ppm, 20 min[31] Spores resistant to chlorine[41]
Ricin	Soluble[42]	Days		Resistant at 10 ppm[31,42]
SEB (staphylococcal enterotoxin B)	Soluble	Unknown		Unknown[31] Contaminated articles disinfected with 0.05% hypochlorite[46]
T-2 (trichothecene mycotoxin)	Practically insoluble	Days		Resistant[31,42]
Aflatoxin	Practically insoluble	Unknown		Probably tolerant[31,42]

TABLE 3.4
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TABLE 3.4 (Continued)
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Target doses for detection were estimated based on the TSS developed for field drinking water by the U.S. Army in collaboration with the LLNL. Because of the terrorist threat against civilian populations, we considered it prudent to include contamination of a daily water consumption of 2 L/day in table 3.2, because this is considered the daily water intake by civilian populations. The amount of allowable contamination in 2, 5, and 15 L/day were based on direct linear extrapolation (Deininger, 2000). Not considered in these calculations, however, was the dilution factor enhancing absorption, as well as the effects on distribution, metabolism (biotransformation) and excretion (ADME) over the 7 day ingestion or exposure period. Table 3.2 also includes estimated oral toxic doses and target doses for detection, based on the lowest concentration of the contaminant, as well as the estimated daily human oral doses ingested, and the estimated human acute oral LD_{50}. In addition, Table 3.2 lists the percentages of the human oral LD_{50} ingested daily.

Because the only toxin that had a previously established TSS was T-2, it was used to determine a constant to calculate estimated TSS for other toxins. Although the relationship between the TSS concentration at the 5 L/day consumption and the minimum reported LD_{50} may not be the same for all toxins, this was the value used in our calculations, that is

$$C = \frac{\text{TSS (5 L/day) mg/L/70 kg}}{\text{Estimated minimum human LD}_{50}\text{/70 kg}} = \frac{0.026}{16.1} = 0.0016.$$

Thus, the estimated TSS for toxins was derived using 0.0016 times the estimated minimum human LD_{50}/70 kg individual for the other toxins in Table 3.2.

If no estimated human oral toxicological data were available, the Army used an uncertainty factor of 10 for interspecies extrapolation from animals to human (NAS, 1995). This same methodology was used to establish the human LD_{50} for T-2 toxin, because there has not been an estimated human LD_{50} by the oral route.

These tables are dynamic documents that will be updated as new data or more precise methods of extrapolation become available. ''Weaponized'' CWAs usually refer to those agents that have been treated or processed to enhance their abilities for dissemination and toxicity by the inhalation

and dermal routes of exposure. ''Weaponization'' can be accomplished by adding stabilizers to agents to prevent degradation and agglomeration, adding thickeners to increase viscosity and persistence, and by developing more effective means of dispersal (Seevers et al., 2002). It is not known if any agents have been weaponized specifically for use against water targets. Historically, nonweaponized agents have been used effectively to contaminate water supplies, thereby reducing unit readiness. Therefore, when considering contamination of water supplies, a more generic definition of weaponized would be needed to include all routes of exposure. Weaponization therefore, would include chemical agents that have been produced for dissemination in sufficient quantities to cause the desired effect by any route of exposure. The desired effect is scenario and mission dependent and may be illness, death, or deniability of life-sustaining drinking water or real estate.

The agents selected for these tables were based on many factors, including technical, political, and social. The technical factors for effectiveness in water were stated above. The threat can be real or perceived and still result in civil and social disruption. If the public suddenly lost confidence in the integrity of the water system, a domino effect could result in a panic run on bottled water and alternative water supplies (Meeks, 2003).

IV. RESULTS AND DISCUSSION

CWAs are chemicals designed for use in military operations to kill, injure, or incapacitate humans through physiological actions. These were classified into categories according to their effects on the body including nerve agents, vesicating or blistering agents, respiratory or pulmonary agents, blood agents, and incapacitating agents. Research into nerve agents began in the 1930s when they were developed in secrecy before and during World War II. Related substances are used in medicine as well as in other fields such as the use of pesticides in agriculture, but lack the potency of the military agents. They are extremely toxic and vary in persistence; some create a short-term battlefield hazard, whereas others linger for days or weeks. The nerve agents include: GA, GB, GD, GF, and VX. The G agents were given the code letter ''G'' because they originated in Germany, whereas the V allegedly stands for venomous. The nerve agents and related pesticides are organophosphates and function through the inhibition of the enzyme acetylcholinesterase (AChE). AChE is the critical regulatory enzyme for the conduction of neuromuscular activity because it breaks down the neurotransmitter acetylcholine. When the nerve agent binds to the active site on AChE, the enzyme can no longer function in destroying the neurotransmitter. Thus, the acetylcholine (neurotransmitter) builds up at the neuromuscular junction and causes continuous nerve impulse generation and organ stimulation. The organs with cholinergic receptors include smooth and skeletal muscles, central nervous system, and exocrine (secretory) glands. The effects of nerve agent exposure depend on dose, route and duration of exposure, and range from miosis through rhinorrhea secretions, muscle fasciculations, and convulsions to death from paralysis of respiratory muscles. These effects can be remembered by the acronym SLUDGE (salivation, lacrimation, urination, diarrhea, gastrointestinal cramping, and emesis) or by DUMBBELS (diarrhea, urination, miosis, bronchorrhea, bradycardia, emesis, lacrimation, and salivation—Salem, 2005).

The data for the nerve agents are presented in Tables 3.2 and 3.3. Table 3.2 includes the TSS or estimated TSS, target dose for detection, daily human oral dose ingested, estimated human oral acute LD_{50}, and average percent of estimated human oral acute LD_{50}. Table 3.3 includes solubility in water, half-life in water, hydrolysis products, and chlorine resistance.

Some of the CWAs (GF, HL, HN_3, and HT) did not have TSS established by LLNL, so they were estimated based on studies in various animal species and by different routes of exposure. These data were based on the most toxic analog or compound in the mixture. For example, the estimated TSS dose equivalent for GF was estimated from GD, its closest analog, and for HL from L which is more toxic than HD, and for HT from HD which is more toxic than HT (Table 3.2). The target dose for detection is based on the lowest concentration found in the water that would not cause degradation of military performance if consumed for up to 7 days.

In a previous publication (Salem et al., 2004), the authors calculated the target dose for detection in terms of mg/day for a 70 kg service member, irrespective of the volume of water consumed. However, reevaluation by the authors determined that the target dose for detection should be based on the daily consumption. Thus, the target dose for detection, in mg/L, in this publication is effectively lower than in the previous publication, because it assumes a daily drinking-water consumption of 15 L. Even so, these numbers are conservative because the entire volume of contaminated water (2, 5, or 15 L/day) is not consumed at one time, but over a period of hours. The calculated percentage of the oral LD_{50} consumed (Table 3.2) assumes that the entire dose of contaminated water is consumed at one time. In the study by Bauer et al. (1949) on the effects of oral ingestion of GB, rats were trained to consume all of their daily drinking water in a 15 min period. The LD_{50} values were also determined by intragastric administration. The authors reported that the total of GB consumed in drinking by the surviving rats can be as much as three times the LD_{50} if the total is received over a period of several days. Apparently, the intervals between consuming the contaminated water allow for metabolism or biotransformation and elimination, thereby diminishing the toxicity.

The estimated oral LD_{50} for botulinum toxin A is 0.07 mg/70 kg person or 0.001 mg/kg. This number is reported by Robinson (1973) and Hickman (1999). This estimated LD_{50} is corroborated in publications by Martin and Adams (2003). The rat oral dose is usually a much higher number (less toxic) and is reported by Gill (1982) as many orders of magnitude less toxic than when administered parenterally (Meyer and Eddie, 1951; Lamanna, 1959).

Botulinum toxin A is a simple protein comprising a single polypeptide chain, readily detoxified by heat, mechanical stress, and oxygen (Stevenson et al., 1947; Lamanna, 1959). In contaminated water, it remains highly toxic for several days but decays rapidly in open air. This toxin inhibits the release of acetylcholine at sites needed to transmit nerve impulse to muscles. Botulinum toxin by ingestion produces nausea and diarrhea, followed by headache, dizziness, fatigue, weakness, vertigo, extreme constipation, convulsions, and death due to paralysis of respiratory muscles.

The nerve agents are liquid under temperate conditions. When dispersed, the more volatile ones (G Agents), constitute both a vapor and liquid hazard. Sarin (GB) is the most volatile, although it evaporates less readily than water. Of the G agents, GA is the least volatile. The much less volatile V agents (such as VX) represent primarily a liquid hazard.

The nerve agents are organophosphorous cholinesterase inhibitors, inhibiting butyryl-cholinesterase in the plasma and AChE in the RBCs and at cholinergic receptor sites in tissue. Acetylcholine accumulates at the nerve in receptor sites and continues to stimulate the affected organs. The clinical effect from nerve agent exposure is caused by excess acetylcholine.

When nerve agents are absorbed into the gastrointestinal (GI) tract, they cause an increased motility of the GI tract, increased secretion by the glands in the wall of the GI tract, and include cramps, salivation, diarrhea, vomiting, anorexia, and involuntary defecation (USAMRICD, 2000; Seevers et al., 2002).

A. Blood Agents (Hydrogen Cyanide)

The cyanides are considered to belong to the ''blood agents'' that were introduced, and used by the French during World War I. No deaths were reported from its military use. It was considered to be militarily useless because of its volatility (Haber, 1986), and because it is rapidly detoxified in the body at the rate of 17 μg/kg body weight/min (USAMRICD, 2000). Hydrogen cyanide and cyanogen chloride affect bodily functions by inactivating the cytochrome oxidase system, which prevents cell respiration and the normal transfer of oxygen from the blood to body tissues (PMCBA, 2005). Cyanide salts in solid form or in solution are readily absorbed from the GI tract (USAMRICD, 2000). Cyanide is primarily an environmental contaminant of industrial processes and usually enters the drinking water as industrial waste (NRC, 1995). Chlorination of

water containing hydrogen cyanide results in the formation of cyanogen chloride, which is also highly toxic, but less toxic than hydrogen cyanide (Cotton and Wilkinson, 1980). Cyanogen chloride has limited solubility in water, persists for more than 24 h, and slowly hydrolyzes to the cyanate ion. All cyanates can persist in aerobic water at pH 7 at 20°C for 10 days (Resnick et al., 1958).

B. Blister Agents (Vesicants)

The blister agents were introduced into warfare in World War I, to produce casualties, to degrade fighting efficiency, and to restrict use of terrain and equipment. The vesicants include sulfur mustard, commonly referred to as mustard. The original mustard (H) was Levinstein mustard that contained about 30% impurities, and thus was distilled to increase the purity (HD) (PMCBA, 2005). The nitrogen mustards (HN_1, HN_2, HN_3), synthesized in the 1930s, were not produced in large amounts for warfare. Mechlorethamine (HN_2, or Mustargen) became the prototypical cancer chemotherapeutic compound and remained the standard compound for this purpose for many years (USAMRICD, 2000). Because of their physical properties, mustards are persistent under cool conditions, but as temperature increases, mustards will evaporate. Persistency can be increased by dissolving them in thickeners (PMCBA, 2005). Lewisite (L) was synthesized during the late stages of World War I, but has probably not been used on the battlefield. The antidote, British Anti-Lewisite (BAL), is used in medicine today as a heavy-metal chelator (USAMRICD, 2000).

C. Incapacitating Agents

Incapacitating agents induce an inability to perform any military task effectively and imply that the condition is achieved with the use of a nonlethal weapon. These agents differ from other CWAs in that their lethal dose is theoretically many times greater than the incapacitating dose. As a result, they do not seriously endanger life except in cases exceeding many times the effective incapacitating dose, and they do not produce permanent injury. Incapacitating agents include deliriants, stimulants, depressants, and psychedelics, and they interfere with higher functions of the brain such as attention, orientation, perception, memory, motivation, conceptual thinking, planning, and judgment. The chemicals that produce delirium demonstrate the incapacitant syndrome of confusion, hallucinosis, and disorganized speech and behavior. The subgroup of anticholinergics are regarded as the most likely to be used as military incapacitating agents. Of these, 3-quinuclidinyl benzilate (BZ) is considered the most likely candidate for military use (PMCBA, 2005). BZ is a glycolate anticholinergic compound related to atropine, scopolamine, and hyoscyamine. It acts as a competitive inhibitor of acetylcholine at postsynaptic and postjunctional muscarinic receptor sites in smooth muscle, exocrine glands, autonomic ganglia, and the brain. In addition, BZ causes peripheral nervous system effects opposite to those seen in nerve agent poisoning. Agent 15 is an (alleged Iraqi) incapacitating agent that is likely to be chemically identical to BZ, or closely related to it (USAMRICD, 2000).

D. Toxins

Toxins are poisonous byproducts of living organisms (microbes, snakes, insects, spiders, sea creatures, and plants). As with chemical agents, toxins have the potential to cause incapacitation as well as lethality. Depending on the goals of the adversary, incapacitation may be more effective than lethality. Large numbers of ill patients might overwhelm the medical and evacuation infrastructure, and create panic and disruption of the affected population (USAMRICD, 2005). The toxins included in this chapter are considered potential water threats, and botulinum toxins and ricin are considered to have been weaponized, whereas staphylococcal enterotoxin B (SEB) is considered to have probably been weaponized (Hickman, 1999).

E. Botulinum Toxins

Toxins of potential concern include botulinum toxins, a group of seven toxins (A through G) produced by *Clostridium botulinum*. The spores are ubiquitous and germinate into vegetative bacteria that produce toxins during anaerobic incubation. They can also be produced in large quantities for use as biological warfare agents by industrial-scale fermentation. Botulinum toxins are the most potent neurotoxins known. They are proteins with molecular masses of approximately 150,000 Da. Each of the seven, distinct but related neurotoxins is produced by a different strain of *C. botulinum* and all act by a similar mechanism of inhibition of presynaptic acetylcholine release. The clinical syndrome ''botulism'' begins with cranial nerve palsies, including ptosis, blurred vision, diplopia, dry mouth and throat, dysphagia and dysphonia, followed by symmetrical descending flaccid paralysis, with generalized weakness and progression to respiratory failure (PMCBA, 2005; USAMRICD, 2005). All seven types of botulinum toxins act by similar mechanisms of inhibition of presynaptic acetylcholine release and produce similar effects when inhaled or ingested and can be used to contaminate food or water. The clinical syndrome produced by these toxins is known as botulism. Although botulinum toxins do not cross the blood–brain barrier and do not cause central nervous system (CNS) disease, the psychological sequelae of botulism may be severe and may require specific intervention (USAMRICD, 2005). Botulinum toxins are inactivated by sunlight within 1–3 h or by heat at 80°C for 30 min or at 100°C for several minutes. Chlorine will cause over 99.7% inactivation of botulinum toxins by 3 mg/L free available chlorine (FAC) in 20 min (USAMRICD, 2005).

F. Ricin

Ricin is a potent protein cytotoxin, easily extracted from the beans of the castor plant (*Ricinus communis*), which is ubiquitous. When ingested, ricin causes severe GI symptoms followed by vascular collapse and death. Ricin is made up of two hemagglutinins and two toxins. The toxins, RCL III and RCL IV, are dimers with molecular masses of approximately 66,000 Da. The toxins are made up of two polypeptide chains (A and B) connected by a disulfide bond. The cytotoxicity of ricin is due to inhibition of protein synthesis, caused when the B chain binds to cell-surface receptors and the toxin–receptor complex is taken into the cell, and the A chain that has endonuclease activity and, at extremely low concentrations, will inhibit DNA replication and protein synthesis (USAMRICD, 2005). Ricin is stable under ambient conditions and can be detoxified by heat at 80°C for 10 min, or 50°C for an hour at a pH of 7.8. Chlorine inactivates over 99.4% by 100 mg/L FAC in 20 min. Low chlorine concentrations, such as 10 mg/L FAC, as well as iodine at up to 16 mg/L will have no effect on ricin (USAMRICD, 2005).

G. Staphylococcal Enterotoxin B (SEB)

SEB is an exotoxin protein of 28,494 Da produced as an extracellular product of *Staphylococcus aureus*. This pyrogenic toxin normally exerts its effects on the GI tract, and is thus called an enterotoxin. It belongs to a class of potent immune stimulants known as bacterial superantigens. Superantigens bind to monocytes at major histocompatibility complex type II receptors rather than the usual antigen-binding receptors. This leads to the direct stimulation of large populations of T-helper cells while bypassing the usual antigen processing and presentation. A brisk cascade of proinflammatory cytokines (tumor necrosis factor, interferon, interleukin-1, and interleukin-2) is induced with recruitment of other immune effector cells and relatively deficient activation of counter-regulatory negative feedback loops. This results in an intense inflammatory response that injures host tissues. Thus, the toxic effects of SEB are considered to be mediated via the released cytokines. Oral exposure results predominantly in GI symptoms including nausea, vomiting, and diarrhea, and may result in toxic shock and death (USAMRICD, 2005).

H. Trichothecene Mycotoxin T-2

The T-2 mycotoxins have a low molecular mass of 250–500 Da, are nonvolatile compounds produced by filamentous fungi or molds of the genus *Fusarium*, and are extremely stable in the environment. Of the multiple mechanisms of actions, many are poorly understood. However, their most notable effect stems from their ability to rapidly inhibit protein and nucleic acid synthesis. Thus, they are markedly cytotoxic to rapidly dividing cells, such as in the bone marrow, GI tract (mucosal epithelium), skin, and germ cells. This cytotoxic effect imitates the hematopoietic and lymphoid effect of radiation sickness, thus the mycotoxins are referred to as ''radiomimetic agents.'' The mycotoxins also alter cell-membrane structure and function, inhibit mitochondrial respiration, and inactivate certain enzymes. Decontamination requires the use of hypochlorite solution under alkaline conditions, such as 1% sodium hypochlorite and 0.1 M NaOH with 1 h contact time (USAMRICD, 2005).

V. CONCLUSIONS

The estimates of human oral toxicities are based primarily on experimental animal studies. Although the data are reported as oral, the contaminants may have been administered by oral gavage or by allowing the animals to drink contaminated water. If this was the case, the results would be different. Also not considered is the dilution factor in 2, 5, or 15 L of consumption per day. Greater dilution of the contaminant could enhance absorption via enhanced surface contact. In addition, since the TSSs were based on 7 day consumption, not only is absorption a factor, but so is distribution, metabolism or biotransformation, as well as excretion. Another factor that complicates the definitive data used in making the extrapolations used in determining the threat is the type of water used in the studies. Some studies report the use of raw, distilled, or seawater. Based on these and other uncontrolled or undefined details, the threat defined in this chapter appears credible based on the available data.

The literature reports that chemical and biological contamination of water supplies has been common in history since at least 600 BCE, some of which have been described in this chapter. The U.S. Army has had a long-standing concern about deliberate contamination of military drinking water and has previously addressed this in collaboration with LLNL by promulgating TSS for CWAs that might be used to contaminate military drinking water.

Although in 1974, Epstein explained that it would be difficult to deposit a harmful amount of nerve agent into large drinking-water systems, Deininger (2000), described potential attack points in our water-supply systems. PCCIP indicated that most water-supply systems in the United States are vulnerable to terrorist attacks through the distribution networks (CIAO, 1997). Senator Bill Frist, the Senate Majority Leader, and the Senate's only physician at the time, stated that our water supply is generally considered safe, but there is anxiety over the threat of bioterrorism on our water supply (Frist, 2002). In addition to the CWAs and toxins discussed in this chapter, contamination with readily available TICs and TIMs can also be a major contamination threat to our water supplies.

Attempts to contaminate our water supply can have unnerving effects, including panic as well as societal and political disruption. The public would lose confidence in our government, the integrity of our water supply, and the resulting domino effect could cause a panic run toward bottled water and alternative water supplies (Meeks, 2003).

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4 Health Effects of Low-Level Exposure to Nerve Agents

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CONTENTS

I. INTRODUCTION

In 2000, when we wrote this chapter for our earlier work, we were influenced by two events: (1) the significant concern for the health of Persian Gulf War veterans as it might have been affected by possible exposure to low levels of chemical warfare agents and (2) an increase in research, both epidemiological and experimental, into the health effects of exposure to nerve chemical warfare agents. In fact, in a note of optimism, we closed the chapter with this statement "The authors close by expressing hope that the recent national investment into additional research will allow a more comprehensive assessment to unfold that will *possibly contribute to better treatment*." In the intervening 6 years, a number of epidemiological and experimental studies have added to the literature regarding health effects of exposure to nerve chemical warfare agents. Additionally, Aas (2003) reviewed current medical therapy for nerve agent intoxication and discussed possible future improvement of medical therapies. Currently, several new approaches to improved treatment of nerve agent intoxication are in full-scale development in the United States. As a result of these developments, this chapter has been updated principally in two areas: (1) inclusion of recent reports on potential chronic health effects from either asymptomatic or symptomatic exposures to nerve agents and (2) discussion of developments leading to potentially improved care for such exposures.

The nerve agents are highly toxic organophosphorous (OP) compounds that are chemically related to some insecticides (parathion, malathion). The five most common nerve agents are tabun (*o*-ethyl *N,N*-dimethyl phosphoramidocyanidate; military designation = GA), sarin (isopropyl methyl phosphonofluoridate; military designation = GB), soman (pinacolyl methyl phosphonofluoridate; military designation = GD), cyclosarin (cyclohexyl methylphosphonofluoridate; military designation = GF), and VX (*o*-ethyl S-2-*N,N*-diisopropylaminoethyl methyl phosphonofluoridate). These compounds exist as colorless and relatively odorless liquids and are meant for use in weapon

systems (shells, rockets, bombs) that are designed to deliver them as aerosols or fine sprays. They exert their toxic effects by inhibiting the cholinesterase (ChE) family of enzymes to include acetylcholinesterase (AChE; E.C.3.1.1.7), a critically important central nervous system (CNS) and peripheral nervous system (PNS) enzyme that hydrolyzes the neurotransmitter acetylcholine (ACh). Although the nerve agents can inhibit other esterases, their potency and specificity for inhibiting AChE account for their exceptionally high toxicity. For example, the rate constants for inhibition of AChE by soman, sarin, tabun, or VX are 2–3 orders of magnitude greater than for the more commonly known OP compounds such as DFP, paraoxon, or methylparaoxon (Gray and Dawson, 1987). Likewise, the rate constants for inhibition of AChE by the nerve agents are also 2–5 times greater than for trypsin (E.C.3.4.21.4), chymotrypsin (E.C.3.4.21.1), or carboxylesterase (E.C.3.1.1.1) (Maxwell and Doctor, 1992) indicative of selective inhibition of this enzyme.

Nerve agents bind to the active site of the AChE enzyme, thus preventing it from hydrolyzing ACh. The enzyme is inhibited irreversibly and the return of esterase activity depends on the synthesis of new enzyme (approximately 1%–3% per day in humans). All agents are highly lipophylic and readily penetrate the CNS. Acetylcholine is the neurotransmitter at the neuromuscular junction of skeletal muscle, the preganglionic nerves of the autonomic nervous system, the postganglionic parasympathetic nerves, as well as muscarinic and nicotinic cholinergic synapses within the CNS. Following nerve agent exposure and the inhibition of approximately >40% of the AChE enzyme pool, levels of ACh rapidly increase at the various effector sites resulting in continuous overstimulation. It is this hyperstimulation of the cholinergic system at central and peripheral sites that leads to the toxic signs of poisoning with these compounds. The signs of poisoning include miosis (constriction of the pupils), increased tracheobronchial secretions, bronchial constriction, laryngospasm, increased sweating, urinary and fecal incontinence, muscle fasciculations, tremor, convulsions/seizures of CNS origin, and loss of respiratory drive from the CNS. The relative prominence and severity of a given sign are highly dependent on the route and degree of exposure. Ocular and respiratory effects occur rapidly and are most prominent following vapor exposure, whereas localized sweating, muscle fasciculations, and gastrointestinal disturbances are the predominant signs following percutaneous exposures and usually develop in a more protracted fashion. The acute lethal effects of the nerve agents are generally attributed to respiratory failure caused by a combination of effects at both central and peripheral levels and are further complicated by copious secretions, muscle fasciculations, and convulsions. There are several excellent reference sources that provide more detailed discussions of the history, chemistry, physiochemical properties, pharmacology, and toxicology of the nerve agents (Koelle, 1963; Sidell, 1992; Somani et al., 1992; Marrs et al., 1996; Taylor, 2001).

Their rapid onset of effects and extreme toxicity has made the nerve agents eminently suitable for use as chemical warfare (CW) agents and, in some cases, many thousands of tons of these agents have been synthesized for military use. Exposure to lethal levels of nerve agents will produce toxicities that are precipitate in onset and catastrophic in effect (Sidell, 1974). For these reasons, the major medical research efforts since the 1940s focused on developing the best possible lifesaving therapeutic interventions, pretreatments, or, more recently, prevention of long-term changes in CNS function following a moderate to severe intoxication using anticonvulsant drugs (Dunn and Sidell, 1989).

II. REVIEW PAPERS, EPIDEMIOLOGICAL RESEARCH, AND EXPERIMENTAL STUDIES OF HEALTH EFFECTS OF EXPOSURE TO NERVE CHEMICAL WARFARE

Due to the focus on lifesaving interventions, it was not until the early 1980s that the question of chronic health effects of low-level exposure to nerve agents was subjected to its first major review. The Committee on Toxicology, National Academy of Sciences, studied the available literature reports from the soldier-volunteer test program of the Army Chemical Center, Aberdeen Proving Ground (formerly Edgewood Arsenal, MD) (National Academy of Science, 1982). Soldier-volunteers

participated in this test program from 1958 to 1975. There were 15 anticholinesterases (anti-ChEs) tested on approximately 1400 subjects during this time frame with the great majority of anti-ChE agents being tested during the 1950s and 1960s.

The National Academy of Sciences' review found that mortality data compiled in 1981 did not indicate increased deaths among soldier-volunteers when compared with soldiers outside the testing program. There was no clear-cut indication of long-lasting CNS effects and no evidence for mutagenicity, carcinogenicity, male reproductive, or cataractogenic effects (1982). The National Academy of Sciences' review committee also reported confidence that its analyses would have had the power to detect any major health effects, had they been present. In general, that viewpoint remained the ''state-of-the-art'' with very little contention until the appearance of Persian Gulf War Illness in the early years of the 1990s. It should be noted that a similar comprehensive review of the exposure to military chemical agents in human volunteers was recently published in the United Kingdom (Ministry of Defense, 2006). Porton Down, the UK chemical test center, had a human volunteer program in place since World War One. Many human exposure tests to nerve agents were conducted at Porton starting in the late 1940s and there is an excellent summary of these and similar US studies by Sidell (1996). Although the Ministry of Defense (2006) study was more to address the ethics of the human volunteer program, it did describe three follow-up studies performed to determine if there were potential long-term health effects of volunteer's exposure to sarin (Technical Notes, 1972, 1973, 1989). All three studies concluded that there was no evidence that ''the exposure of volunteers to low doses of nerve agents results in any adverse medical sequelae.'' There were no statistical differences between the number of hospital admissions, out-patient appointments, and incidents of reporting sick or days lost through illness before or after the men's visit to Porton for those exposed to sarin. Nor were there any differences on these measures between those exposed to sarin and control groups of volunteers who were not exposed. Likewise, there was no evidence of long-term psychiatric symptoms or in the type of illnesses that the exposed versus control groups experienced.

As a result of concern regarding a high incidence of undiagnosed illness among veterans of Operation Desert Shield/Storm, a Presidential Advisory Committee was formed to analyze the full range of the Federal Government's outreach, medical care, research, and coordinating activities pertinent to Gulf War Veterans' Illness (GWI). The Presidential Advisory Committee also looked at short- and long-term health effects of selected Gulf War risk factors, for example, chemical/biological weapons, depleted uranium, infectious diseases, anti-biological warfare vaccines, pyridostigmine bromide, and so on. The Presidential Advisory Committee gave specific and serious attention to the question of health effects of low-level exposure to nerve agents. The Committee urged DoD to support additional research on the potential long-term health effects of low-level exposures to CW agents, the nerve agents in particular (Presidential Advisory Committee on Gulf War Veterans' Illnesses, 1996). Such an increased level of research has already been initiated and some elements of it are discussed throughout this chapter.

Correspondingly, this work had a proactive component to it. Much of the work within the DoD core research program addressed the question, ''Is the current US military medical treatment doctrine, as well as physical protective measures (protective masks, clothing, and support systems), adequate to protect soldiers in future deployments from effects of exposure to low-levels of CW agents?'' (GAO/NSIAD, 1998).

Since the end of the first Gulf War there has developed a substantial literature, in the form of review papers, on potential long-term health effects from low-level exposure to nerve chemical warfare agents. These papers have presented slightly different analyses of this issue and, not surprisingly, they have reached slightly different conclusions. We do not intend to review this entire body of literature in this chapter. However, we do highlight several papers to show the controversies within the field, as well as areas of consensus. Brown and Brix (1998) argued that for nearly all accidental or wartime exposures to nerve agents or OP compounds, it is difficult to obtain reliable exposure data. Thus, they argued that exposures could be characterized as high,

intermediate, or low, depending upon factors such as intensity of cholinergic signs (e.g., rhinorrhea, salivation, neuromuscular effects, etc.), level of ChE inhibition, and type of medical treatment required. Clearly identified long-term effects have been noted at or above their defined intermediate level exposure. Long-term health effects, according to Brown and Brix (1998), are not reported in individuals experiencing repeated low-level exposure alone.

In his brief review of chronic effects of low-level exposure to anti-ChEs, Roy (1998) concluded that ''concerns about major adverse health effects of low-level exposure to anti-ChEs in general seem entirely unwarranted on the basis of currently available literature, but the data are at present insufficient to reflect the possibilities of subtle, agent-specific effects.'' Riddle et al. (2003) in their review of potential Gulf War nerve agent exposures found (1) difficulties in documenting symptomatic exposures to nerve agents, (2) corresponding lack of specific exposure-related health effects, yet (3) commented that feelings of helplessness in face of an uncertain exposure could be overwhelming. In Section IV, we will also review the scientific basis for these health concerns.

It is a common practice for toxicologists to differentiate exposure to chemicals based on the dose and the duration of exposure. Four time frames have been used to define duration of exposures: acute, subacute, subchronic, and chronic. It is useful in light of today's interest in ''long-term, low-level'' exposures to clarify these terms. Acute exposure is defined as exposure to a chemical for less than 24 h. When referring to an inhalation exposure, the exposure duration most frequently used is 4 h. Subacute exposure refers to an exposure of 1 month or less, subchronic for 1–3 months, and chronic for more than 3 months. These exposures can be by any route; for most chemicals, it is the oral route with the chemical given in the diet (Klaasen and Eaton, 1991). All are intermittent. The limited animal studies using nerve agents have usually employed parenteral administration of the agent and virtually all of them involve acute or subacute durations of exposure.

It is equally important to clearly define the term ''low-level exposure.'' This term has seen many different usages in the papers reviewed by these authors. These appear to range from any nonlethal exposure through ''subtoxic'' (defined by DeMenti [1999] as no clinical signs) to ''subclinical'' (defined by DeMenti [ibid.] as no clinical signs and no significant depression of ChE). Exposure, then, is any contact with a chemical that may induce a biochemical effect. Each definition suffers from arbitrariness and we see no way around this. For the purposes of this review, we will attempt to characterize each paper in terms of presence/absence of either clinical signs or symptoms (in the case of human studies) and level and type of ChE inhibition.

With respect to epidemiological research studies, Smith, Gray, and colleagues reported on continuing efforts to assess plausible relationships among potential Gulf War exposures and health effect outcomes. In 1999, they issued their first report, suggesting there was no evidence that Gulf War veterans, possibly exposed to CWA at Khamisiyah, Iraq, were suffering increased hospital morbidity through September, 1995. Between 1999 and 2003 there were improvements and refinements of exposure estimates, as well as improvements in regression modeling of hospitalizations. Thus, in 2003, Smith and colleagues were able to determine the relative risk (RR) of hospitalization, using Cox regression modeling, for an extended sample of possibly exposed veterans versus a cohort not likely to have been exposed through the period ending 31 December, 2000.

Recently, this same group reported that RR for hospitalization was actually slightly greater for post-Gulf War deployments to Southwest Asia or to Bosnia than for deployment to the first Gulf War (Smith et al., 2006). In summary, even after employing refined exposure estimates and up to a greater than 10 year postwar hospitalization window, these authors were unable to conclude that Gulf War veterans are at greater risk of hospitalization due to a specific exposure-related disease.

III. CHRONIC HEALTH EFFECTS OF ACUTE EXPOSURE

Much of the data regarding long-term neurological sequel to exposures to ChE inhibitors in man have been gathered following accidental exposures to organophosphate pesticides. While pertinent, extrapolation from these exposures to predictions of effects from nerve agents may be subject to

risk. Several phenomena appear to differentiate nerve agent exposure from exposure to OP pesticides. These include: (1) the fact that the cholinergic crisis caused by acute, severe intoxication with the OP pesticides is generally much longer than that caused by OP nerve agents (days to weeks for pesticides versus hours for nerve agents), (2) many OP pesticides produce delayed peripheral neuropathy, a phenomenon known for more than 50 years, whereas nerve agents have caused polyneuropathy in animals only at doses manifold greater than the LD_{50}—a phenomenon only seen in the presence of massive pretreatment and therapy with atropine and oxime (Davis, Holland and Reumens, 1960), and (3) the ''intermediate syndrome,'' a delayed manifestation of OP poisoning seen in perhaps up to 100 accidentally poisoned patients (Sidell and Hurst, 1997), has not been described after administration of nerve agents to animals nor in the instances of nerve agent poisoning in man (Sidell, 1997).

Grob et al. (1947) described the effects of acute to subacute short-term exposure of humans to DFP (1–2 mg, i.m., daily for up to 7 days) on electroencephalographic (EEG) and psychological parameters. The changes produced by DFP included increases in EEG potential, frequency (especially noted was an increase in beta rhythm), more irregularities in rhythm, and by the intermittent appearance of abnormal waves similar to those seen in patients with grand mal epilepsy (high voltage waves of 3–6 Hz, usually most marked in frontal leads, and increased by hyperventilation). The CNS symptoms that were noted were excessive dreaming, insomnia, jitteriness and restlessness, increased tension, emotional lability, subjective tremulousness, nightmares, headache, increased libido, giddiness, drowsiness, paresthesias, mental confusion, and tremor. The EEG changes usually followed the onset of CNS symptoms. CNS symptoms and EEG changes were correlated with the depression of red-blood cell ChE to 70% and 60% of original activity, respectively. CNS symptoms disappeared within 1–4 days after exposure was stopped while the EEG changes persisted in a diminishing degree from 8 to 42 days (average 29 days). Holmes and Gaon (1956) described essentially similar CNS symptoms and EEG changes but occurring acutely in OP pesticide exposed workers. They also noted that the more severely exposed individuals or those with multiple exposures tended to display persistent symptoms that included forgetfulness, irritability, and confused thinking, although the duration of these persistent symptoms was never clearly defined.

These CNS symptoms and EEG changes are virtually identical to those that have been reported to occur following symptomatic exposure to different nerve agents. Grob and Harvey (1953, 1958) described extensive studies of the effects of sarin in man, to include effects on ChE, EEG, and behavior. They noted behavioral and EEG effects virtually identical to those reported for DFP. These effects began coincident with the depression of plasma and red-blood cell ChE activity to approximately 60% and 50% of original activity, respectively, following a single i.v. dose, or 34% and 22% of original activity, respectively, following oral administration. These differences between i.v. and oral administration of sarin highlight the effect of rate of ChE inhibition, and consequently the rate of increase in CNS ACh, as a factor in the development of symptoms of exposure. Bowers et al. (1964) studied the effects of the nerve agent VX in man and described behavioral symptoms of anxiety, psychomotor depression, general intellectual impairment consisting of difficulties in concentration and retention, and sleep impairments generally involving insomnia due to excessive dreaming. Psychological/behavioral effects were typically evident before the occurrence of physical symptoms. These effects were associated with whole blood ChE inhibitions of $>60\%$.

There have been descriptions of the acute toxic effects in humans that follow high-dose exposure ($>LD_{50}$) to the nerve agents soman (Lekov et al., 1966; Sidell, 1974), sarin (Sidell, 1974; Inoue, 1995; Nakajima et al., 1998), and VX (Nozaki et al., 1995). The same cluster of behavioral symptoms that are reported following lower doses (anxiety, psychomotor depression, intellectual impairment, and sleep disturbances) dominate the clinical picture in the immediate period following resolution of the acute toxic signs of intoxication and then slowly fade with time, sometimes taking months to fully resolve.

There have been a number of investigations as to the possible long-term consequences of an acute symptomatic exposure to OP compounds. For the nerve agents, Burchfiel et al. (1976)

evaluated the long-term effects of an acute high dose (5 μg/kg, i.v.) of sarin on the EEG of rhesus monkeys. The animals were paralyzed and artificially respirated during exposure since this dose of sarin produced generalized seizure activity on the EEG that lasted an average of 2.5 h. At both 24 h and 1 year following the exposure there was a significant increase in the relative voltage in the beta frequency bands (13–22 Hz = beta-1; 22–50 Hz = beta-2) in the occipital–temporal EEG lead while the animals were awake in darkness. Similar EEG effects were seen in other animals in this study that were exposed to high doses of the chlorinated hydrocarbon, dieldrin. Functional behavioral tests of other rhesus monkeys exposed to sarin under identical conditions revealed no deficits in performance of a previously learned delayed response test 24 h after the exposure (Lattal et al., 1971). Duffy et al. (1979) performed a similar analysis of EEGs of manufacturer workers accidentally exposed to the nerve agent sarin at doses that produced clinical signs and symptoms of exposure and produced a reduction of erythrocyte ChE at least 25% below the individual's preexposure baseline. Within the exposed group there was a maximally exposed subgroup that had experienced three or more such exposures. The study was performed at least 1 year after the last exposure. Univariate and multivariate analysis of the data show that the exposed group, especially the maximally exposed subgroup, displayed (1) elevated amounts of spectral energy in high-frequency beta activity, (2) visual inspection of the EEGs showed decreased amounts of alpha (9–12 Hz) activity along with increased amounts of slow activity (0–8 Hz, delta and theta) and an increased amount of ''nonspecific'' abnormalities in the EEG background, and (3) increased amounts of rapid eye movement (REM) sleep. The functional consequences of these EEG changes were not established, but this group reportedly had a high incidence of self-reported memory disturbances, difficulty maintaining alertness, and appropriate focusing of attention (Metcalf and Holmes, 1969).

Several studies of the long-term effects of the sarin-exposure victims from Japan have been published. Murata et al. (1997) and Yokoyama et al. (1998a, 1998b) evaluated 18 victims of the Tokyo subway incident 6–8 months after the exposure. All but three of these victims had plasma ChE values below normal values on the day of exposure. Sarin-exposed individuals scored significantly lower than controls on a digit symbol substitution test; they scored significantly higher than controls on a general health questionnaire (GHQ) (psychiatric symptoms) and a profile of mood states (POMS) (fatigue) as well as having elevated scores on a posttraumatic stress disorder (PTSD) checklist; they had significantly longer P300 latencies on event-related brain evoked potentials and longer P100 latencies on brain visual evoked potentials. The P300 is a late positive wave that occurs between 250 and 800 ms after the onset of a meaningful stimulus and is related to the subject's psychological reaction to the stimuli and not the physical characteristics of the stimulus. In contrast, the P100 latency and magnitude is directly linked to the physical characteristics of the stimulus. Female-exposed cases were reported to have significantly greater indexes of postural sway. Although exposed individuals of both sexes displayed greater R–R interval variability in their electrocardiograms, the magnitude correlated with degree of ChE inhibition on the day of exposure. The elevated scores on the GHQ and POMS were positively related to the increased PTSD scores and were considered to be due to PTSD and were not related with degree of ChE inhibition on the day of exposure. Some of the Tokyo subway victims have been studied as far out as 7 years after the exposure. Nishiwaki et al. (2001) and Miyaki et al. (2005) reported on rescue workers and police officers who were exposed, and these could be further broken down into high and low exposure groups. The earlier noted changes in postural sway failed to achieve conventional statistical levels of significance in these later studies. There were persistent significant, dose-dependent, changes in psychomotor function (reduced tapping frequency, dominant hand), and a memory test (e.g., shorter backward digit span) in comparison with a referent group. Nakajima et al. (1997, 1998) performed a cohort study of victims of the Matsumoto City sarin exposure 1 and 3 years following the incident. At 1 year following the exposure they report that 20 victims still felt some symptoms (fatigue, asthenopia, blurred vision, asthenia, shoulder stiffness, and husky voice), and that they had lower erythrocyte ChE activity than did those who did not have symptoms and had all lived close to the sarin

release site. (Note: Not all the symptoms seen at 1 year have been related to nerve agent exposure historically.) At 3 years, some victims still complained of experiencing these symptoms, although with a reduced degree and frequency. There have been two brief reports of severely poisoned nerve agent victims (one sarin, one VX) in Japan that experienced retrograde amnesia, possibly due to prolonged periods of seizures and hypoxia (Nozaki et al., 1995; Hatta et al., 1996). Additionally, one of the Matsumoto victims that experienced prolonged seizure activity was followed up for at least 1 year and was found to have sporadic sharp wave complexes in the EEG during sleep and frequent premature ventricular contractions on Holter monitoring of the electrocardiogram (Sekijima et al., 1995).

In addition to these neuropsychological changes that could be attributed to the sarin exposure alone, many of the Japanese victims suffered PTSD (Kawana et al., 2001). There have been a number of reports that have dealt specifically with neurological and psychological changes that appear to be specifically attributed to that condition. Matsuo et al. (2003) reported Tokyo subway sarin attack victims with diagnosed PTSD showed significantly greater activation of the prefrontal cortex as assessed by near-infrared spectroscopy and skin conductance response to stimuli directly related to the exposure incident than did victims without PTSD or controls. Yamasue et al. (2003) reported that victims of the sarin attack with the clinical diagnosis of PTSD showed a significant reduction in grey matter volume of the left cingulate cortex as assessed by voxel-based magnetic resonance imaging (MRI) than did victims of the attack that did not develop PTSD. A similar reduction in anterior cingulate cortex volume has been reported in combat veterans with PTSD (Woodward et al., 2006) and in individuals with PTSD of other etiologies. It has been hypothesized that the anterior cingulate cortex may become hypofunctional as a consequence of repeated glucocorticoid activation during the exaggerated fear responses of PTSD victims and that as this condition goes on the volume of this area may physically shrink (Villarreal and King, 2001; Nutt and Malizia, 2004; Shin et al., 2006). Araki et al. (2005) followed up on the previously reported P300 event-related potential deficits and PTSD. They showed PTSD victims had significantly greater reductions in P300 amplitude than did victims with no PTSD. Moreover, within the group of victims with PTSD there was a positive correlation between P300 amplitude and anterior cingulated cortex grey matter density. Given these findings, it might appear that reduced brain volume or other physical neurologic changes of some of the sarin victims might be confounded by changes in brain function that accompany severe cases of PTSD. Most of these changes in brain volume and functioning that accompany PTSD have been reported over the last 8 years and it is clear that this is still an active area of research to decipher the exact neurophysiological etiology of the changes. Romano et al. (2007) look closely at the stress component of CWA exposure and its possible health effects, to include PTSD (see Chapter 22).

Finally, Yanno and Musiychuk (1997) published a short summary of 209 acute poisonings by sarin, soman, or VX in Russian nerve agent production facilities. Twenty-eight percent of the victims required hospitalization that ranged from a few to 120 days. Long-term consequences of these exposures were described as memory loss, signs of asthenia, sleep disorders, diencephalic paroxysms, vegetative changes in the cardiovascular system, and microorganic disorders of the CNS. It was noted that CNS symptoms were most prominent and persistent following soman poisoning, confirming observations made by Sidell (1974).

Outside of accidental exposures to nerve agent at military facilities and the Japanese terrorist incidents, the other notable instance of exposure to high doses of nerve agent occurred during the Iran–Iraq war (1981–1987). From 1984 to 1987 there were numerous instances of Iranian troops suffering exposure to supralethal doses of tabun or sarin and surviving due to prompt medical intervention. Experiences from this war in treating nerve agent casualties were described by Dr. Syed Foroutan who published his reminiscences of nerve agent and sulfur mustard casualty care in a series of articles in the Farsi-language Kowsar Medical Journal in the late 1990s. These treatment protocols and general experiences have been summarized in an English-language review paper (Newmark, 2004). It is surprising that there has been no published follow-up medical studies

of any long-term effects in the many thousand of nerve agent casualties that survived, although there have been a number of studies published on long-term persistent adverse health effects of victims of sulfur mustard exposure from that conflict (Balali-Mood and Hefazi, 2006; Hashemian et al., 2006).

In one of the major OP pesticide studies, Savage et al. (1988) retrospectively (approximately 9 year after the poisoning) examined 100 individuals with documented acute OP pesticide poisoning and compared them with matched-pair nonpoisoned controls. They reported no differences between the two groups in visually inspected EEGs or a number of neurological tests. There were, however, significant differences between the two groups in their performance on a number of neuropsychological tests, as well as self- and family-assessment of functioning ratings. They stated that their results showed subtle long-term neuropsychological sequel to acute OP poisoning that are difficult to detect with standard neurological exams that stress sensory and motor function. Rosenstock et al. (1991) performed a retrospective neuropsychological study of OP-poisoned agricultural workers and compared them with a matched control group. They found that when tested 2 years after exposure, poisoned workers self-reported significantly higher numbers of neuropsychological difficulties and had significantly lower test scores than controls on tests of verbal attention, visual memory, and visuomotor and motor functions, as well as tests of visuomotor sequencing and problem solving. Likewise, Steenland et al. (1994) found deficits in vibrotactile sensitivity and sustained attention among previously intoxicated subjects versus controls. These effects showed a rough dose–response relationship in that there were significant trends to worse performance on other neurobehavioral tests by those subjects who were more severely poisoned (longer hospitalization, took more days off from work). However, as with other studies, nerve conduction tests and neurological examinations were negative. There was no evidence of changes in postural sway in poisoned subjects as was reported for the sarin-exposed subjects from Tokyo indicating, perhaps, a difference between OPs and nerve agents with regard to effects on motor activity (Yokoyama et al., 1998a, 1998b).

Wesseling et al. (2002) assessed long-term neurobehavioral effects of mild OP or *n*-methyl carbamate poisoning in banana workers. The subjects (81 exposed, 130 controls) were tested on average after 27 months exposure and assessed on measures of psychomotor and visuomotor performance and neuropsychiatric symptoms. The exposed group showed a modest yet significant decreased performance on a visuomotor (digit symbol substitution) test and an increase in neuropsychiatric symptoms. These effects were especially pronounced in the OP exposed subgroup, and those exposed individuals that reported a subsequent exposure to OPs within 90 days of testing showed even poorer performance. This same research group has followed individuals that were acutely poisoned with OPs up to 2 years after exposure (Delgado et al., 2004). Fifty-three poisoned individuals and 28 controls were given a standardized neurobehavioral test battery at the time of hospital discharge, 7 weeks later and 2 years later. The authors reported decreased visuomotor performance (digit symbol substitution) and verbal memory at the time of discharge, whereas only visuomotor performance was decreased at the 7 week test. No effects were detected at the 2 year testing point, although the exposed subjects reported significantly greater neuropsychiatric symptoms at that time.

Studies in animals of long-term effects of acute, nonlethal exposures to nerve agent are numerous in the literature since 1980. Following high-dose exposure (approximately 0.6 LD_{50} and higher), seizures are a prominent sign of nerve agent intoxication and these prolonged seizures can produce both neural and cardiovascular lesions if not promptly treated (McDonough and Shih, 1997). Neurological, behavioral, and cardiac deficits are predictable long-term effects following exposure to such doses. Animals exposed to convulsant doses of nerve agent can develop spontaneous seizures, display hyper-reactive and aggressive behavior (rats), and display profound deficits in learning and performance of a variety of behavioral tasks. In fact, animal studies have demonstrated deficits in acquisition of several types of operant tasks (differential reinforcement of low rates, alternation), performance of serial probe recognition task, acquisition and performance of several maze tasks, and passive avoidance learning following acute intoxication with nerve

agents (McDonough, Smith and Smith, 1986; Raffaele et al., 1987; Modrow and Jaax, 1989; Castro et al., 1991; McDonough and Shih, 1997; Raveh et al., 2002, 2003). Invariably, animals displaying such behavioral changes also are shown to have brain lesions in cortical and subcortical limbic structures. Exemplifying the relationships seen between such neuropathology and deficient behavior is the demonstration of EEG and performance changes following near LD_{50} challenge with soman (GD) in a comprehensive paper by Phillippens et al. (1992). In this study, rats were intoxicated with an LD_{50} of soman and immediately treated with an antidotal combination of atropine and diazepam (described as a ''low-dose combination''). These rats had been trained to an overlearned criteria of 80% correct avoidance response (i.e., avoidance of a signaled foot shock). After a period of recovery of motor capacity, animals demonstrated impaired performance of the conditioned response for three test sessions before they approached the prechallenge performance level. Similarly, electrographic correlates of lesions and ultimately, light microscopic observation of lesions suggested the neuroanatomic basis for this deficit. By contrast, animals exposed to the same challenge dose of agent and receiving a ''high-dose combination'' of atropine and diazepam performed at or near the level of prechallenge performance and were somewhat (but not completely) protected from electrographic and neuropathologic changes. This report demonstrates a general theme found in most of these ''high-dose'' exposure studies; animals exposed to nerve agents that develop seizures that are not promptly controlled develop brain damage and consequent neurobehavioral problems, animals that do not develop seizures or those that seize and are rapidly and effectively treated with drugs that stop the seizures suffer no brain lesions, and display no long-term neurobehavioral deficits.

In the case of acute ''low-level'' exposures to nerve agents, exposures that produce minimal or no acute CNS signs of intoxication, an earlier study by Burchfield et al. (1976) suggested that a ''clinically sign-free'' dose of sarin (1 μg/kg, i.m.) given repeatedly (1 time/week for 10 weeks) to nonhuman primates (three rhesus monkeys) resulted in subtle, but persistent, EEG changes (increases in the percentage of high-frequency beta activity) that were virtually identical to those already described above that are seen after an acute high-dose exposure (5 μg/kg, i.v.) that provoked seizures. Pearce et al. (1999) followed behavioral and electrographic outcomes in nine marmoset monkeys for up to 15 months following exposure to a single low dose of sarin (2.5–3 μg/kg, i.m.). Although the dose of sarin caused 36%–67% inhibition of RBC–AChE, there were no acute behavioral signs of intoxication (thus, the exposure was subtoxic), there was no significant change or decrement in performance on a series of touch-screen mediated discrimination tasks either immediately or over a 12–15 month period following the exposure. There were also no significant long-term changes in EEG patterns in this study. Although there were changes in beta-2 amplitude that approached significance ($p = 0.07$), this was entirely due to a long-term change in the EEG of a single subject out of the nine animals that were exposed. The parameters chosen in this study were employed because they had been used to demonstrate deficits caused by cholinergic lesions or ChE inhibitors in previous studies (Roberts et al., 1992; Sohakian and Coull, 1993). More recently, van Helden et al. (2004) reported that the lowest observable adverse effect level (LOAEL) for vapor exposure to sarin to modify EEG activity in marmoset monkeys produced LOAEL values (0.2 and 0.1 $mg/min/m^{-3}$) for untreated and pyridostigmine pretreated monkeys, respectively, at least an order of magnitude lower than the previously established LOAEL for miosis. When evaluated 1 year after exposure, vehicle pretreated animals had significant increases in total power within each EEG band compared with their recordings a year earlier. A similar, but more attenuated effect was seen in the pyridostigmine pretreated animals. Visual examination of the EEG records at 1 year showed that both groups of sarin-exposed animals had greater number of bursts within the alpha frequency that resembled sleep spindles than did the controls. These results are different than those reported by Pearce et al. (1999) or by Burchfield et al. (1976) that reported changes in the high-frequency beta bands.

Genovese and colleagues (Genovese et al., 2007a, 2007b) have reported on several studies on the potential for long-term behavioral effects of acute exposure to several nerve agents. In a study of nonhuman primates, rhesus and African green monkeys were trained to perform a serial probe

recognition task (a test of short-term memory similar to list learning) and then exposed to doses of approximately 0.25 LD_{50} of sarin. The animals were tested for up to 3 months following exposure and showed no decrements or changes in performance over that time even though there were significant reductions in blood ChE over that time. These results are similar to the reports of Lattal et al. (1971) and Pearce et al. (1999). In two other studies (Genovese et al., 2007a, 2007b), rats were trained preexposure to perform on a food-motivated variable interval 56 s (VI 56; the first lever press response after a variable interval that averages 56 s produces food reinforcement; this schedule produces steady intermediate rates of lever press responding) operant task. The rats were then exposed to varying doses of cyclosarin or VX using a whole-body inhalation exposure system and then tested repeatedly for up to 3 months on the VI 56 task. In addition, they were tested for their ability to acquire normal performance on a radial arm maze, a task that assesses spatial memory. In both studies, the highest levels of exposure produced transient mild signs of nerve agent intoxication (miosis, fasciculations, mild ataxia) and transient decreases in VI 56 performance at these high doses; doses that produced no signs were without immediate behavioral effects. Across all doses, there were no lasting effects of nerve agent exposure on VI 56 performance or on the acquisition or performance on the radial arm maze task. The authors concluded that doses of nerve agent that produce acute mild signs of intoxication can produce transient disruptions in performance on a well-learned task, but these effects quickly dissipate and there were no evident long-term effects; asymptomatic exposure produces no behavioral effects.

In an extensive series of studies, Kassa and colleagues have reported changes in immune function, biochemical parameters, protein and nucleic acid metabolism, and various aspects of neurobehavioral function and learning following single or repeated exposure to sarin or soman in mice and rats. Following a single asymptomatic exposure to sarin, changes in immune function (Kassa et al., 2003, 2004a, 2004b) were seen up to one week after exposure. This group reported that a single inhalation exposure of rats to sarin produced increases in stress markers (plasma cortisol levels, liver tyrosine aminotransferase activity), decreases in DNA synthesis, increases in CNS excitability, and impairments in spatial discrimination (T-maze, Y-maze) that persisted as long as 3 months after exposure (Kassa et al., 2004c). Many of the effects were dose-dependent, animals that were exposed to the highest dose and developed moderate signs of poisoning were most affected, asymptomatic animals showed attenuated and less persistent effects, whereas animals that received repeated asymptomatic exposures showed more long-lasting effects.

Many of these effects on immune function have also been reported by Henderson et al. (2001, 2002) and others in that research group (Kalra et al., 2002; Penna-Philippides et al., 2007). They have shown that F344 rats exposed either once or multiple times to asymptomatic levels of sarin vapor had decreases in immune function that typically recovered over weeks after the exposures stopped. Pretreatment of the rats with chlorisondamine attenuated the effects of sarin on immune response, implicating the autonomic nervous system in these sarin-induced changes. They also reported that there were reductions in serum adrenal corticotrophic hormone (ACTH) and corticosterone levels that were still evident after 4 weeks exposure. It is quite possible that many of the effects on the immunological system reported for sarin exposure may be due to the central nicotinic effects of ACh stimulation, since this same group has reported similar immunological function changes following chronic nicotine exposure (Langley et al., 2004; Razani-Boroujerd et al., 2004).

Marrs et al. (1996) and Sidell (1996) have provided an extensive overview of human studies of nerve agent exposures conducted by the US and the UK military as well as accidental exposures that occurred at production or test facilities. Although this report does not come to any conclusions about long-term effects, there is no indication that asymptomatic exposures to nerve agents have produced long-term, adverse health effects. This is the same conclusion reached by the National Academy of Sciences, committee that reviewed the then available literature, to include the EEG studies of Burchfield et al. (1976) and Duffy et al. (1979). They stated that while there may be subtle

long-term EEG changes, the clinical significance and functional relevance of such changes had not been demonstrated (National Academy of Science, 1982).

IV. CHRONIC HEALTH EFFECTS OF REPEATED LOW-LEVEL EXPOSURE

For chronic or repeated subclinical exposures to OP compounds, whether they are CW nerve agents or OP pesticides, the data in regard to long-term health effects are less consistent. As regards to the nerve agents, the report of Burchfield et al. (1976) about the effects of repeated low doses of sarin to rhesus monkeys producing a long-term increase in relative power in the EEG beta frequency bands is the most cited study in support for a long-term health effect. There are no human studies known to these authors, other than the National Academy of Science report (1982) on the volunteer program mentioned earlier, which directly address the possible adverse long-term health effects of repeated subclinical exposures to nerve agents. Workers exposed to small amounts of nerve agents that produced mild, nonthreatening medical signs of exposure, reported CNS effects such as headache, insomnia, excessive dreaming, restlessness, drowsiness, and weakness (Marrs et al., 1996). The medical officers describing these patients suggested that ''mental processes used in making judgments and decisions were also affected'' (Marrs et al., 1996). Out of 53 patients with mild exposures not requiring antidotal therapy, CNS symptoms often were fully resolved within 3 days after exposure. However, Sidell cautions that psychological symptoms are probably more common than usually recognized and may persist in more subtle forms for much longer days or weeks than physical symptoms (Sidell, 1974).

Reports in the literature of animal studies show that nerve agents can be administered repeatedly with minimal overt neurobehavioral effects if care is taken in choosing the dose and the time between doses (Sterri et al., 1980, 1981). Blood and brain AChE levels can be reduced to $>20\%$ of normal with no observable signs of toxicity with appropriate dosing schedules. Animal studies performed at the USAF School of Aerospace Medicine have demonstrated a progressive and long-lasting inhibition of ChE in the CNS following repeated administration of low doses of the nerve agent soman, a finding recently corroborated by Olson using the nerve agent sarin (Hartgraves and Murphy, 1992; Olson et al., 2000). There appear to be differential sensitivities among various brain regions, with frontal and pyriform cortex being most sensitive to the ChE inhibiting effects of CW nerve agents, whereas the neostriatum and the hypothalamus are relatively less sensitive. These studies and others did not demonstrate a tolerance to the CNS ChE inhibiting effects of repeated administration of low levels of CW nerve agents.

Recovery from the ChE inhibition produced by chemical warfare nerve agents or other OP compounds is not a simple matter, however. The recovery of CNS ChE does not parallel the recovery of plasma ChE, with plasma ChE often recovering much more rapidly than RBC–AChE, which more closely parallels the recovery of brain AChE (McDonough et al., 1983; Russell et al., 1986) Thus, these and other tolerance studies (Clement 1989, 1991) suggest that behavior has often recovered to near baseline, while AChE is still significantly lowered. This has been noted clinically and Sidell (1992) cautions, ''Analysis of blood for ChE is useful for occupational monitoring, but in an exposed patient, one treats the patient, not the ChE activity.''

Recently, Olson et al. (2000) and Benschop et al. (1998) have provided reports of animal studies of effects of repeated low-level exposure to nerve CWA. In rats, Olson et al. determined the LOAEL and NOEL of subacute dosages of sarin, administered, i.m. They found that the dose of sarin (GB) needed to produce a low but measurable blood ChE inhibition was 0.75 μg/kg once a day for 4 days. Thus, the exposure in Olson's study would be described as subclinical. GB was paired with a variety of other chemicals to include chlorpyrifos, DEET (*N,N*-diethyl-*m*-toluamide), carbaryl, and PB. No neurobehavioral or neuropathologic effects could be attributable to dosing with GB alone or in any combination with the other chemicals. Rats were also evaluated using a functional observational battery (FOB) and a Figure 8 Activity Monitor with no significant behavioral effects reported. Benschop et al. (1998) reported on the toxicokinetics of low-level inhalation exposure to soman in

atropinized guinea pigs. Animals were exposed to 200 ppb for 5 h of a toxic steroisomer of soman, which resulted in a gradual inhibition of RBC–AChE to approximately 10% of baseline. This level of exposure resulted in an insignificant reduction of AChE activity in brain and diaphragm although it was equivalent to Ct values of 0–48 mg min/m^3, a dose well above one sufficient to cause an incapacitating miosis. The observed lack of inhibition of AChE in brain and diaphragm at the end of the long-term, low-level exposure was interpreted to mean that systemic intoxication is unlikely despite extensive inhibition of blood AChE. Furthermore, Benschop et al. argued that the development of persistent neuropsychological disorders under these conditions would be unlikely. The authors cautioned that studies in animals without the benefit of carboxylesterase binding sites, such as primates, would most probably reflect a different outcome. This last study points out the influence of dose rate in determining whether a given exposure would be ''nonlethal,'' ''subtoxic,'' or ''subclinical,'' a point made as long ago as 1975 by Sim. The latter wrote that a patient appearing in a clinic without measurable ChE, yet not appearing to be intoxicated, ''emphasizes that the poison is cumulative and if taken into the body slowly, can be accommodated without the appearance of critical illness.''

The most notable effect of repeated low doses of nerve agents seen in animal experiments is the development of tolerance to the disruptive effects of each acute exposure on certain behaviors (Russell et al., 1986; van Dongen and Wolthuis, 1989; Hymowitz et al., 1990; Wolthius et al., 1990; Clement, 1991). This is primarily thought to be brought on by downregulation (i.e., reduction in the number) of muscarinic receptors in the brain, which will remain lowered (maximal reduction 30%–40%) for the duration of the exposure and then recover in parallel with the erythrocyte ChE activity following the cessation of exposure (McDonough et al., 1983; Churchill et al., 1984). During the period of reduction in muscarinic receptor numbers, the animals are subsensitive to anti-ChEs or direct acting muscarinic agonists and suprasensitive to the effects of antimuscarinic drugs (e.g., atropine) (Modrow and McDonough, 1986). In this respect, nerve agents act much like other OP compounds and the possibility and mechanisms of tolerance development have been addressed in several studies (see the review by Russell and Overstreet [1987] for an overview of these animal studies). The question of behavioral tolerance is seen by some authors as a masked toxicity in a vulnerable organism (DeMenti, 1999) or as an adaptive response to a changed internal physiologic state (Young et al., 1999). Bignami et al. (1985) have suggested that while some of the tolerance developed to OPs may be attributable to cholinergic receptor changes, behavioral (test) variables may also play a role. These authors studied feeding and drinking responses and examined the role of practice factors in tolerance to paraoxon. Specifically, they measured the decrease in food consumption and the strength of a conditioned flavor aversion (CFA) produced by repeated doses of low levels of paraoxon. They qualified their finding of reductions in the depression of food intake or extinction of CFA by stating that treatment–behavior interactions may produce apparent attenuations of toxicity, which are often not maintained when the situation is changed, leaving open entirely the nature of the purported response.

Munro et al. (1994) reviewed both animal and human studies of the nerve agents tabun, sarin, and VX and paid special attention to the phenomenon of organophosphorous induced delayed neuropathy (OPIDN). Reproductive toxicity and carcinogenicity tests were reviewed as well as in vitro studies of mutagenicity. Munro et al.'s findings can be summarized as follows: (1) for the nerve agent tabun, no evidence of subchronic toxicity was observed at any dose other than effects on ChE activity. No evidence of teratogenicity was found and tabun was a weakly active in an in vitro assay as a mutagen, (2) for nerve agent sarin, no evidence of acute or chronic toxicity was found at low intermittent exposure levels, sufficient to significantly depress AChE levels. No evidence for carcinogenicity, teratogenicity, or mutagenicity was found for sarin, but a data gap in the area of reproductive toxicity was noted, and (3) for nerve agent VX, no evidence for likely development of OPIDN was found. VX exposure sufficient to significantly depress RBC–AChE activity produced no subchronic toxicity, no evidence of carcinogenicity was found, and based on multiple studies VX was not considered a potential mutagen or a teratogen. Thus, the authors concluded that the

overriding concern with regard to exposure to tabun, sarin, or VX was their extraordinarily high acute toxicity (Munro et al., 1994).

Since the original preparation of this chapter, a number of other animal studies have been published that involve repeated low level, asymptomatic, exposure to nerve agents, primarily sarin. In a series of reports, Scremin and colleagues used a standard exposure paradigm to evaluate the effects of repeated sarin exposure, chronic pyridostigmine exposure, or repeated sarin + pyridostigmine exposure on a variety of physiological and behavioral parameters at 2, 4, and 16 weeks after the conclusion of the exposures. They gave rats regular drinking water or water adulterated with pyridostigmine (to produce ~30% reduction in RBC–AChE) and then exposed them to $0.5 \times LD_{50}$ of sarin or saline by SC injection three times a week for 3 weeks. In the first study (Scremin et al., 2003), the animals were then evaluated on various neurobehavioral tests (passive avoidance, acoustic startle response, open-field locomotion, nocioceptive threshold) at 2, 4, and 16 weeks after the cessation of exposure. The sarin exposure produced no acute observable signs of OP intoxication. At 2 weeks after exposure, acoustic startle was enhanced while locomotor activity was decreased in sarin-exposed animals; these effects were not seen in the sarin + pyridostigmine or pyridostigmine groups. At 4 weeks after exposure, there were no differences between groups. At 16 weeks, the sarin animals had reduced habituation in the open field while there was more rapid habituation and an increase in nocioceptive threshold in the sarin + pyridostimine group. Brain AChE was not affected by sarin exposure, although they did measure a reduction in brain muscarinic receptors at 2 weeks after exposure. In a second report (Scremin et al., 2005), regional cerebral blood flow and glucose utilization were measured. There were significant increases in regional cerebral blood flow, but not glucose utilization, in neocortex at 2 weeks after exposure for the sarin + pyridostigmine group, with a similar pattern of change being seen in the sarin group at 4 weeks after exposure. Changes seen at 16 weeks after exposure were few and random. The authors concluded that there were no long-term changes in cerebral activity as indexed by cerebral glucose use or blood flow produced by repeated sarin exposure or any of the other treatments. Likewise, this group has recently reported (Scremin et al., 2006) that such chronic sarin and sarin + pyridostigmine treatments produced lower heart rates and changes in heart rate power spectrum that were prominent at different times of the daily circadian cycle in freely moving rats 2 weeks posttreatment. These differences were not seen at the 4 and 16 week observation times, nor were there changes in locomotor activity at any time. In a final report (Shih et al., 2006), levels of both endogenous and synthesized choline (Ch) and ACh were determined in a variety of brain regions at the three postexposure time points. There were significant differences in endogenous levels of ACh and Ch at the 2 and 4 week time points between controls and exposed groups; there was no difference in the ability to synthesize ACh at any of the test times. There were no group differences on any of these measures at the 16 week observation time, leading the authors to conclude that there were no persistent changes in these measures of cerebral cholinergic function.

A study by Atchison et al. (2004) provided information on the initial validation of a guinea pig model of low-dose exposure to the nerve agents sarin, soman, and VX. This model was then used in a number of subsequent studies that evaluated physiological, electrophysiological, biochemical, and behavioral effects of this exposure paradigm. In the Atchison et al. (2004) study, male guinea pigs were injected (sc) once a day, 5 days a week (M–F), for 2, 4, or 13 weeks with 0.2, 0.4, 0.6, and $0.8 \times$ acute LD_{50}s previously established of sarin, soman, or VX. These exposure times (2, 4, and 13 weeks) were designed to cover the subacute to chronic durations of exposure as defined by Klaassen and Eaton (1991). Signs of cholinergic toxicity (for 2 h after exposure) and body weights were monitored throughout the exposure period. After the initial study, a second study was performed using asymptomatic doses (vehicle, 0.2 and $0.4 \times LD_{50}$) administered for 2 or 4 weeks according to the same protocol. In these animals, red-blood cell AChE was measured 1 h after the last injection, then 3 days later, the animals were sacrificed and diaphragm tissue was collected for measurement of AChE activity, blood was sampled for standard chemistries and hematology, and tissues (brain, heart, lung, liver, gallbladder, kidney, adrenal gland, skeletal muscle, sciatic nerve,

and testes) were collected for histopathology (hematoxylin and eosin stain) examination by a board certified veterinary pathologist. Doses of 0.2 or 0.4 × LD_{50} of any of the three agents were tolerated with no lethality or toxic signs throughout the 13 week exposure. In contrast, 0.8 × LD_{50} of the three agents were lethal to all animals exposed to this dose, and 0.6 × LD50 of sarin was also lethal while these same dose fractions of soman or VX were survived by ~20% of the animals. In the 2 and 4 week exposure study with 0.2 and 0.4 × LD_{50} of each agent, blood AChE was inhibited from 75% to 91% of control activity on the last day of exposure depending upon the agent and the dose regardless of whether the animals were exposed for 2–4 weeks. Diaphragm AChE measured 3 days after the last exposure was inhibited 18%–30% depending upon the agent, but the differences were not statistically significant compared with controls. There were no differences in body weight gains, body temperature, blood chemistries, hematology, or histopathology of any of the tissues collected between exposed guinea pigs and the controls. It should be noted that in the 4 week exposure study, animals received cumulative doses of 4 and 8 × the acute LD_{50} of each of these nerve agents.

The 2 week repeated exposure paradigm in guinea pigs has been used in subsequent studies of the effects of repeated nerve agent exposure on learned and unlearned behaviors, EEG activity, and brain neurochemistry. Hulet and colleagues (Hulet et al., 1993; Shih et al., 2006) used this exposure protocol with guinea pigs to determine any adverse effects of repeated doses (saline, 0.4 × or 0.5 × LD_{50}) of sarin. The animals were assessed for changes in body weight, RBC–AChE levels, neurobehavioral reactions to an FOB, cortical EEG power spectrum, and intrinsic ACh neurotransmitter regulation over the 2 weeks of sarin exposure and for up to 12 days postinjection. Animals receiving the 0.5 × LD_{50} showed signs of cholinergic toxicity and gained significantly less weight than the 0.4 × LD_{50} or saline animals, which did not differ from each other. RBC–AChE levels had dropped to 11% and 10% of control values for the 0.4 and 0.5 × LD_{50} groups, respectively, by the end of the sarin-exposure period, although the rate of AChE depression was faster in the 0.5 × LD_{50} group. Both the 0.4 and 0.5 × LD_{50} groups failed to display evidence of habituation to aspects of being handled over the period of exposure, unlike the controls which showed a significant decline in these scores on the FOB test. Power spectral analysis of the EEG showed that saline-injected animals tended to fall asleep after exposure and showed a progressive increase in the power of the lower (delta, theta) bandwidths. The high-dose sarin-exposed animals (0.5 × LD_{50}) failed to show any change in distribution in EEG power over this time while the 0.4 × LD_{50} sarin animals were intermediate between the controls and the high-dose group. These changes persisted for 6 days after the exposure ended for the high-dose group. Six days after the end of the exposure, sarin-exposed animals showed a greater increase in striatal ACh output than controls, as measured by microdialysis, in response to a pharmacological challenge with atropine. The authors concluded that the results suggested subtle, but transient, effects on neurobehavioral function, EEG, and brain neurochemistry for the asymptomatic animals that received the 0.4 × LD_{50} sarin, and possibly more persistent changes in the symptomatic 0.5 × LD_{50} sarin group.

Langston et al. (2005) trained dietary restricted guinea pigs responding for food on progressive ratio (PR) schedule. Once performance had stabilized, the animals were exposed daily (5 day/week) to 0.1, 0.2, or 0.4 × LD_{50} sarin for 2 weeks and performance was measured during the 2 weeks of exposure and for 2 weeks following the exposure. The two lower doses (0.1 and 0.2 × LD_{50}) produced no effects on performance during or after the period of exposure. However, the 0.4 × LD_{50} dose group showed decreases in body weight and concurrent decreases in response rates and break points that was due to increases in pausing following receipt of food reinforcement. These performance changes reverted to preexposure levels following termination of the exposure.

There are several other reports that have demonstrated the disruptive effects of the 0.4 × LD_{50} chronic sarin, soman, or VX exposure in food-restricted animals, which has also been reported by others. Sipos et al. (2001; and in Thompson et al., 2004, 2005) evaluated the effects of repeated low-dose exposure of guinea pigs to sarin, soman, or VX using this same procedure and evaluate acoustic startle response and active avoidance (two-way; air puff) performance. Chronic low-level exposure to sarin, soman, or VX enhanced acoustic startle. This enhancement started after several

days of exposure with sarin ($0.4 \times LD_{50}$) and abated over the weekend when sarin was not administered; this effect on acoustic startle was more robust (occurred earlier) in animals exposed to soman or VX. Only soman produced any effect (decreased avoidance) on active avoidance performance.

There are numerous studies of humans that have addressed the issue of chronic or repeated subclinical (asymptomatic) exposures to OP pesticides, but there is no consensus among the results and this has been reflected in several recent reviews (Colosio et al., 2003; Kamel and Hoppin, 2004). Korsak and Sato (1977) reported that pesticide workers with relatively high occupational levels of exposure to OP pesticides, in comparison with workers with low levels of exposure, tended to have increased EEG power within the beta frequencies, primarily in frontal areas of the brain. In addition, the high exposure level group had lower performance on a Trail Making Test and the Bender Visual Motor Gestalt test of a neuropsychological test battery. The authors suggested that these results indicated subtle frontal lobe dysfunction in the exposed subjects. Stephens et al. (1995), studied a population of 146 sheep dippers with an average of 15 years of potential exposure to several OPs (diazinon, propetamphos, chlorfenvinphos) found these individuals compared with a control group (quarry workers) has slower simple reaction time latencies, slower latencies in a symbol digit substitution test and slower correct reaction times in a syntactic reasoning test. Only the syntactic reasoning effect showed a significant dose–effect relationship when analyzed with an analysis of covariance. Tests of memory and learning showed no effect and there were no self-reported drops in intellectual performance. On the other hand, Ames et al. (1995) surveyed 45 pesticide applicators, each of whom had at least one documented episode of asymptomatic exposure. He reported no CNS or PNS effects. Rodnitsky et al. (1975) evaluated 23 workers chronically exposed to a mild degree to pesticides (farmers and commercial pesticide applicators) using a battery of neurobehavioral tests. They found no differences between the exposed versus a control group on tests of memory, signal processing, vigilance, language, and proprioceptive feedback performance even though plasma ChE levels of the exposed group were lowered below the control values. Similarly, Daniell et al. (1992) studied 49 pesticide applicators over a 7 month period of spraying. Performance of the cohort was compared with a control group of 40 subjects (slaughterhouse workers). Both groups were given a computerized neuropsychological test battery that consisted of visuomotor coordination tests, memory and cognition tests, and tests of motor coordination. The pesticide applicators were known to have generally well-controlled, low, intermittent exposure, as part of a program of occupational health training and monitoring. The authors found no evidence for clinically significant decrements in neuropsychological performance following the 7 month season of such exposure among the applicators, the main pesticide being Guthion (azinphosmethyl). Maizlish et al. (1987) examined 99 pest control workers (46 exposed workers versus a group of nonapplicators). The 46 workers applied diazinon to residential lawn properties. Both applicators and nonapplicators were monitored for the appearance of diethylthiophosphate (DETP) in their urine. Subjects were given a comprehensive neurobehavioral test battery, and had urine samples taken, before and after their 8 h work shift. The application season lasted 39 days. The following tests were included in the neurobehavioral battery: Continuous Performance test (measures attention/vigilance), Finger Tapping (motor speed), Digit Symbol Substitution (visual/motor speed), and so on. Median diazinon exposure per workday for applicators versus nonapplicators was 2.1 versus 0.03 mg, respectively. No adverse DETP-related changes were found in pre- or postshift neurobehavioral function. The authors concluded that there were no demonstrable behavioral effects of short-term, low-level diazinon exposure in a pest control program characterized by adequate personal protective equipment and direct supervision (Maizlish, 1987).

More recently Bazylewicz-Walczak et al. (1999) published their study of greenhouse workers occupationally exposed to pesticides. They gave greenhouse workers and a matched control group neuropsychological tests (simple reaction time, digit symbol, digit span, Benton visual retention test, Santa Ana test, aiming test, POMS) and a subjective symptom questionnaire before and then 4 months later after the heaviest period of OP pesticide application. Overall, when compared with

the controls, the exposed subjects showed slower simple reaction times, lower hand movement efficiency on the aiming test, and reported a higher degree of anxiety, anger, depression, and fatigue-inertia. In addition they also reported more complaints relating to absent-mindedness and neurological symptoms. There were no differences in the exposed group over the one season. There was no change or an improvement in scores on the neuropsychological tests across the season while there was an improvement in mood and general feeling scores between the pre- and the postseason tests. The authors concluded that even low, long-term OP pesticide exposure may be associated with subtle adverse behavioral effects, and that they are characterized by increased tension and anxiety states, depression, fatigue, and a slow-down of perceptual-motor functions.

Since we first reviewed the above studies, several other reports have been published that further examined the relationship between low-level asymptomatic exposure to pesticides and changes in neuropsychological function. Farahat et al. (2003) evaluated neurobehavioral performance on a battery of tests given to unexposed controls and Egyptian workers that applied pesticides (OPs, carbamates, insect growth regulators, pyrethroids) to cotton crops during the height of the application season. The pesticide applicators had slightly (20%) lowered serum ChE levels compared with the controls, but these levels were still within the normal range. The exposed group showed significantly poorer performance than the controls on six of the neurobehavioral battery tests (similarities, digit symbol substitution, trailmaking parts A and B, letter cancellation, digit span, and Benton visual retention). In addition, exposed subjects reported higher instances of the neurological symptoms of dizziness and numbness and significantly higher neuroticism (nervousness, anxiety) scores than controls. Serum ChE was not significantly correlated with task performance, but duration of pesticide exposure was. The authors suggested that the deficits in a wider array of neurobehavioral tests observed in this than in other studies, coupled with the neurological signs, indicated a higher level of exposure in this study population. Roldan-Tapia et al. (2005) studied neurobehavioral performance of greenhouse workers exposed occupationally to both OPs and carbamate pesticides. Individuals with a high cumulative exposure risk (''years working with pesticides'') displayed worsened perceptive function performance, visuomotor praxis, and integrative task performance times. These findings were taken as evidence that long-term use of these pesticides has adverse effects on neurobehavioral functioning. Rothlein et al. (2006) reported that neurobehavioral performance of Hispanic farm workers with high risk for exposure to pesticides was lower than similar Hispanic immigrants in nonagricultural jobs. Within the exposed farm worker population there was a positive correlation between severity of exposure, as measured by urinary OP metabolite levels, and poorer performance on some of the neurobehavioral tests. This same group has also used a computerized test battery to examine whether adolescent farm workers exposed to pesticides are any more susceptible to these neurobehavioral effects than adults (Rohlman et al., 2006). Although there was no evidence of selective sensitivity between adolescents and adults in this study, the results showed that cumulative exposure to low levels of pesticides is associated with neurological impairment as measured by tests of selective attention, digit symbol substitution, and reaction time. In contrast, Eckerman et al. (2007) have assessed neurobehavioral function in rural adolescents involve with agriculture and urban controls. When level of pesticide exposure was factored into a multiple linear regression analysis of performance, it was found that poorer performance on finger tapping, digit span, and selective attention was associated with high levels of exposure and that this relationship was especially strong for the youngest (10–11 years) age group.

In summary, the above studies show that neurobehavioral evaluation of pesticide workers reveal increased prevalence of neurologic symptoms and changes in neurobehavioral performance indicative of mild cognitive and psychomotor dysfunction. These effects appear to be more prevalent during the period of exposure, but also persist beyond the period of exposure. Total cumulative exposure seems to be a greater risk factor for poor performance than any other covariant. Deficits in visuomotor speed as indexed by the digit symbol substitution test and the trailmaking tests have been shown to be consistently effected as well as tests of selective attention and memory

(digit span). Many of these same neurobehavioral functions were also reported to be long-term effects of OP pesticide poisoning (Savage et al., 1988; Rosenstock et al., 1991; Steenland et al., 1994; Wesseling et al., 2002; Delgado et al., 2004) as well as sequel of sarin poisoning (Murata et al., 1997; Yokoyama et al., 1998a, 1998b;. Nishiwaki et al., 2001; Miyaki et al., 2005).

V. DEVELOPMENT OF IMPROVED MEDICAL COUNTERMEASURES

The major medical research efforts since the 1940s focused on developing the best possible lifesaving therapeutic interventions and pretreatments for nerve agents. More recently, focus has been on prevention of long-term changes in CNS function following a moderate to severe intoxication using anticonvulsant drugs. This refocusing on the health effects of nonlethal exposures while improving protection from otherwise lethal exposures has resulted in several new approaches to improved treatment of nerve agent intoxication. A number are in full-scale development in the United States today. The major efforts are described subsequently.

A. Advanced Anticonvulsant System

In 1990, the benzodiazepine drug diazepam was introduced by the US military as an anticonvulsant to control nerve agent-induced seizures that are associated with severe intoxication. Diazepam was fielded in automatic injectors with a dose of 10 mg per injector and up to three injectors were authorized to be administered to a convulsing casualty in the field by a combat medic or corpsman. A prodrug formulation of diazepam, known as Avizafone, is used by the UK as the anticonvulsant in their nerve agent treatment kit (McDonough et al., 1999; Lallement et al., 2000; Taysse et al., 2003, 2006). Diazepam had been shown to be capable of controlling nerve agent-induced seizures and to prevent or reduce associated brain damage that resulted from prolonged seizure activity (Lipp, 1972, 1973; Martin et al., 1985; Hayward et al., 1991; Philippens et al., 1992; McDonough and Shih, 1997). Just as importantly, diazepam was already recognized as effective and approved for the acute treatment of seizures by the Food and Drug Administration (FDA). However, animal studies have shown control of nerve agent seizures by diazepam is slow when the drug is administered by the intramuscular route and full control of seizure activity may require higher doses than are currently approved (Philippens et al., 1992; Murphy et al., 1993; McDonough et al., 1999). The later the control of nerve agent seizures, the greater the probability that an individual may experience neurological damage (Lallement et al., 1994; McDonough et al., 1995). Because of these concerns there were continuing research efforts to identify a more effective anticonvulsant treatment for nerve agent-induced seizures. A number of animal studies have shown that the benzodiazepine midazolam is more rapidly acting and more potent in controlling nerve agent seizures than diazepam (Bokonjic and Rosic, 1991; Anderson et al., 1997; McDonough et al., 1999; Shih et al., 2003, 2007). This is apparently due to the greater potency and more rapid pharmacokinetics of midazolam compared with diazepam when administered via the intramuscular route (as with an autoinjector). This, in turn, equates to more rapid anticonvulsant effectiveness of midazolam (Capacio et al., 2001, 2004). In addition to these animal studies, human clinical data from a variety of investigators have shown that midazolam is highly effective in controlling generalized convulsive status epilepticus seizures that result from a variety of etiologies (Fountain and Adams, 1999; Towne and DeLorenzo, 1999; Galdames-Contreras et al., 2006; Kyrkou et al., 2006). This anticonvulsant effectiveness has been reported in both adults and children and when midazolam was given by intravenous, intramuscular, intranasal, or buccal routes of administration. It is for these reasons that midazolam has been chosen for advanced development as an anticonvulsant treatment to eventually replace diazepam. Efforts are currently under way to provide data demonstrating the effectiveness of midazolam against nerve agent-induced seizures in two animal species to satisfy the ‘‘two animal’’ rule of the FDA. Other formulation, stability, pharmacokinetic, and safety studies are also planned to support this effort.

B. Improved Oxime Therapies for Nerve Agents

Like other OP anti-ChE compounds, the nerve agents react with AChE by phosphylating the active site of the enzyme. Oximes are nucleophilic compounds that are capable of splitting off the phosphorus atom from the active site, thereby restoring enzyme activity. However, oxime treatment of a nerve agent casualty is complicated by several factors. First, the ability of a given oxime to reactivate nerve agent-inhibited enzyme is highly dependent on the specific nerve agent, owing to individual differences in the structures of the enzyme–inhibitor complex. For example, 2-PAM Cl, the only oxime approved for use in the United States, is highly effective in reactivating enzyme inhibited by the agents sarin and VX, but is substantially less effective in reactivating enzyme inhibited by the agents tabun, cyclosarin or soman. Second, the phosphylated enzyme can undergo a dealkylation reaction, termed ''aging,'' which makes the resultant enzyme–inhibitor complex totally resistant to oxime reactivation. The different agents vary considerably in their rates of aging, with soman (GD) being the fastest ($t^{1/2}$ = several min) and the others considerably slower ($t^{1/2}$ = several h). Third, oximes are quaternary drugs, do not penetrate the blood–brain barrier, and thus provide minimal reactivation of CNS-inhibited enzyme. For these reasons, no single oxime has been developed that provides equivalent therapeutic efficacy against all nerve agents (Dawson, 1994).

By combining enzyme kinetics (inhibition, reactivation, aging) with OP toxicokinetics and oxime pharmacokinetics many investigators have developed dynamic in vitro models to calculate AChE activities at different scenarios. These models were validated with data from pesticide-poisoned patients and simulations were performed for intravenous and percutaneous nerve agent exposure and intramuscular oxime treatment. These dynamic models have led to the conclusion that reactivation of inhibited ''non-aged'' AChE sufficient to preserve life may be possible. Two conditions must be met: (1) the exposure to the nerve agent must not be supralethal and (2) the oxime must be administered early and in sufficient titration. Thieman et al. (2006), reported that when RBC–AChE activity is kept at 30% or restored to that level, neuromuscular transmission is maintained at near normal levels. Using these data a number of oximes have been identified as more effective than 2-PAM Cl against soman or several other OPs. In fact, an oxime is currently in advanced development in the US Department of Defense. This oxime would be the first developed in the United States in more than 30 years. This oxime development program is known as the Improved Nerve Agent Treatment System (INATS). The goal of INATS is to develop a treatment system that offers optimal protection against a broad spectrum of nerve agents. Of course, it will not eliminate the need for other protective and therapeutic systems. The new oxime candidate (MMB4) transitioned to advanced development in 2005. In 2005, initial preclinical studies were completed and cGMP manufacturing efforts continued.

C. Carbamate Pretreatment

Pyridostigmine (bromide) has been employed as a pretreatment drug against possible nerve agent exposure. It has been suggested that its effectiveness is due to carbamylation of a portion of tissue AChE that protects it against irreversible inhibition by nerve agents. Spontaneous decarbamylation of pyridostigmine produces sufficient AChE to preserve life when followed by prompt atropinization. Pyridostigmine is most appropriate for anticipated exposure to nerve agents capable of rapid ''aging, such as soman.'' PB was approved in 2003 for pretreatment use by military personnel under the threat of exposure to nerve agents, specifically soman (Couzin, 2003).

The use of PB is always to be accompanied by immediate treatment after OP exposure with atropine and an oxime such as pralidoxime (2-PAM), the current doctrinal treatments after exposure. PB was chosen not to cross the blood–brain barrier to avoid possible CNS impairment or incapacitation, an effect particularly undesirable in soldiers engaged in combat, at least those not being exposed to OPs. Several other anti-ChEs that do cross the blood-barrier, including physostigmine,

tacrine, and huperzine A, have been found to offer better protection than pyridostigmine, but at the cost of undesirable central effects. These carbamate anti-ChEs are discussed by Pereira et al. in Chapter 10 and by Saxena et al. in Chapter 7.

Pereira et al. (Chapter 10) suggest that galantamine preferentially blocks peripheral AChE, but does bind to central AChE and this may account for part of the superiority of galantamine over other centrally acting anti-ChEs in terms of side effects. Unlike pyridostigmine, galantamine inhibits BuChE poorly, leaving intact this scavenger site for OPs in blood. In fact, galantamine is approximately 50-fold more potent as an AChE inhibitor than a BuChE inhibitor. It also interacts directly with nAChRs, potentiating their activation by subsaturating concentrations of classical agonists. Additionally, Pereira et al. suggest that galantamine is reported to have anticonvulsant and neuroprotective properties, both of which are promising in the context of OP poisoning.

D. Enzymes as Scavengers

Recent research has explored approaches leading to a transient ''immunity'' or drugs that would provide protection against lethal nerve agents, yet be devoid of side effects. It has been suggested that human BuChE from plasma participates in the endogenous scavenging of naturally occurring drugs (e.g., physostigmine, cocaine), man-made therapeutics (e.g., succinylcholine). A novel approach is to use enzymes, whether wild type or altered through directed mutation, to scavenge these highly toxic nerve agents before they attack their intended targets. The accumulated work has shown that if a scavenger is present at the time of nerve agent exposure, rapid reduction of toxicant levels is observed (Lenz et al., 2001). This reduction is so rapid and profound that the need to administer a host of pharmacologically active drugs as antidotes is, in theory, eliminated. The promise afforded by the use of scavenger enzymes is so great and their applications so diverse that three different chapters in this text (see Chapters 7, 8, and 9) are devoted to their discussion.

VI. SUMMARY AND CONCLUSIONS

Following the first Gulf War, reports of unexplained illness in returning US veterans, and episodes of chemical agent terrorism, a renewed interest in the health consequences of nerve agent chemical weapons took place. The US government increased its investment into studies of the health of Persian Gulf War veterans as it might have been affected by possible exposure to low levels of chemical warfare agents. It also increased its emphasis on research, both epidemiological and experimental, into the health effects of exposure to nerve chemical warfare agents, with the intent on improving medical response to chemical agents. We believe that a comprehensive assessment of the health consequences of nerve agent chemical weapons is unfolding and has already begun to improve our understanding of this problem. Finally, it has already begun to improve our ability to deal with these health consequences effectively.

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5 Toxicokinetics of Nerve Agents

Marcel J. van der Schans, Hendrik P. Benschop, and Christopher E. Whalley

CONTENTS

I. INTRODUCTION

Toxicokinetic studies, together with toxicodynamic studies of nerve agents, provide a quantitative basis for the design of new strategies against intoxication with nerve agents. There is a long tradition of investigations on the toxicodynamics of nerve agents since their introduction as potential agents of chemical warfare during World War II. These studies have led to strategies in which phosphorylated cholinesterase is reactivated with oximes, often in combination with the administration of a central nervous depressant suppress convulsions and other central effects, and through administration of the muscarinic cholinergic antagonist, atropine.

Toxicokinetic studies were initiated in the last two decades of the last century. The reasons for the late development were twofold. First, it was assumed that nerve agents react so quickly and are so rapidly degraded that it is impossible to measure these low levels of agent. Second, the expected levels were so low that the analytical capability was not sufficient to detect the nerve agent at relevant levels. With the advent of sophisticated sensitive analytical capabilities using capillary gas chromatography combined with sensitive detectors such as Nitrogen Phosphorus Detection (NPD), Flame Photometric Detection (FPD), and Mass Selective Detection (MSD), it is possibile to measure these extremely low levels of nerve agent. Moreover, there was reason to believe that the nerve agents might be more persistent than anticipated. Wolthuis et al.[1] showed in 1981 that rats initially surviving a challenge with a supra-lethal dose of soman by immediate treatment with atropine and the oxime HI-6 became fatally reintoxicated 4–6 h later. Hence, soman appeared to be far more persistent than anticipated. The persistence is even more pronounced in the case of percutaneous VX intoxication, which is caused by a slow penetration through the skin and slow enzymatic hydrolysis. Especially in the case

intoxication with VX, toxicokinetic studies are of ultimate importance because the time period of the intoxication might span several hours, which means that the timing of the antidote administration has to be adapted to the toxicokinetic process.

The toxicokinetics of soman and sarin has also been thoroughly described by Benschop and De Jong in the previous version of this book, *Chemical Warfare Agents: Toxicity at Low Levels*.[2] This chapter is not meant as a duplicate of the contribution in the first edition. However, the intravenous (i.v.) toxicokinetics of soman and sarin will be presented again because they serve as a reference for the toxicokinetics of VX after i.v. administration and percutaneous application that will be presented. Additionally, the effect of human butyrylcholinesterase as scavenger on the toxicokinetics of nerve agents will be mentioned. The discussion of the toxicokinetics will be preceded by a section discussing the importance of distinguishing the stereo-isomers of nerve agents. Finally, the distribution of sarin over tissues will be discussed in the last section of this chapter as a reasoning for the elimination pathways of nerve agents. Readers who are interested in the inhalation toxicokinetics of sarin and soman are referred to Chapter 2 in the first edition of the already-mentioned *Chemical Warfare Agents* book in this series.

II. NERVE AGENT STEREO-ISOMERS: CHIRAL ANALYSIS

Interpretation and understanding of the toxicokinetics of nerve agents would not be possible without taking into consideration that these agents consist of mixtures of stereo-isomers, which are often extremely different in their toxicokinetic and toxicodynamic properties.[3] A common feature of these agents is the chirality on the phosphorous atom. (±)-Sarin, C(±)P(±)-soman, and (±)-VX consist of an equimolar mixture of P(−)- and P(+)-stereo-isomers. In the case of sarin and soman, the P(−)-stereo-isomers appeared to be far more toxic and persistent in vivo than the P(+)-stereo-isomers. In contrast, the difference in rates of inhibition of AChE and lethality between the stereo-isomers of VX are only moderate. In addition, soman consists of four stereo-isomers denoted as C(+)P(+), C(+)P(−), C(−)P(−), and C(−)P(+), in which C stands for asymmetry in the pinacolyl moiety and P for asymmetry at phosphorus atom. The enantiomeric pairs [C(+)P(+) + C(−)P(−)] and [C(+)P(−) + C(−)P(+)] are present in synthetic C(±)P(±)-soman in a ratio of 45:55, with equal amounts of the two enantiomers within each pair. Separation of the various stereo-isomers became feasible with optically active stationary phases in chromatography. The four isomers of soman can be separated on a ChiraSil-Val column and the isomers of sarin on a Cyclodex column, whereas the isomers of VX can be separated by HPLC using Chiracel OD-H column.[4–6] With the analytical methodology available, it is possible to monitor the progress of the isolation and purification of the stereo-isomers. In the case of soman, synthetic resolution of the pinacolyl alcohol and subsequent synthesis of soman from these stereo-isomers yielded C(+)P(±) and C(−)P(±)-soman.[3] After incubation with α-chymotrypsin, which binds the P(−)-isomers of soman, the P(+)-isomers could be isolated. Incubation with rabbit plasma hydrolyzes the C(±)P(+)-isomers and yields therefore the C(±)P(−)-isomers. Similarly, the incubation of sarin with rabbit plasma gave (−)-sarin. The two stereo-isomers of VX are easily obtained synthetically from optically resolved precursors.[7–9] With sufficient amounts of the stereo-isomers available, it is possible to study the affinity for binding with AChE, rate of hydrolysis, and finally the lethality of the different isomers.[10–13] As expected, the degree of lethality correlated with the binding constant of AChE (see Table 5.1). Apparently the P(−)-isomers of soman and sarin inhibit AChE with rate constants that are 3–4 orders of magnitude higher than those of the corresponding P(+)-isomers. The determination of the binding constant of the P(+)-isomers requests a high concentration of the inhibitor, whereas it cannot be excluded that small impurities of the P(−)-isomer bias the bimolecular reaction. In contrast to sarin and soman, the difference of the binding constant of the P(−)-isomer of VX is only two orders of magnitude higher than the P(+)-isomers. In this case, the LD50 of both isomers could be determined, which verified that the P(−)-isomer is only eightfold more toxic than the P(+)-isomer. This

TABLE 5.1
Stereoselectivity in Binding to AChE and Acute Lethality of Nerve Agent Stereo-Isomers

Nerve Agent	Rate Constant (M^{-1} min^{-1})[a]	LD50 Mouse (μg/kg)	References
C(+)P(−)-Soman	2.8×10^8	99[b]	[9]
C(−)P(−)-Soman	1.8×10^8	38[b]	[9]
C(+)P(+)-Soman	$<5 \times 10^3$	>5000[b]	[9]
C(+)P(+)-Soman	$<5 \times 10^3$	>2000[c]	[9]
C(±)P(±)-Soman		156[b]	[9]
(−)-Sarin	1.4×10^7	41[c]	[3,10]
(+)-Sarin	$<3 \times 10^3$		[10]
(±)-Sarin		83[c]	[3][d]
(−)-VX	4×10^8	12.6[c]	[3,11]
(+)-VX	2×10^6	165[c]	[3,11]
(±)-VX		20.1[c]	[3][d]

[a] Electric eel AChE (pH 7.5, 25°C) for soman stereo-isomers; bovine erythrocyte AChE for sarin and VX stereo-isomers (pH 7.7, 25°C).
[b] Subcutaneous administration.
[c] Intravenous administration.
[d] Estimated from an experiment with optically enriched sarin (64% enantiomeric excess).

data make clear that it is essential to distinguish the isomers of the nerve agents from each other in toxicokinetic studies, because one is mostly interested in the elimination rate of the toxic compound.

III. EXPERIMENTAL

Toxicokinetic studies are only interesting if the agents can be determined at levels that are toxicologically relevant. The relevant levels should be related to the binding constant of the agent with AChE. Since nerve agents inhibit AChE with rates up to $10^8 M^{-1} min^{-1}$, it can be derived that blood levels down to a few picograms per milliliter can still cause a significant inhibition over a period of hours. Only the most sophisticated gas chromatographic techniques combined with sensitive detection methods such as NPD, FPD, or MS are sensitive enough to fulfill this task. During the period of time in which the toxicokinetic studies mentioned in this chapter were performed, only the NPD detector was available.[5,14] Nowadays, organophosphofluoridates are analyzed by means of mass spectrometry with chemical ionization using ammonia as a reaction gas.[15,16] This ionization mode is efficient for ionization of organophosphofluoridates but also more selective than electron impact ionization since it is a softer ionization mode. As mentioned in the previous section, it is also essential that the isomers can be distinguished from each other in view of the extreme difference in toxicity of the isomers. The combination of chiral-sensitive analysis was realized by two-dimensional gas chromatography in combination with large volume sample introduction (up to 400 μL) by means of thermal desorption. This allows the detection of low concentration nerve agent, that is, in the picogram per milliliter range. The two-dimensional chromatography utilized the heart-cutting technique. The configuration comprises two GC-columns, that is, a precolumn and a second analytical chiral column, which are connected in series. The sample is introduced on the precolumn. After separation on the precolumn, a small fraction of the effluent is injected on the second analytical chiral column. The reason for utilization of two-dimensional chromatography is twofold. First, the combination of two stationary phases creates

additional selectivity that is required for the detection of nerve agents at trace levels in biological samples. Second, the configuration preserves the chiral column, which has a fragile stationary phase that deteriorates by the formation of liquid phases onto the column wall and elution of interfering matrix components. With this configuration, it was possible to detect amounts of soman and sarin down to 1–5 pg when separating all isomers of sarin and soman.[21–23]

The separation of the VX isomers was only successful using HPLC on a Chiracel OD-H column with a mixture of *n*-hexane:ethanol (95:5) as a mobile phase.[6,17] Detection was accomplished by electrochemical detection, which is possible by oxidation of VX at the sulfur or nitrogen atom. The mobile phase leaving the chiral column was postcolumn mixed with a 0.1 M potassium perchlorate in ethanol solution to ensure conductivity, which is necessary for electrochemical detection. The method was workable for testing optical purity of the isomers or analysis of biological samples at high concentrations of VX (>10 ng/mL). The selectivity of the separation was not sufficient for the analysis of VX at lower concentrations in biological samples. As an alternative, it appeared possible to separate the isomers of VX on the Chiracel OD-H column and fractionate the VX isomers.[17] Next, the collected samples were off-line analyzed with GC-NPD. VX itself can be analyzed with GC-NPD using a rather straightforward technique.[18] Due to the low volatility of VX, the sample can be concentrated in a vial with a gentle stream of nitrogen. Typically, a volume of 8 μL VX in hexane can be injected onto the column, whereas the detection limit of VX was down to 2 pg. The lowest concentration of VX isomers that could be detected using off-line separation of isomers and detection with GC-NPD was 1 ng/mL. Recently, the method was further improved using chiral LC-MS/MS. The combination of chiral LC-MS was already published by Smith,[19] albeit at relatively high concentrations of VX. Recently, we analyzed the isomers of VX on an LC-MS/MS configuration using APCI conditions. It appeared possible to analyze the two stereo-isomers down to 100 pg/mL. At the time that the toxicokinetics of VX was studied, this analytical configuration was not available yet, which means that the isomers of VX were measured using the off-line LC-GC-NPD method.

The sample preparation of the nerve agent samples appeared to be a critical step as well. In vivo, the stereo-isomers of soman and sarin are subject to a rapid process of elimination by binding to AChE, BuChE, or CaE and enzymatic hydrolysis. It is essential that these processes should be frozen once the biological sample has been taken for the time period that is required for the further sample preparation. It appeared that enzymatic hydrolysis can be sufficiently suppressed by immediate acidification of the sample using an acetate buffer at pH 4. A second essential step is the saturation of available binding sites such as AChE, BuChE, and CaE. The enzymes were blocked by addition of excess of another organophosphate such as neopentyl sarin. Finally, it appeared that the presence of a significant amount of fluoride ions from natural sources or from hydrolysis of the nerve agent itself results in regeneration of the organophosphofluoridate, which leads to substantially higher levels of soman or sarin in the samples.[14] This complication was effectively suppressed by addition of aluminum sulfate, which binds the fluoride ions, mostly in the complex $[AlF_2]^+$. Next, the stabilized mixture was extracted into ethyl acetate using a solid-phase extraction procedure with Nexus or Seppak C18 cartridges.

Some experiments described in this chapter refer to the analysis of regenerated sarin from regenerable binding sites. In that case, aluminum sulfate is not added to the sample. On the contrary, the regeneration of sarin is even accelerated by the addition of a high concentration (250 mM final concentration) of fluoride ions.[20] The regenerated sarin is also extracted into ethyl acetate by solid-phase extraction. Next, the sample is analyzed without the use of a chiral column: regeneration with a high concentration of fluoride ions occurs with rapid racemization of the organophosphofluoridate, which means that a chiral analysis is redundant.

The isolation of (±)-VX from a biological matrix is even simpler.[17] The sample is first stabilized by the addition of an excess of organophosphate, for example, neopentyl sarin, made alkaline to deprotonate the nitrogen atom and extracted with a mixture of *n*-hexane:methanol (95:5). The extract appeared to be clean enough to be analyzed directly with GC-NPD or LC-MS/MS.

Additionally, preconcentration of the sample to a smaller volume was possible by evaporation of the solvent with a gentle stream of nitrogen and reconstitution of the sample in a small volume of *n*-hexane.

IV. INTRAVENOUS TOXICOKINETICS OF SOMAN, SARIN, AND VX

A. Soman

Initial investigations on the toxicokinetics of nerve agents were performed after i.v. administration at dosages corresponding with multiple LD50 values to obtain the toxicokinetic data at 100% bioavailability.[21–24] This data can be used in the interpretation of the results from more relevant exposure routes such as the respiratory and percutaneous route. Animal species selected for the investigations were the rat, guinea pig, and marmoset, with the latter species serving as model for man. In order to be able to perform the experiments, animals were anesthetized, atropinized, and mechanically ventilated through a tracheal cannula. Blood samples were drawn from a carotid cannula. Intravenous injection was performed in the dorsal penis vein or in the vena jugularis. The LD50 values of soman are highly species dependent since the amount of CaE in blood is species dependent.[25] These enzymes act as scavengers of nerve agents by irreversibly binding to the nerve agent. The enzyme is present in large amounts in blood of rats, in significantly smaller amounts in guinea pigs, and almost absent in blood of marmoset and man. Accordingly, the LD50 values decrease in the order rat > guinea pig > marmoset. Blood levels of the C(+)P(−) and C(−)P(−)-isomers are shown in Figures 5.1 through 5.6. The relatively nontoxic C(±)P(+)-isomers were eliminated from the blood stream within 5 min, which is mainly caused by the rapid enzymatic hydrolysis of C(±)P(+)-soman. In contrast with the C(±)P(+)-isomers, the C(±)P(−)-isomers can be measured for several hours. Almost all curves were best described with a three exponential curve. Areas under the curve have been calculated using the coefficients of these equations. The toxicokinetic data of the i.v. soman experiments are summarized in Table 5.2. The derivation of the time period during which acutely toxic levels of soman stereoisomers is based, somewhat arbitrarily, on a scenario of intoxication in which an animal resumes spontaneous respiration presumably due to about 5%–7% reactivation by oxime (or protection by carbamate) of completely inhibited AChE in diaphragm. Since the concentration of AChE in

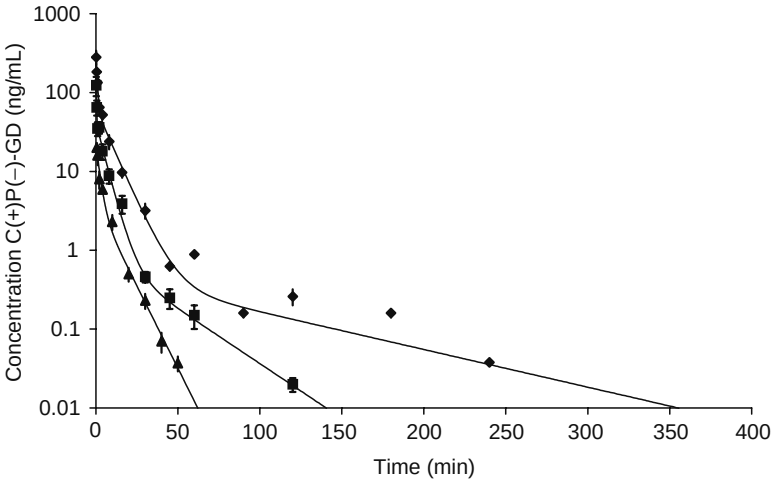

FIGURE 5.1 Semi-logarithmic plot of the concentrations in blood of C(+)P(−)-soman (GD) versus time after i.v. administration of 0.8 × LD50 (66 μg/kg, ▲), 3 × LD50 (■), and 6 × LD50 (◆) of C(±)P(±)-soman to anesthetized, atropinized, and mechanically ventilated rats. (From Benschop, H.P. and De Jong, L.P.A., *Neurosci. Biobehav. Rev.*, 15, 73, 1991. With permission.)

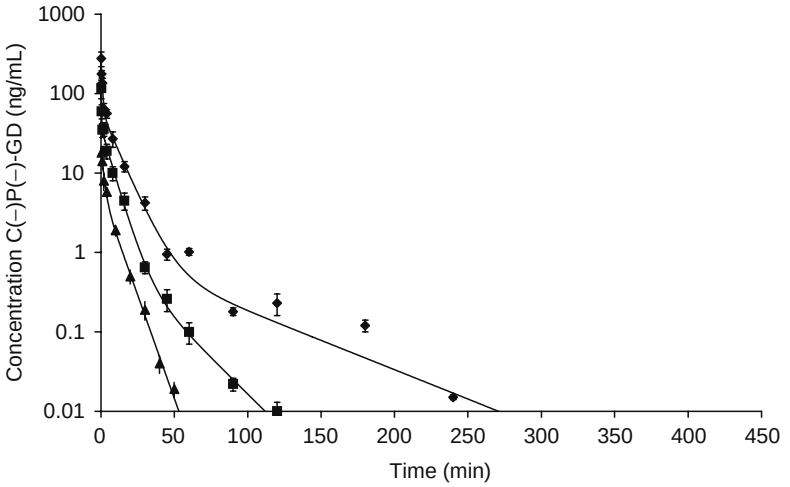

FIGURE 5.2 Semi-logarithmic plot of the concentrations in blood of C(−)P(−)-soman (GD) versus time after i.v. administration of 0.8 × LD50 (66 μg/kg, ▲), 3 × LD50 (■), and 6 × LD50 (◆) of C(±)P(±)-soman to anesthetized, atropinized, and mechanically ventilated rats. (From Benschop, H.P. and De Jong, L.P.A., *Neurosci. Biobehav. Rev.*, 15, 73, 1991. With permission.)

diaphragm of guinea pigs is approximately 2–2.6 nM, this reactivated fraction corresponds with approximately 150–200 pM AChE. Based on a bimolecular rate constant of AChE by soman of about $10^8 M^{-1}$ min^{-1}, it is calculated that this reactivated fraction of AChE can be reinhibited by 150 pM (approx. 30 pg/mL) soman with a half-life of about 1 h. An order of magnitude lower concentration soman can only cause insignificant inhibition. Therefore, it is assumed that 150 pM soman represents the lowest concentration having toxicological relevance. In a more generalized way, it may be reasoned that an area under the curve of 30 pg/mL × 60 = 1.8 ng min mL^{-1} in the last part of the blood level curve is needed for toxicological relevance. The period of time in between intoxication and the point on the time axis at which this area starts can be regarded as the period of time in which

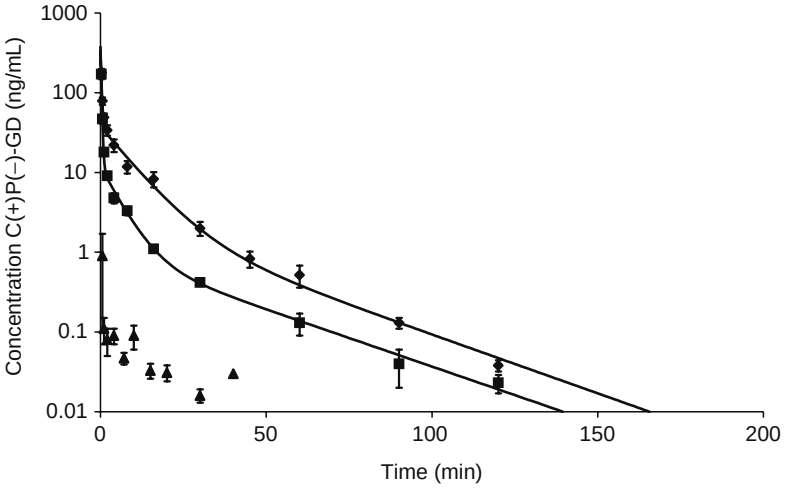

FIGURE 5.3 Semi-logarithmic plot of the concentrations in blood of C(+)P(−)-soman (GD) versus time after i.v. administration of 0.8 × LD50 (22 μg/kg, ▲), 3 × LD50 (■), and 6 × LD50 (◆)of C(±)P(±)-soman to anesthetized, atropinized, and mechanically ventilated guinea pigs.

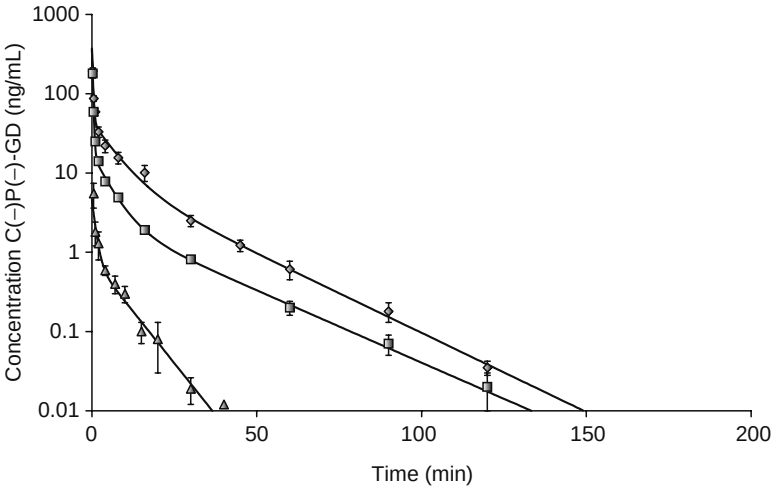

FIGURE 5.4 Semi-logarithmic plot of the concentrations in blood of C(−)P(−)-soman (GD) versus time after i.v. administration of 0.8 × LD50 (22 μg/kg, ▲), 2 × LD50 (■), and 6 × LD50 (◆) of C(±)P(±)-soman to anesthetized, atropinized, and mechanically ventilated guinea pigs.

toxicologically relevant levels of soman are present. Table 5.2 shows that these time periods in rat, guinea pig, and marmoset range between 50 and 100 min after receiving a dose corresponding with 2 × LD50.

Based on the earlier-mentioned ratio of the C(+)P(−) and C(−)P(−)-isomers of soman it would be expected that the AUC of the C(+)P(−)-isomer is 20% higher than that of the C(−)P(−)-isomer. Instead, the AUC of C(+)P(−)-isomer is often equal to or even lower than C(−)P(−)-isomer. This outcome can be explained by taking into consideration the difference in binding rate constant with CaE, which is 30-fold higher for the C(+)P(−)-isomer.[26,27] The relative effect of differential binding of C(+)P(−)- and C(−)P(−)-soman with CaE becomes more pronounced at lower doses of soman, because the elimination route of binding to a finite amount of CaE is relatively more

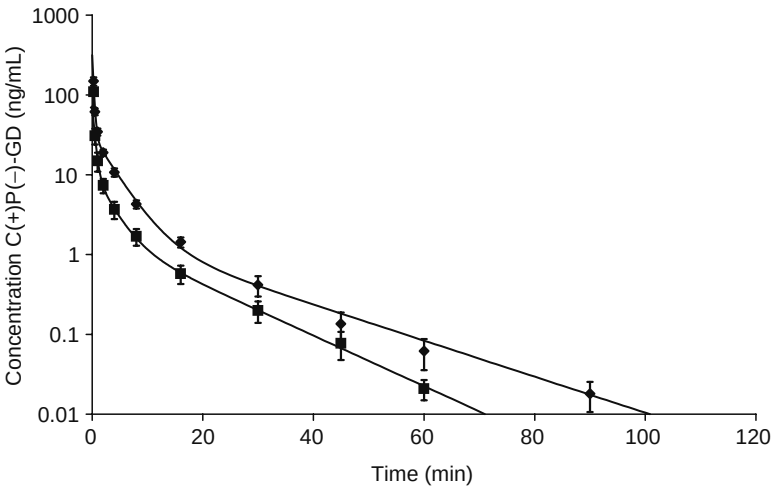

FIGURE 5.5 Semi-logarithmic plot of the concentrations in blood of C(+)P(−)-soman (GD) versus time after i.v. administration of 2 × LD50 (20 μg/kg, ■) and 6 × LD50 (◆) of C(±)P(±)-soman to anesthetized, atropinized, and mechanically ventilated marmosets.

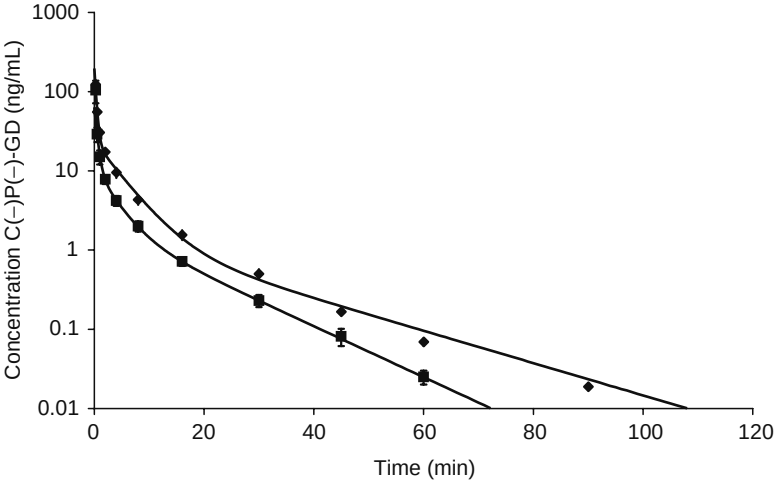

FIGURE 5.6 Semi-logarithmic plot of the concentrations in blood of C(−)P(−)-soman (GD) versus time after i.v. administration of $2 \times$ LD50 (20 μg/kg, ■) and $6 \times$ LD50 (◆) of C(±)P(±)-soman to anesthetized, atropinized, and mechanically ventilated marmosets.

important at lower doses of soman. Indeed, it was observed that the differences of the AUC between the two isomers increase with decreasing dose.

B. Sarin

In comparison with soman, little work has been done so far on the toxicokinetics of sarin.[23,28] In order to obtain reference data for inhalational studies, the i.v. toxicokinetics of sarin was investigated at doses corresponding with $0.8 \times$ LD50 (19.2 μg/kg) and $2 \times$ LD50. Blood levels of (−)-sarin are shown in Figure 5.7. The (+)-isomer of sarin was not detectable in the blood of the guinea pigs after an i.v. bolus of a dose corresponding with $0.8 \times$ LD50 and $2 \times$ LD50. The half-life of distribution appears to be 0.15 min after a dose of $0.8 \times$ LD50 and 0.98 min after $2 \times$ LD50, whereas the terminal half-lives resulting from these doses are 58 and 389 min, respectively. The calculated AUC values are 15.3 ng min mL^{-1} for $0.8 \times$ LD50 and 109 ng min mL^{-1} for $2 \times$ LD50, indicating nonlinearity of the toxicokinetics with the dose, as was also observed for $0.8 \times$ LD50 and $2 \times$ LD50 of C(±)P(±)-soman (Table 5.3).[28] It can be observed that (−)-sarin is more persistent in guinea pigs than C(±)P(−)-soman at an i.v. dose corresponding with $0.8 \times$ LD50 and $2 \times$ LD50. It is remarkable that the concentrations of (−)-sarin remain rather constant at a relatively low level for a long period of time. Interestingly, we hardly ever have seen the concentrations of either of the P(−)-isomers of soman to persist in the terminal phase. Tentative explanations for the leveling-off or increase of the (−)-sarin concentration in the terminal phase of the toxicokinetics are release from rather aspecific, noncovalent binding sites, or reactivation from covalent binding sites induced by endogenous fluoride ions. The latter reaction proceeds more readily in the case of sarin than in the case of soman.

C. VX

Data on toxicokinetics of (±)-VX are very scarce. However, it can be anticipated that the toxicokinetics of (±)-VX differs substantially from that of the phosphofluoridates, since:

1. (±)-VX circulates in vivo as a protonated amine ($pK_a = 9.4$).[17]
2. (±)-VX is hydrolyzed much more slowly than phosphonofluoridates.[29,30]

TABLE 5.2

Toxicokinetic Parameters of C(+)P(−)- and C(−)P(−)-Soman in Anesthetized, Atropinized, and Mechanically Ventilated Rats, Guinea Pigs, and Marmosets after i.v. Administration

	Rat						Guinea Pig						Marmoset			
	6 LD50		3 LD50		1 LD50		6 LD50		2 LD50		0.8 LD50		6 LD50		2 LD50	
Parameter	C(+) P(−)	C(−) P(−)	C(+) P(−)	C(−) P(−)	C(+) P(−)	C(−) P(−)	C(+) P(−)	C(−) P(−)	C(+) P(−)	C(−) P(−)	C(+) P(−)	C(−) P(−)	C(+) P(−)	C(−) P(−)	C(+) P(−)	C(−) P(−)
Dose (μg/kg)	136	111	68	56	22.7	18.6	45.4	37.1	15.1	12.4	6	5	16.5	13.5	5.5	4.5
A (ng/mL)	253	233	301	259	18	15	339	406	318	354		3.8	285	172	61	52
B (ng/mL)	63	61	41	37	3.9	5.9	35	40	11	15		0.8	30	22	9.9	9.1
C (ng/mL)	0.55	1	0.9	1.1			2.8	9.9	1	1.7			1.9	1.6	1.8	2.1
a (min^{-1})	1.3	1.2	5.0	4.7	0.45	0.57	3.8	4.3	3.8	3.9		0.95	3.9	3.0	2.2	2.0
b (min^{-1})	0.11	0.10	0.19	0.15	0.096	0.12	0.12	0.19	0.19	0.21		0.12	0.27	0.22	0.35	0.30
c (min^{-1})	0.011	0.017	0.032	0.042			0.034	0.046	0.033	0.042			0.052	0.047	0.073	0.073
Terminal half-life (min)	64	40	22	16	7	6	20	15	21	16.5		5.8	13	15	9.5	9.4
AUC (ng min mL^{-1})	806	877	308	320	81	76	458	520	169	228		10.6	218	191	81	85
Acute toxic levels until (min)[a]	317		95		37		126		104				74		49	

Note: Toxicokinetic parameters are calculated on the basis of 55:45 ratio of the [C(+)P(−) + C(−)P(+)]-stereo-isomers and the [C(+)P(+) + C(−)P(−))]-stereo-isomers. The concentration of each isomer at time *t* ($conc_t$) is described by: $conc_t = Ae^{-at} + Be^{-bt} + Ce^{-ct}$.

[a] After administration of C(±)P(±)-soman. It is assumed that the area under the curve of 1.8 ng/mL for C(±)P(−)-soman is the minimum area with toxicological relevance.

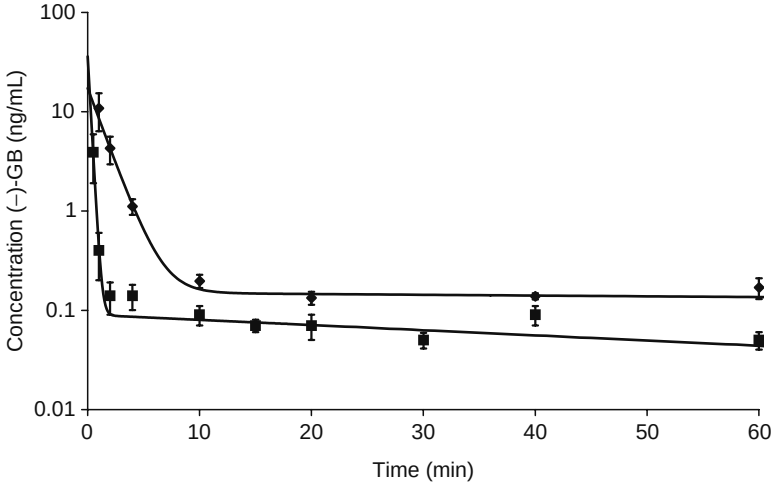

FIGURE 5.7 Semi-logarithmic plot of the concentrations in blood of (−)-sarin (GB) versus time after i.v. administration of 0.8 × LD50 (■) and 2 × LD50 (◆) of (±)-sarin to anesthetized, atropinized, and mechanically ventilated guinea pigs. (From Spruit, H.E.T., Langenberg, J.P., Trap, H.C., Van der Wiel, H.J., Helmich, R.B., Van Helden, H.P.M., and Benschop, H.P., *Toxicol. Appl. Pharmacol.*, 169, 249, 2000. With permission.)

3. (±)-VX is probably also metabolized by other routes than hydrolysis, for example, by means of oxidation reactions at nitrogen and sulfur, and by undefined anaerobic mechanisms.[31–34]
4. (±)-VX reacts hardly with carboxylesterases (TNO-PML, unpublished results), which may imply that the differences in its toxicokinetics in various species, for example, rats,

TABLE 5.3
Toxicokinetic Parameters of (−)-Sarin and C(±)P(−)-Soman after i.v. Administration of 0.8 × LD50 of (±)-Sarin and 0.8 × LD50 of Soman to Anesthetized, Atropinized, and Mechanically Ventilated Guinea Pigs

	Sarin		Soman	
Parameter	**0.8 LD50**	**2 LD50**	**0.8 LD50**	**2 LD50**
Dose (μg/kg)	9.6	24	4.95	27.5
A (ng/mL)	35.9	17.1	3.75	683
B (ng/mL)	0.09	0.151	0.8	27
C (ng/mL)				3.8
a (min^{-1})	4.6	0.705	0.95	3.9
b (min^{-1})	0.012	0.00178	0.12	0.21
c (min^{-1})				0.039
Terminal half-life (min)	58	389	5.8	18
AUC (ng min mL^{-1})	15.3	109	11	397
Acute toxic levels until (min)[a]	10	480		104

Note: The concentration of each isomer at time *t* ($conc_t$) is described by: $conc_t = Ae^{-at} + Be^{-bt} + Ce^{-ct}$.

[a] After administration of C(±)P(±)-soman or (±)-sarin. It is assumed that the area under the curve of 1.8 ng min mL^{-1} for C(±)P(−)-soman or 6.9 ng min mL^{-1} for (−)-sarin is the minimum area with toxicological relevance.

guinea pigs, and marmosets, are much smaller than that for phosphonofluoridates. This assumption is also supported by the rather small range of LD50 values for (±)-VX in various species when compared with, for example, soman.[25,35]

In order to obtain the basic data, the toxicokinetics of VX was investigated in anesthetized, atropinized, and mechanically ventilated hairless guinea pigs and marmosets after i.v. administration at doses corresponding with 1 × LD50 (28 μg/kg) and 2 × LD50.[17] The LD50 of VX in marmosets is not known but is assumed to have the same value as that in guinea pigs because the influence of CaE on the toxicokinetics is expected to be minimal. In the case that the expected (±)-VX levels were higher than 1 ng/mL, the sample was split and one part was analyzed with the off-line chiral LC-GC-NPD method. In the case that the expected concentrations of (±)-VX were lower than 1 ng/mL, the samples were analyzed with GC-NPD, without off-line separation of the stereo-isomers. It appeared that the ratio of the enantiomers [(+)-VX/(−)-VX] never exceeded the value of 1.5, indicating that the stereoselectivity in the sequestration of the VX isomers is not as pronounced as with sarin and soman. Some in vitro experiments with plasma and liver homogenates from hairless guinea pigs confirmed that the sequestration of the enantiomers is lower than that observed with the G-agents. For lack of better alternatives, we have assumed that equal amounts of (+)-VX and (−)-VX are present in all phases of our toxicokinetic experiments and calculated the toxicokinetic parameters for (±)-VX rather than for the individual isomers.

The curve of the toxicokinetics of VX in hairless guinea pigs is shown in Figure 5.8. For comparison, analogues data for soman are also given in Figure 5.8. The toxicokinetic data of the VX and soman are shown in Table 5.4. The value of the AUC experiments at doses corresponding with 1 × LD50 and 2 × LD50 shows that the toxicokinetics is reasonably linear with the dose. Evidently, the detoxification processes in the animal are not saturated with an excess of VX. The outcome is not surprising since the binding of VX to CaE is very slow, although this route is a major elimination route for the G-agents. The AUCs of the toxicokinetic experiments are summarized in Figure 5.9. This figure shows clearly the nonlinearity of the AUC as function of the dose in the

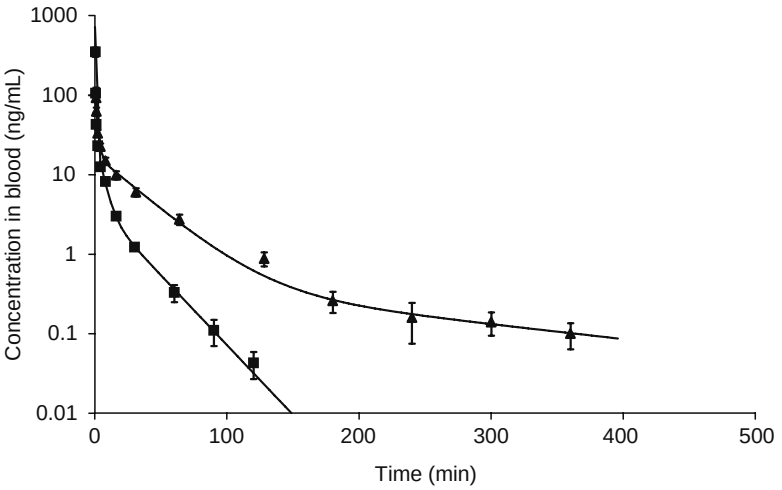

FIGURE 5.8 Semi-logarithmic plot of the nerve agent concentrations in blood versus time after i.v. administration of 2 × LD50 soman (■) and 2 × LD50 VX (▲) to anesthetized, atropinized, and mechanically ventilated guinea pigs. VX was administered to hairless guinea pigs. (From Van der Schans, M.J., Lander, B.J., Van der Wiel, H., Langenberg, J.P., and Benschop, H.P., *Toxicol. Appl. Pharmacol.*, 191, 48–62, 2003.)

TABLE 5.4
Toxicokinetic Parameters of VX and C(±)P(−)-Soman after i.v. Administration of 1 × LD50 and 2 × LD50 of Soman and VX to Anesthetized, Atropinized, and Mechanically Ventilated Guinea Pigs or Marmosets

Parameter	HGP VX	HGP VX	Marmoset VX	GP Soman	Marmoset Soman
Dose (μg/kg)	56	28	28	27.5	10
Dose (nmol/kg)	210	106	106	151	54
A (ng/mL)	77	48	14	683	113
B (ng/mL)	17	8.7	12	27	19
C (ng/mL)	0.48	0.39	1.1	3.8	3.9
a (min^{-1})	0.67	0.71	0.42	3.9	2.1
b (min^{-1})	0.033	0.045	0.039	0.21	0.33
c (min^{-1})	0.0042	0.0071	0.0062	0.039	0.073
Terminal half-life (min)	165	98	111	18	9.5
AUC (ng min mL^{-1})	744	318	504	397	166
AUC (nmol min mL^{-1})	2.8	1.2	1.9	2.2	0.91
Acute toxic levels until (min)[a]	1153	583	841	104	49

Note: The concentration of each isomer at time *t* ($conc_t$) is described by: $conc_t = Ae^{-at} + Be^{-bt} + Ce^{-ct}$. HGP = hairless guinea pig, GP = guinea pig.

[a] After administration of C(±)P(±)-soman or (±)-VX. It is assumed that the area under the curve of 1.8 ng min mL^{-1} for C(±)P(−)-soman or 0.9 ng min mL^{-1} for (±)-VX is the minimum area with toxicological relevance.

experiments with rats, guinea pigs, and marmosets. The curves with a straight line through the origin pertain to experiments with a minor influence of CaE on the toxicokinetics because of the absence of this enzyme (in case of marmosets) or a low affinity of this enzyme with the nerve agent (in case of VX).

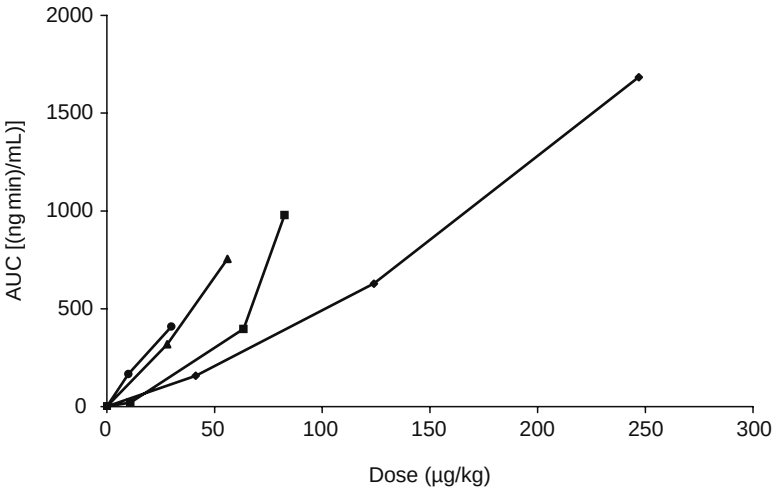

FIGURE 5.9 AUC as function of the dose of nerve agent after i.v. administration to anesthetized, atropinized, and mechanically ventilated animals. Soman-rat (◆), soman-guinea pig (■), soman-marmoset (▲), and VX-guinea pig (●).

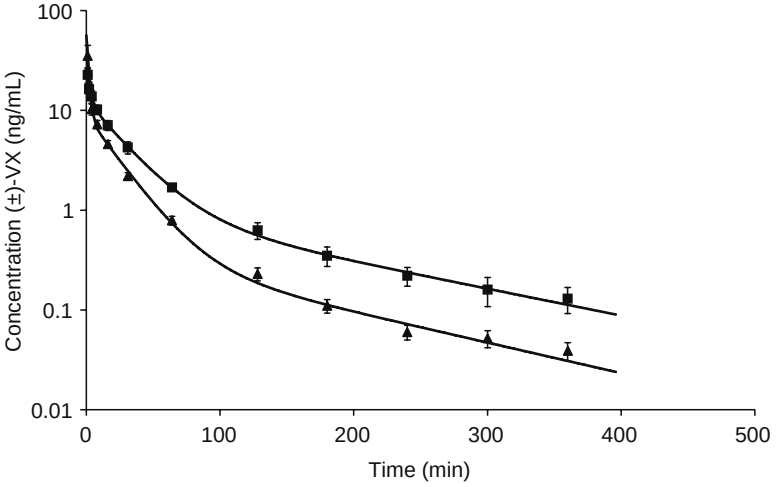

FIGURE 5.10 Semi-logarithmic plot of the concentrations in blood of VX versus time after i.v. administration of $1 \times$ LD50 to anesthetized, atropinized, and mechanically ventilated hairless guinea pigs (▲) or marmosets (■). (From Van der Schans, M.J., Lander, B.J., Van der Wiel, H., Langenberg, J.P., and Benschop, H.P., *Toxicol. Appl. Pharmacol*., 191, 48, 2003. With permission.)

Figure 5.8 shows also that the elimination rates of the two types of agents differ widely. Both the terminal half-life and the period of time that toxicologically relevant concentrations are present are approximately one order of magnitude longer for VX than for soman.

As shown in Figure 5.10, the toxicokinetics of VX in marmosets and guinea pigs at a dose corresponding with $1 \times$ LD50 in hairless guinea pigs reveals that after a short time, the blood levels in the marmoset are in between the levels found in blood of guinea pigs at dosages corresponding with $1 \times$ LD50 and $2 \times$ LD50. Correspondingly, the AUC and period of time in which toxicologically relevant concentrations of VX are present are for the marmoset in between the values of $1 \times$ LD50 and $2 \times$ LD50 for guinea pigs. Nevertheless, the data show that the difference between marmoset and guinea pig is not as large due to the lack of affinity of VX for CaE.

V. PERCUTANEOUS TOXICOKINETICS OF VX

Due to the low volatility of VX, the main porte d'entrée for this agent is by penetration through the skin. The toxicokinetics of VX was investigated in hairless guinea pigs after percutaneous application of VX in 2-propanol at a dose corresponding with $1 \times$ LD50 (125 μg/kg).[36–38] Blood samples were taken up to 7 h after the application. Unfortunately, it was not possible to take blood samples for a longer period of time for technical reasons. Therefore, the experiments pertain only to the first phase of the toxicokinetic curve, where the levels of VX just start to decrease after increasing for several hours. The concentration of VX in blood versus time after percutaneous application is shown in Figure 5.11.

Evidently, a large variation is observed in the data of the levels of VX in blood. Presumably, this spread is caused by the variation in thickness and permeability of the skin in the various animals.[39] The curve through the data points has been drawn by the eye. The AUC was estimated at 43 ± 8 ng min mL^{-1} and was calculated as the mean of the individual curves. The bioavailability can be calculated as the ratio of the AUC after percutaneous application and the extrapolated AUC that would have been derived after i.v. injection of the same dose. The bioavailability after percutaneous (p.c.) application appeared to be only 2.5%. In view of

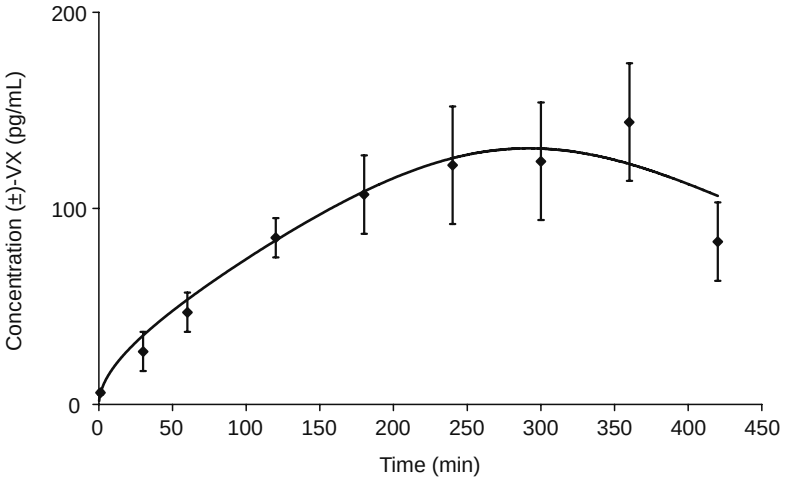

FIGURE 5.11 Concentration of VX in blood of anesthetized, atropinized, and artificially ventilated guinea pig after percutaneous application of VX at a dose of 125 μg/kg, corresponding with 1 × LD50. (From Van der Schans, M.J., Lander, B.J., Van der Wiel, H., Langenberg, J.P., and Benschop, H.P., *Toxicol. Appl. Pharmacol.*, 191, 48–62, 2003.)

the low bioavailability, it is possible that a significant part of the administered dose of VX is still present in depot in the skin. The blood levels of VX build up slowly and reach a maximum level of approximately 140 pg/mL at 6 h after the application of the toxicant. Figure 5.12 shows the degree of AChE inhibition for three individual animals, together with the corresponding individual blood levels of VX. A qualitative agreement between the two sets of data was observed since a rapid

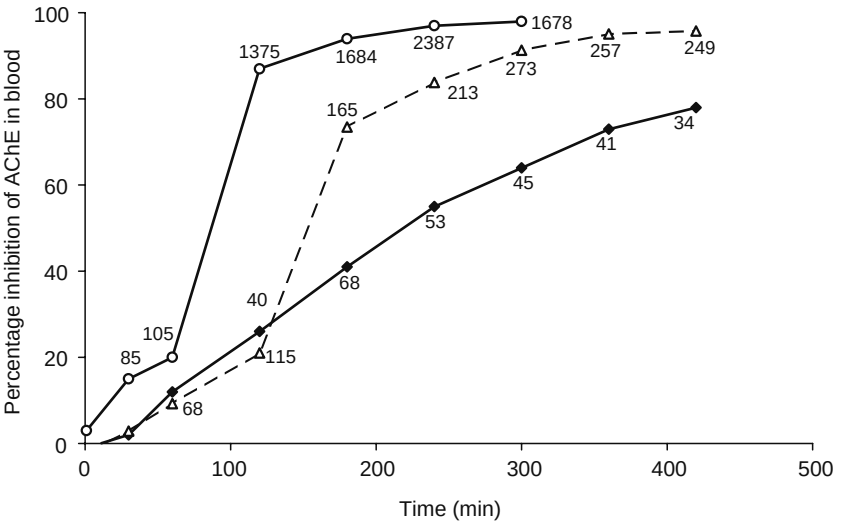

FIGURE 5.12 Progressive inhibition of AChE of anesthetized, atropinized, and artificially ventilated hairless guinea pigs after percutaneous application of VX at a dose of 125 μg/kg, which corresponds with 1 × LD50. Numbers at the data points correspond with the concentration (pg/mL) VX in blood in that particular sample. (From Van der Schans, M.J., Lander, B.J., Van der Wiel, H., Langenberg, J.P., and Benschop, H.P., *Toxicol. Appl. Pharmacol.*, 191, 48–62, 2003.)

decrease of AChE activity corresponds with relatively high levels of VX and a slow decrease of AChE corresponds with relatively low levels of VX. The experiment in which the most rapid decrease of AChE activity and high levels of VX were observed was not used in the construction of the toxicokinetic curve (Figure 5.11). This result was considered as an outlier. As shown in Table 5.4, the period of time that toxicological relevant levels of VX circulate after i.v. administration is 10–20 h, but this time period might be considerably longer after p.c. exposure. Moreover, these time periods are considerably longer than those observed with G-agents, that is, at most 2 h for an i.v. dose corresponding with $2 \times$ LD50. This difference in combination with the gradual build-up of the blood levels after p.c. exposure may cause specific problems in the diagnosis and treatment of intoxications with V-agents. These findings are in agreement with the description of the symptoms of a victim who was attacked with VX by a member of the AUM Shinrikyo sekt.[40] With G-agents, the most important porte d'entrée is the respiratory route, where the symptoms occur shortly after the exposure. With p.c. exposure of liquid VX, there will be a certain lag time between exposure and symptoms, which may complicate the timely diagnosis. The traditional treatment of nerve agent intoxication with atropine and oximes might be hampered by the rapid pharmacokinetics of the oxime and slow elimination of the VX, which might still circulate at toxic relevant levels when the blood levels of the oxime have decreased to ineffective levels. In that case, oxime therapy must be continued until the VX levels drop below the toxicologically relevant level. In accordance with the latter scenario Clarkson et al.[41] treated VX-intoxicated guinea pigs with repetitive oxime administration and was able to treat the animals up to challenges of $10 \times$ LD50. However, the animals were euthanized at 24 h after the application of the agent. Presumably, this period is too short to judge whether the treatment has been truly effective because it is possible that animals would have died after that period of time. Interestingly, the paper mentioned also a positive effect of pyridostigmine in treatment. The reports in the literature contradict each other. Gordon et al.[42] reported a positive effect of pretreatment with carbamates in case of a s.c. challenge of guinea pigs with VX. However, Koplovitz et al.[43] reported a negative influence of pretreatment with pyridostigmine on the efficacy of treatment with an oxime. It should be mentioned that a treatment with scavengers such as BuChE would be more effective in this respect, since these scavengers have a longer half-life time than oximes.[44]

VI. ELIMINATION PATHWAYS OF SOMAN, SARIN, AND VX

The rapid decrease of soman levels in blood after i.v. administration or respiratory exposure is due to three processes, that is, distribution to various tissues, spontaneous or enzymatic hydrolysis, and covalent binding.[45–49] It has been established that the toxic C(±)P(−)-isomers react rapidly with covalent binding sites. The less toxic C(±)P(+)-isomers are hydrolyzed several orders of magnitude faster than the C(±)P(−)-isomers. The low toxicity of the C(±)P(+)-isomers is primarily due to a low intrinsic reactivity toward AChE and rapid hydrolysis.

The elimination of VX deviates from that of soman. As observed in the toxicokinetic studies, the elimination of VX proceeds much slower than that for the G-agents. In addition to the major detoxification route of VX, which leads to the formation of *O*-ethyl methylphosphonic acid, it cannot be excluded that toxic metabolites will be formed. Possible toxic metabolites are the dealkylated form of VX or desethyl-VX and the N-oxide of VX.[31–34] Neither metabolite has been found in blood samples taken for toxicokinetic experiments. In vitro experiments, in which high concentrations of VX (10 μg/mL) were incubated in plasma or liver homogenate, did not yield any of these toxic metabolites either. However, desethyl-VX could be detected in reasonable amounts when plasma was derived from blood that was drawn in tubes with EDTA as anticoagulant. EDTA binds metal ions that are essential for the enzymatic hydrolysis of VX, which leads to the formation of EMPA. When that route is inhibited, the formation of desethyl-VX may increase.[50]

Elimination products such as hydrolyzed organophosphate or covalently bound organophosphate are the biomarkers of choice for detection of exposure, since the persistence of the intact nerve agents, although sufficiently long to interfere with therapeutic measures, is too short for this

purpose.* Incubation of inhibited CaE or BuChE with fluoride ions can regenerate the phosphyl moiety from the protein, thus creating the phosphofluoridate, which can be analyzed with gas chromatography. The method can also be used to study the distribution of the nerve agent over the tissues, provided that the nerve agent can be regenerated with fluoride ions, which is the case for sarin and VX but not for soman.†

Whalley et al.[51] exposed several guinea pigs to sarin through the respiratory route and by subcutaneous administration. Next, the animals were euthanized at different time points and the amount of regenerable sarin in the tissues was determined. Figure 5.13 shows the amount of sarin

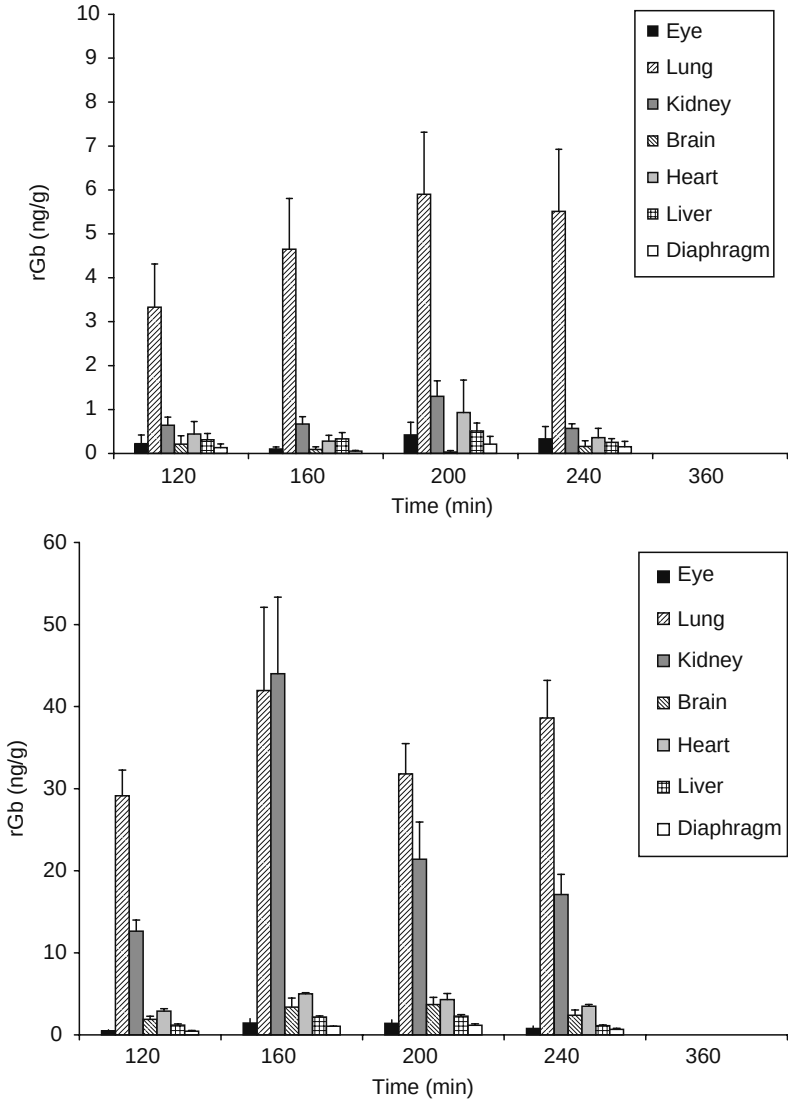

FIGURE 5.13 Concentration (ng/g) of regenerated sarin (GB) in various tissues of guinea pigs after subcutaneous administration of sarin at dosages corresponding with 0.1 × LD50 (4.2 μg/kg, left) and 0.4 × LD50 (16.8 μg/kg, right).

* For a comprehensive review of methods for diagnosis of exposure to CW-agents see Noort et al.[78]

† Soman cannot be regenerated with fluoride ions because dealkylation of the pinacolyl group of soman once bonded to BuChE (a process known as aging) prevents the regeneration of soman.

that could be regenerated from the tissues after subcutaneous administration of doses corresponding with 0.1 × LD50 and 0.4 × LD50. The figures show that after subcutaneous administration of sarin corresponding with 0.1 × LD50, the majority of sarin adducts is found in the lungs and only a small portion in the other organs.

After administration of sarin at a dose corresponding with 0.4 × LD50, the kidney appears to be also an important site for binding of sarin. The amount of regenerable sarin seems to correlate with the flow of blood through that organ. Intact sarin is supplied by the blood stream and reacts with the available binding sites. The lungs are the only organ where 100% of the cardiac output flows through, which may be an explanation for the high amount of regenerable sarin in that particular organ. The liver is an organ with an extremely high number of binding sites, but the limited blood flow through that organ causes a moderate amount of bonded sarin.

Figure 5.14 shows the amount of regenerable sarin in tissues of guinea pigs after whole-body exposure to sarin at dosages corresponding with 0.1 × LCt50 and 0.4 × LCt50. It is obvious that the amount of regenerable sarin in the eyes is relatively high, because the uptake of sarin takes place directly from the air into the eyes. It cannot be excluded that the amount of measured sarin partly consists of intact sarin that is not necessarily regenerated from binding sites. At a higher exposure level, the amount of regenerable sarin is highest in the lungs.

It is remarkable that the amounts of regenerable sarin in the lungs after whole-body exposure or subcutaneous administration at comparable dosages (0.4 × LCt50 or 0.4 × LD50) are roughly the same. It might be expected that the amount of bonded sarin upon whole-body exposure is higher because sarin is inhaled. However, the absorption of sarin does not occur in the lungs but proceeds most likely in the upper airways rather than in the lungs. In this way, intact sarin has to be transported by blood to the lungs, where it can react with the binding sites in that organ.

VII. EFFECT OF HUMAN PLASMA BUTYRYLCHOLINESTERASE AS SCAVENGER ON THE TOXICOKINETICS OF NERVE AGENTS

In the previous section, it was discussed that binding is one of the most effective elimination routes for nerve agents. The difference in lethality of various nerve agents in rats, guinea pigs, and marmosets is a result of the presence of different amounts of CaE, which reacts as a scavenger in blood of these species.

Therefore, attention has been paid to pretreatment with highly reactive scavengers, which would intercept or destroy the nerve agent before it could reach its target site, when entering the blood stream. It is anticipated that effective scavengers offer protection against both lethal and incapacitating effects of an acutely toxic dose. In addition, if a scavenger remains in circulation at an effective concentration during a relatively long period of time, the pretreatment will, a fortiori, protect against a long-term exposure to low doses of a nerve agent.[52] It is further anticipated that bioscavengers do not induce adverse physiological effects, particularly when bioscavengers from human origin are applied.

As early as 1957, Cohen and Warringa[53] achieved some protection in rats against a lethal subcutaneous dose of diisopropyl phosphorofluoridate and sarin by pretreatment with an enzyme capable of hydrolyzing organophosphates.[54] In more recent years, the feasibility of using bioscavengers that can rapidly bind nerve agents has been studied. For this purpose, monoclonal antibodies,[55,56] serum AChE,[57–60] and human plasma butyrylcholinesterase (HuBuChE)[61–71] have been investigated.

Very promising results were obtained with HuBuChE as a scavenger. The enzyme is rapidly distributed in laboratory animals, such as mice, rats, guinea pigs, and rhesus monkeys, after i.v. administration, followed by a slow elimination.[61,63,69] In addition, the enzyme is sufficiently absorbed following an intramuscular (i.m.) administration to provide therapeutically significant blood levels over a time period of 10–70 h in laboratory animals, which is a prerequisite for practical

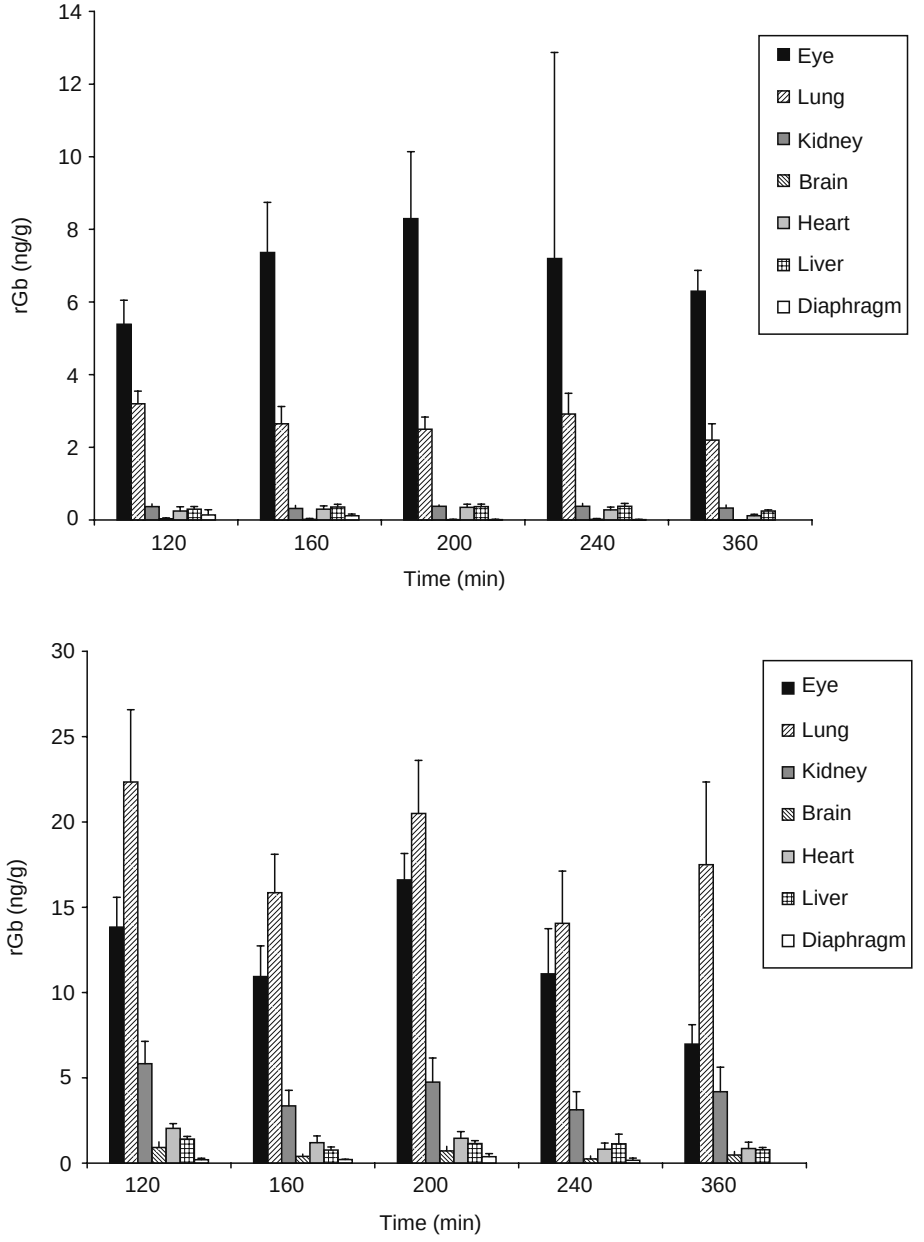

FIGURE 5.14 Concentration (ng/g) of regenerated sarin in various tissues of guinea pigs after respiratory exposure to sarin (GB) at dosages corresponding with $0.1 \times$ LCt50 (0.4 mg m^{-3} for 60 min, left) or $0.4 \times$ LCt50 (1.6 mg m^{-3} for 60 min, right).

application.[61,63,69] The peak level in blood after i.m. administration amounted to 50%–60% of the concentration obtained immediately after i.v. administration of the same amount of enzyme. Pretreatment with the enzyme resulted not only in an increase in survival of mice, rats, and rhesus monkeys intoxicated (i.v.) with C(±)P(±)-soman or other nerve agents, but also in a significant alleviation of postexposure incapacitation. An effective protection of guinea pigs against respiratory exposure to C(±)P(±)-soman has been reported.[63] Since the efficacy of HuBuChE as a scavenger

is based on the inhibitory properties of the challenging agent, it can be expected that such scavengers will be effective against nerve agents having a wide variety of chemical structures. Moreover, the stereoselectivity of HuBuChE as scavenger is in favor of the P(−)-isomers, which means that the toxic isomer of the nerve agents is preferentially eliminated. The ultimate goal is to use these scavengers as pretreatment to protect humans against nerve agent intoxication. Toxicokinetic studies can be an adequate tool for the quantitative description of the protective mechanism, which is needed for registration of the enzyme as pretreatment drug for application in humans.

The toxicokinetics of soman in anesthetized, atropinized, and artificially ventilated naive and HuBuChE-pretreated guinea pigs was studied.[72] Animals were pretreated with HuBuChE by an i.m. administration at 24 h before administration of the nerve agent. The enzyme was administered i.m., since this route is much more appropriate than i.v. administration for application of a scavenger in a realistic scenario. The ratio of the dose of HuBuChE relative to the dose of the nerve agent was chosen on the basis of results reported by Ashani et al.[61] or Allon et al.[63] in order to obtain sufficient protection in a similar experiment. The dose of HuBuChE was a 0.7-fold fraction of the molar dose of soman, which corresponds with $0.7 \times 55/182 = 211$ nmol HuBuChE/kg.

The levels of HuBuChE in blood at 24 after i.m. administration of HuBuChE was approximately 690 nM, which is over an order of magnitude higher than the baseline level of BuChE, which is approximately 20 nM.

In order to study the effect of the scavenger we compared the toxicokinetic data of the HuBuChE-pretreated and nonpretreated animals. Figure 5.15 shows the concentration–time curves of C(−)P(−)-soman after i.v. administration of soman in naive and HuBuChE-pretreated animals. Evidently, the concentrations of C(−)P(−)-soman in HuBuChE-pretreated animals are much lower than those in naive animals. This effect of the scavenger is also clearly reflected in the values of the AUC shown in Table 5.5. The difference in AUC of the P(−)-isomers between naive and HuBuChE-pretreated animals is $65.9 - 2.9 = 63$ ng min mL^{-1}, which is equal to 0.34 nmol min mL^{-1}. The period of time that acutely toxic levels of soman exist is reduced from 40 to 1 min.

After one injection of soman only the C(−)P(−)-isomer of soman could be detected in the HuBuChE-pretreated guinea pigs. The C(+)P(−)-isomer had completely disappeared from the blood stream. In a qualitative sense, this result was expected in view of the higher binding constant of C(+)P(−) with BuChE compared with the binding constant of the C(−)P(−)-isomer with BuChE.

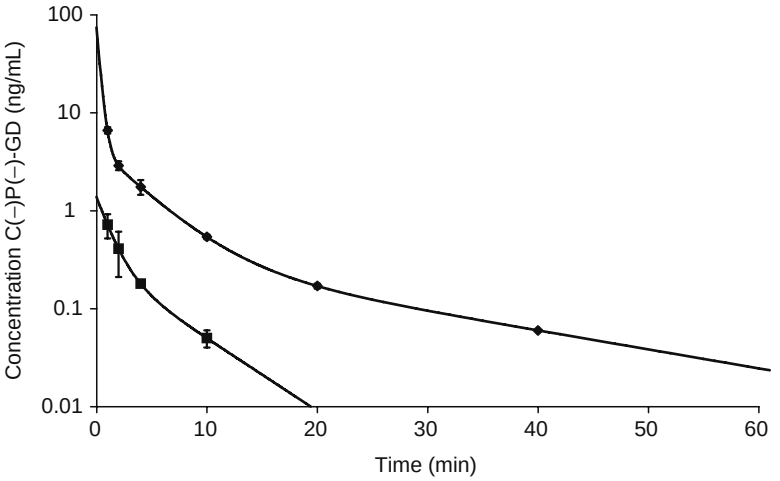

FIGURE 5.15 Mean concentration ± SEM (ng/mL) of C(−)P(−)-soman (GD) in blood of individual anesthetized, atropinized, and mechanically ventilated naive (◆) and HuBuChE-pretreated (200 nmol/kg, ■) guinea pigs after i.v. bolus administration of a dose of C(±)P(±)-soman corresponding with 2 × LD50 (55 μg/kg).

TABLE 5.5
Effect of Pretreatment (i.m.) with HuBuChE on the Toxicokinetic Parameters for Soman and VX in Anesthetized, Atropinized, and Artificially Ventilated Guinea Pigs after i.v. Administration

Parameter	Soman	Soman	Soman	Soman	VX	VX
Isomer	C(+)P(−)	C(−)P(−)	C(+)P(−)	C(−)P(−)	(±)	(±)
Dose scavenger (nmol/kg)	0	0	200	200	0	200
Dose nerve agent (μg/kg)	15.1	12.4	15.1	12.4	56	56
A (ng/mL)	2.05	70.0	—	1.11	77	54.2
B (ng/mL)	0.658	4.05	—	0.274	17	11.0
C (ng/mL)	—	0.356	—	—	0.48	—
a (min^{-1})	0.277	3.11	—	0.815	0.67	0.817
b (min^{-1})	0.054	0.256	—	0.179	0.033	0.0257
c (min^{-1})	—	0.0445	—	—	0.0042	—
Terminal half-life (min)	14.7	15.5	—	3.8	165	26.9
AUC (ng min/mL)	19.6	46.3	0	2.89	744	494
Acutely toxic levels until (min)		40	—	1	1153	230

Note: The concentration of each isomer at time *t* ($conc_t$) is described by: $conc_t = Ae^{-at} + Be^{-bt} + Ce^{-ct}$. It is assumed that the area under the curve of 1.8 ng min mL^{-1} for C(±)P(−)-soman or 0.9 ng min mL^{-1} for (±)-VX is the minimum area with toxicological relevance.

Experiments analogous to those performed with soman were also performed with VX, that is, the effect of pretreatment of HuBuChE at 0.7 × LD50 the molar dose of VX corresponding with 2 × LD50 of VX on the toxicokinetics of the agent was investigated. As shown in Figure 5.16, the VX levels in blood of HuBuChE-pretreated guinea pigs are somewhat lower. However, this effect seems not to be very large during the first 180 min. A more pronounced difference between the two curves in the time period beyond 200 min is observed, but the contribution of this part to the AUC of

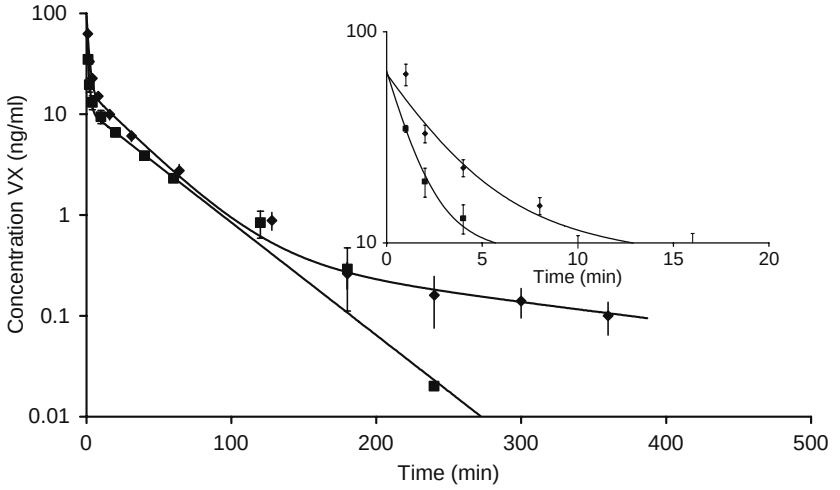

FIGURE 5.16 Mean concentration ± SEM (ng/mL) of (±)-VX in blood of individual anesthetized, atropinized, and mechanically ventilated naive (◆) and HuBuChE-pretreated (200 nmol/kg, ■) guinea pigs after i.v. bolus administration of a dose of (±)-VX corresponding with 2 × LD50 (56 μg/kg). Inset shows the data for the first 20 min plotted on an expanded scale.

TABLE 5.6
AUC (nmol.min.ml^{-1}) of (±)-VX and C(±)P(−)-Soman in Blood of Naive or HuBuChE-Pretreated Anesthetized, Atropinized and Mechanically Ventilated Guinea Pigs. Guinea Pigs were i.v. Injected with VX (56 μg/kg) or C(±)P(±)-Soman (55 μg/kg)

	AUC of (±)-VX (nmol.min.ml^{-1})			AUC of C(±)P(−)-Soman (nmol.min.ml^{-1})		
Time (min)	Without HuBuChE	HuBuChE (200 nmol/kg)	Difference as Result of HuBuChE Treatment	Without HuBuChE	HuBuChE (200 nmol/kg)	Difference as Result of HuBuChE Treatment
0	0	0	0	0	0	0
0–30'	1.69	1.11	0.58	0.337	0.014	0.32
0–60'	2.18	1.51	0.67	0.356	0.015	0.34
0–90'	2.39	1.69	0.70	0.360	0.015	0.35
0–120'	2.49	1.78	0.81			
0–180'	2.58	1.84	0.74			
0–240'	2.62	1.85	0.77			

the toxicokinetic curve is less than 7%. Table 5.5 shows a summary of the toxicokinetic parameters derived from the blood levels of soman and VX.

The decline of the AUC for the summated stereo-isomers of VX on pretreatment with 200 nmol/kg HuBuChE was $744 - 494 = 260$ ng min mL^{-1}, which is equal to 0.97 nmol min mL^{-1}. By comparison, the decrease of the AUC of C(±)P(−)-soman in guinea pigs challenged with 55 μg/kg C(±)P(±)-soman was 0.34 nmol min mL^{-1}. The P(+)-isomers of soman were not detected but it cannot be excluded that these P(+)-isomers consumed a part of the scavenger.[72] Although it cannot be excluded that the P(+)-isomers consumed a part of the scavenger, this will not suffice to explain the lesser decrease of the AUC for soman than for VX. The AUCs of the VX curves are calculated over a time span of 360 min, whereas the AUCs of the soman curves are calculated over a time span of 60 min. Table 5.6 Please provide "Table 5.6". Please provide "Table 5.6" shows the AUCs of both experiments expressed in nmol min mL^{-1} for different time spans. The decrease of AUC as result of HuBuChE pretreatment is larger for VX than for soman. However, the relative decline of the AUC of the soman curve (97% at $t = 90$ min) is more pronounced than that for VX (29% at $t = 90$ min).

The elimination of soman in nonpretreated animals is caused by enzymatic hydrolysis and binding to CaE, whereas the elimination of VX in such animals is driven by slow enzymatic hydrolysis and slow binding to CaE. Additional binding sites such as BuChE will be consumed rapidly. Addition of fast binding sites to soman will only increase the number of multiple fast binding sites. It is therefore explicable that the scavenger will not be consumed completely by soman, which means that the effect of the scavenger on the toxicokinetics of soman is less in this case. Table 5.5 shows clearly that the time period in which acutely toxic levels of VX circulate is reduced from 1153 to a still considerable 230 min after administration of 200 nmol/kg HuBuChE. HuBuChE acts as a stoichiometric scavenger for nerve agents, which means that the efficacy of the protection by HuBuChE depends on the balance between the amount of nerve agent and the amount of scavenger. In these experiments, the amount of HuBuChE was not sufficient to decrease the concentration of VX below the acutely toxic level within a reasonable time window.

VIII. CONCLUDING REMARKS

With the analytical methodology available, it appeared possible to measure nerve agents at the toxicologically relevant level. This capability allowed to measure the toxicokinetics of nerve agents after a variety of exposure routes at several doses, and to determine the time period for which

acutely toxic levels exist. The relevance of such studies has been shown since the persistence of nerve agent in vivo is longer than that anticipated, with the percutaneous exposure to VX as being the most remarkable. It is evident that these studies can be used for the development of strategies for timely administration of antidotes in case of nerve agent intoxication. For example, the efficacy of HuBuChE as a scavenger for nerve agents was demonstrated by toxicokinetic studies.

Analysis of regenerated sarin from binding sites has provided more insight into the elimination pathways of nerve agents. This is especially true for the lungs and the eyes, which appeared to contain many binding sites for nerve agents following whole-body exposure. Both organs are also the porte d'entrée for the agent, and for the eyes it cannot be excluded that the amount of measured sarin partly consists of intact sarin. Exposure studies at lower levels (occupational exposure) are near the lower limit of what can be reached with regard to toxicokinetics based on in vivo measurement of the intact nerve agent. Ultra low level exposure can only be studied after analysis of fluoride-regenerated nerve agents.[73] With these studies, it is possible to quantify the link between external and internal dosages, which is highly relevant in the area of regulatory toxicology.

Eventually, the toxicokinetic data, together with the distribution data, are very useful for the validation of physiologically based pharmacokinetic modeling (PBPK).[74–77] These models are needed because the experiments that were discussed in this chapter can never be performed in humans, whereas extrapolation of the results obtained in these animal experiments to man is still the ultimate goal of these investigations.

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6 Application of Genomic, Proteomic, and Metabolomic Technologies to the Development of Countermeasures against Chemical Warfare Agents

Jennifer W. Sekowski and James F. Dillman III

CONTENTS

I. INTRODUCTION

Since World War I, chemical warfare agents (CWAs) have been manufactured and stockpiled in numerous countries and remain a threat to both civilian and military personnel. CWAs have been used both in the context of military battle (e.g., World War I, the Iran–Iraq War) and in terrorist plots (e.g., Aum Shinrikyo attack on the Tokyo subway system) (Smart, 1997). CWAs are categorized into several classes based on chemical composition and toxicological effects. These include vesicants or blistering agents (sulfur mustard [HD], Lewisite [L]), blood agents (cyanide), choking

or pulmonary agents (phosgene [CG], chlorine [CL]), and nerve agents (tabun [GA], sarin [GB], soman [GD], cyclosarin [GF], VX). To protect military and civilian personnel from the threat posed by CWAs, research and development is ongoing into CWA detection methods, diagnosis of CWA exposure, treatment of CWA-exposed personnel, and decontamination of CWA-exposed personnel and equipment.

Early CWA detectors were quite primitive and included primarily chemical-reactive dyes in paints. These were insensitive and therefore unreliable (Smart, 1997). Later technology used more sensitive chemical dyes in other formats such as paper tickets. Modern detectors now consist of fieldable IR spectrometers and an alarm system designed to warn of the presence of CWAs on the battlefield or in an enclosed space. Several reliable tests for the diagnosis of CWA exposure have been developed. To detect HD exposure, the level of thiodiglycol, a metabolite of HD, is quantitated in the urine using a gas chromatography/mass spectrometry (GC/MS) analytical method (Jakubowski et al., 1990; TB MED 296, 1996). Nerve agent exposure is detected in the field by the use of a fieldable Ellman assay to determine cholinesterase inhibition in the blood (Ellman et al., 1961; TB MED 296, 1996).

In addition to the fieldable Ellman assay, the metabolites of GB, GD, and GF can be quantitated in urine using GC/MS analytical methods (Shih et al., 1991; TB MED 296, 1996). Cyanide levels in the blood can be detected using a fluorometer (TB MED 296, 1996). Early medical treatments for CWA exposure were primarily supportive in nature, as there was no therapeutic regimen for the first CWAs used in war (i.e., blister agents and choking agents). British anti-Lewisite was developed for Lewisite exposure. However, there is as yet no approved therapeutic treatment for cutaneous, pulmonary, or ocular HD exposure. For systemic HD exposure, granulocyte colony-stimulating factor, which is Food and Drug Administration (FDA) approved for treatment of neutropenia following chemotherapy, has been shown to be effective in the treatment of HD-induced neutropenia (Anderson et al., 2006). In the case of the nerve agents, atropine was in use as an antidote from the time nerve agents were first uncovered after World War II (Smart, 1997; Maynard, 2000). Oxime was added to the treatment regimen after the development of oxime therapy in the 1950s (Maynard, 2000). The oxime 2-PAM was available at the battalion aid station starting in the late 1960s. The United States first put an oxime in an autoinjector in 1975 with the TAB antidote (T = TMB-4, A = atropine, B = benactyzine). The TAB antidote received an Investigational New Drug (IND) status from the FDA. Although it was deployed, it was never used. In 1979, work began to obtain approval of 2-PAM for autoinjector use; it was approved by the FDA in the early 1980s. The anticonvulsant therapeutic diazepam was fielded in 1990 to alleviate the seizures induced by nerve agent intoxication (Lipp, 1972, 1973; Departments of the Army, Navy, Air Force, and Commandant Marine Corps, 1995; Sidell, 1997; Maynard, 2000).

Although these advances have served to protect personnel from the threat of CWAs, research and development are moving into new paradigms to improve on these current countermeasures. By gaining a greater understanding of the effects of CWA exposure on biological systems at the molecular and cellular levels, we can identify new therapeutic targets, develop high throughput drug screening assays, and identify biomarkers of exposure and effect for improved diagnosis, detection, and risk assessment. In addition, the detection, diagnosis, and treatment of subacute, low-level, and chronic exposure to CWAs are of concern to the military. Given that these are primarily asymptomatic exposures, any deleterious effects will need to be observed at the molecular and cellular levels rather than at the organism level.

In the past, molecular and cellular biology techniques allowed investigators to focus on only one or a small number of genes or proteins at a time. However, large-scale genome sequencing projects, such as the Human Genome Project, have revolutionized applied biology and toxicology. Over the past decade, there has been a move away from a reductionist approach of studying one gene or protein at a time toward a more global approach of studying molecular and cellular networks and how these networks integrate information and respond to the environment (Farr and Dunn, 1999). Recent technological developments allow researchers to study the function of a single gene or

protein in the context of cellular and molecular networks, or to study the response of numerous genes, proteins, or metabolites to an environmental stimulus (e.g., CWA exposure). These new molecular techniques allow for higher throughput studies and are well suited to elucidating the mechanisms of cellular responses to toxicants (e.g., CWAs) (Steiner and Anderson, 2000). This has resulted in the development of several disciplines focused on global analysis of gene expression (genomics, transcriptomics), global analysis of protein expression, modification, and function (proteomics), and global analysis of metabolism and metabolites (metabonomics, metabolomics). Within the Army life science research community, we are following the lead of the pharmaceutical and biotechnology industries in applying these global approaches and associated technologies to the problem of CWA countermeasures. In this chapter, we provide an overview of each of these technologies and their current state of the art, and provide examples of how these approaches are being applied to the development of CWA countermeasures.

II. GENOMICS, TRANSCRIPTOMICS, AND GENE EXPRESSION PROFILING

Genomics, transcriptomics, and gene expression profiling are all related to studying gene expression of hundreds to thousands of genes simultaneously from a single biological sample. The primary tool that advanced these fields is the nucleic acid microarray (oligonucleotide microarray, cDNA microarray). The development of microarrays resulted from the convergence of several disparate technologies. One was high throughput DNA sequencing, which led to the complete sequencing of the genome of a number of model organisms (e.g., human, rat, mouse, yeast). Having this information publicly available provided the material that was needed to develop techniques for assaying a large number of genes simultaneously. Advances in robotics made simultaneous, parallel assaying of numerous genes possible on a large scale. Application of integrated circuit manufacturing processes and inkjet/micropin fabrication technology to nucleic acids made microarray fabrication possible. The widespread use of confocal microscopy and associated optical systems made microarray scanners possible.

From these foundations, two basic types of microarray formats have emerged. One format is the oligonucleotide-based microarray. These are primarily commercial products consisting of 20–60 base pair oligonucleotides synthesized directly onto a chip surface (e.g., silicon, glass) using one of several different processes. One widely used oligonucleotide microarray format utilizes photolithography techniques used in the manufacture of integrated circuits combined with the use of masks to synthesize oligonucleotides onto a silicon wafer (Fodor et al., 1991, 1993; Pease et al., 1994). Another format utilizes a digital light processor to synthesize oligonucleotides onto a derivatized chip surface using only light focused by rotating mirrors (Singh-Gasson et al., 1999; Nuwaysir et al., 2002). In a different approach, modified piezoelectric (inkjet) processes deposit nucleic acid directly onto a derivatized glass surface, and oligonucleotides are subsequently synthesized on the chip using phosphoramidite chemistry (Hughes et al., 2000). Other oligonucleotide platforms use micro-electric fields (Sosnowski et al., 1997), microelectrodes (Dill et al., 2004), or polymers (Ramakrishnan et al., 2002) in the manufacture or application of oligonucleotide microarrays. Oligonucleotide microarrays use a single fluorophore to label a sample (single channel fluorescence) and can provide complete or near complete genome coverage for model organisms (e.g., human, rat, mouse). Typically, the mRNA or total RNA extracted from the test tissue is amplified by means of two in vitro reactions. First, the mRNA is reverse transcribed into cDNA. Next, the resultant cDNA is in vitro transcribed into cRNA. This reaction also incorporates a label, often biotin, into the cRNA. The labeled cRNA is then hybridized to the oligonucleotides on the microarray. After several washing steps, the array is then stained. In the case where biotin was incorporated into the sample cRNA, the stain is composed of a fluorescently labeled streptavidin, which binds with high affinity to the biotin label. The fluorescent signal is detected by means of a confocal laser array scanner. The levels of fluorescence of the experimental cRNA hybridized to the oligonucleotides on one microarray are compared with those of the control cRNA hybridized

to the oligonucleotides on another microarray. This comparison determines which genes are up- or down-regulated. Control oligonucleotides within the array are used to normalize the signal between arrays. Due to the hardware infrastructure required and the costs associated with manufacture, oligonucleotide microarrays are available primarily as commercial products.

The other microarray format is the cDNA microarray (Schena et al., 1995; Shalon et al., 1996). These were the first type of array, and are still primarily noncommercial products generated in a laboratory or core facility. They are composed of 200–300 base pair long cDNA clones printed onto specially treated glass microscope slides (Schena et al., 1996). The building blocks for these arrays are homemade or commercially available cDNA libraries from the organism of interest. The control sample and the experimental sample (usually cDNA) are labeled separately, each with a different fluorescent dye (e.g., Cy3 and Cy5), pooled together in equal amounts, and hybridized to the same microarray. The fluorescent signal is detected using a confocal laser scanner with dual fluorescence capability. The ratio of the two fluorescent dyes at each cDNA spot on the microarray (control sample compared with experimental sample) is used to calculate the level of expression of each gene in the study and determine which genes are up- and down-regulated.

The applications for microarrays have grown since their initial introduction in the mid-1990s. One of the first uses of microarrays was to compare the gene expression profile of a healthy cell or tissue with a diseased one (DeRisi et al., 1996; Heller et al., 1997; Golub et al., 1999; Iyer et al., 1999; Alizadeh et al., 2000; Bittner et al., 2000; Perou et al., 2000). This provided a description of the genes expressed in a disease state and provided insight into the molecular pathways involved in the disease state. The field of toxicology in particular realized the potential of microarrays to aid in understanding toxicant mechanisms at the molecular level (Yang et al., 2004). From this, the field of toxicogenomics was developed. Toxicogenomics seeks to characterize the changes in gene expression between a control sample and a sample exposed to a toxicant of interest, to identify genes up- and down-regulated due to the toxicant, to map regulatory pathways activated or antagonized by the toxicant, and in some cases (such as in the development of CWA medical countermeasures) to identify potential therapeutic targets in these pathways. Toxicogenomics has also been used as a tool for biomarker discovery, whereby microarrays are used to screen for alterations in gene expression that consistently correlate with a given exposure or effect. This technique provides a ''net'' with which to ''catch'' potential genes (and thus, their expressed polypeptide) to be evaluated for their utility as biomarkers. Both individual protein biomarkers (e.g., adipsin, Searfoss et al., 2003) and gene expression profiles or signatures (e.g., Thomas et al., 2001; Hamadeh et al., 2002) have been identified using microarrays.

Although microarrays can provide a global view of an organism's response to a toxicant, their primary limitation lies in the fact that they can only assess gene expression changes. Any effects due to changes in protein function or modification will not be observed using these techniques, and techniques from the field of proteomics must be used.

III. PROTEOMICS

Proteomics seeks to characterize the protein complement of a cell, tissue, or organism. Unlike genomics and transcriptomics, in which the target molecules (nucleic acid) are chemically more homogeneous, proteomics techniques must deal with a set of target molecules (proteins), which are chemically more heterogeneous and exist in many diverse states. Not only can each gene have multiple splice variants, each transcribed into slightly different polypeptides, but each of those polypeptides can be modified by posttranslational processes such as phosphorylation, glycosylation, ribosylation, and myristylation (Guo et al., 2007). Given the myriad modifications and variations of proteins coded from the genome, the number of chemically distinct proteins in a cell at any given point of time for a certain genetic composition has been estimated to be in the range of 1 million (Jensen, 2004). This is much greater than the number of genes in the genome, which is currently estimated to be 20,000–25,000 genes (Stein, 2004). Given that the final functional proteins in a

system are much more complex than the basic DNA code from which they are translated, a study of the complete proteome, on the scale of what was accomplished in the Human Genome Project, is practically impossible given the current technology. With the present state of the art, the best approach for understanding the proteome is to carefully dissect it using the latest tools of proteomic investigation to identify key proteins (or protein networks) important in response to precisely defined environmental conditions, life stages, or disease processes. Regardless of whether the goal is to understand one protein among many, or to reveal an overall ''profile'' of a large set of proteins, many of the same techniques are used. Current proteomic analyses rely on four key technologies: (1) Fractionation techniques (e.g., anion-exchange chromatography, differential centrifugation) are used to separate the complex mixture of proteins into biochemically or size-distinct fractions with fewer components. (2) Whole protein analysis methods such as liquid- and gel-based two-dimensional (2D) separation methods are useful to elucidate larger structural changes, including some posttranslational modifications (PTMs) where antibody-based detection is possible. (3) Fragmented polypeptide analysis methods, such as tryptic digestion followed by electrospray ionization (ESI) or liquid chromatography (LC)–tandem mass spectrometry (ESI-MS-MS or LC-MS-MS). These ''bottom-up'' approaches are better suited for quantitating increases or decreases in protein abundance. (4) Databases and computer algorithms that are able to predict protein identity, folding, and 3D structure. These can correlate mass spectrometry data with the latest published protein sequence databases.

One of the first preparative steps in a proteomics investigation involves fractionation. This step is often necessary to obtain adequate resolution in a complex mixture of polypeptides (i.e., whole cell homogenates, cellular and subcellular fractions, protein complexes, etc.). Fractionating a protein mixture can be accomplished by size exclusion chromatography or differential centrifugation (i.e., functioning as a molecular ''sieve'' to separate proteins based on size) and other methods, such as anion-exchange chromatography or isoelectric focusing (i.e., separate proteins based on protein pH or charge). Typically, the analytical resolution of a protein mixture is much greater when fractionation is used before a two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) or a mass spectrometry-based investigation.

The traditional workhorse technique of proteomics is 2D-PAGE (or 2DE) (Klose, 1975; O'Farrell, 1975; Bjellqvist, 1982). In this method, proteins in a mixture are separated first by isoelectric focusing. In this step, the proteins migrate in a liquid or gel matrix by electric current along a pH gradient (isoelectric focusing gradient) to a point where their charge is neutral. The point along the pH gradient where a protein has a neutral charge is termed the isoelectric point (p*I*). In the second dimension, the proteins, held at their isoelectric points, are separated by mass. The separated polypeptides are then stained (e.g., silver, Coomassie blue, Sypro Ruby) in the gel and located by their isoelectric point and mass. Identification can be made by Western blotting (in the case where an antibody is available for a protein of interest), or staining and excision (manual or robotic) followed by mass spectrometry in the case where a protein is unknown. Western blotting can be a useful method of identifying large shifts in protein abundance or shifts in isoelectric point, if antibodies that recognize the proteins of interest are available. In most cases of discovery, however, shifts in either intensity or position on the gel are detected by staining all proteins and using image analysis software to identify differences in position and abundance of spots. After the locations of these spots of interest are mapped, the spots can be excised manually or robotically for identification by mass spectrometry.

Although this technique has been used for years to successfully identify proteins altered by a particular disease state, toxicant exposure, or drug or other treatment, it does have several limitations. There is a good deal of art involved, and even for the most practiced, it is extremely difficult to solve the problem of gel to gel variability. To resolve some of the uncertainty created by gel variability, multiple gels (i.e., $n = 5–10$) can be combined (''piled up'') to create a consensus image for a given condition. Moreover, the problem of solubilizing certain proteins (i.e., highly lipophilic membrane proteins) in a buffer suitable for running through the gel can be very challenging.

One last problem that is yet to be completely solved is that the dynamic range (both in pH and protein abundance) is limited. The isoelectric focusing dimension requires that a range of separation be predetermined (e.g., pH 3–10). With the most sensitive staining methods it is possible to detect as little as 1–10 ng for silver-stained proteins and 1–100 pg for radioactive-labeled proteins (Jungblut and Thiede, 1997). However, very low abundance proteins can go undetected, or are masked by higher abundance proteins. Given the limitations associated with 2D-PAGE, the field of proteomics has developed newer techniques with greater reproducibility and sensitivity.

Mass spectrometry (MS) techniques have been long used as follow-on techniques after 2D-PAGE separation to identify shifted or new spots discovered on the gel. However, several MS-based techniques are now being used in ways that are rapidly superseding 2D-PAGE as a means to rapidly, accurately, and reproducibly measure and identify proteins and polypeptides in mixtures.

There are many other methods that can be used to separate proteins before characterization by mass spectrometry. Often in characterizing unknown proteins in a mixture, the constituent polypeptides are first enzymatically digested to yield a mixture of peptides. The peptides then are analyzed by MS either with or without some prior chromatographic or electrophoretic separation. With the aid of database search algorithms, the MS data are then evaluated to identify the proteins represented in the mixture (Abersold and Mann, 2003; Elia et al., 2005).

Matrix-assisted laser desorption ionization–time of flight (MALDI–TOF) MS employs laser energy to generate peptide ions from co-crystallized mixtures of peptides and ultraviolet-absorbing organic acids. MALDI–TOF analyses yield mass measurements of peptides, which can then be searched against databases with algorithms to identify the proteins from which the peptides were derived. This technique, termed peptide mass fingerprinting, has been shown to accurately identify proteins, particularly in organisms for which completed genome sequences or extensive protein sequence databases are available (Gevaerrt and Vandekeckhove, 2000). However, this approach is very difficult in organisms for which there is little or no reliably annotated genome and protein sequence.

Electrospray ionization–tandem mass spectrometry (ESI-MS-MS) has emerged as the second major MS technique for analytical proteomics (Loo et al., 1999). In ESI-MS-MS, peptides are ionized under high voltage at atmospheric pressure and then analyzed with ion trap, triple-quadruple, or quadruple-TOF mass analyzers. In these analyses, the peptide ions are subjected to collision-induced dissociation, which generates fragment ions that are detected by the tandem mass analyzer. The main advantage of this approach is that is does not require crystallization in a matrix before ionization. This method is also a more ''gentle'' ionization, allowing the possibility to capture the mass of native (nondenatured) proteins. Lastly, the mass accuracy is generally $\pm 0.5\%$ or better, allowing accurate measurement of dilute samples (Loo et al., 1999).

The resulting MS-MS spectra represent the fragmentation patterns produced. The spectra provide fingerprints that define the sequence of peptide ions. Searching algorithms such as SEQUEST (http://fields.scripps.edu/sequest), Peptident (http://ca.expasy.org/tools), and MASCOT (http://matrixscience.com) makes the identification of proteins possible by correlating peptide MS-MS data with predicted MS-MS data generated from protein and nucleotide sequence databases. Automated liquid chromatography–tandem mass spectrometry (LC-MS-MS) scanning acquires MS-MS spectra for hundreds to thousands of peptides in a single LC analysis. The combination of data-dependent scanning and the use of search algorithms facilitates the identification of proteins from complex peptide mixtures using LC-MS-MS data. Combining MS-MS with reverse-phase or tandem LC (e.g., ion-exchange reverse phase) makes LC-MS-MS the most powerful approach for protein identification and characterization. Moreover, fragmentation of peptides using MS-MS provides unambiguous confirmation not only of sequence but also of the location and character of posttranslational modifications, protein adducts, and sequence variants (Loo et al., 1999).

The tools of proteomics allow us to gain both breadth and depth in our perspective of the complexities of the molecular response to CWA exposure. However, there is another more complex layer of responsive molecules that is beginning to open a new window into the entirety of the molecular-level impact of CWA exposure.

IV. METABOLOMICS AND METABONOMICS

The metabolome is defined as the entirety of the rapidly changing metabolic processes and molecules present in the body at any given point of time (Lindon et al., 2006). As such, the entirety, or profile, of these molecules is very sensitive to subtle changes in study variables. Every process in the body, from transcription, translation, protein modification, and transport to active physiological metabolism, influences the tissue-level and global metabolomic composition at any given time point. Thus, the ability to carry out meaningful measures and interpretation of the metabolome relies on both sound experimental design and sensitive measurement techniques.

Sometimes referred to as ''biochemical profiling,'' metabolomics is a parallel and complementary discipline to transcriptomics and proteomics. Although transcriptomics and proteomics can generate hypotheses for both molecular mechanisms of toxicity and potential biomarkers of exposure, they are limited to identifying the presence or structure of the transcript or protein. Certainly, these end points serve their own purposes, such as defining a protein target for therapeutics or revealing a blood-based biomarker indicative of an exposure. However, they are not able to assess the overall functional significance of these changes within the widely interconnected system of biochemical processes in the body (Lindon et al., 2006).

The goal of metabolomics is to fully understand the dynamic complexity and identities of the metabolites present in a given condition. Metabonomics, on the other hand, takes a global view of the data, striving to capture the overall pattern or signature of the MS or nuclear magnetic resonance (NMR) peaks (Waters et al., 2001; Holmes and Antti, 2002; Lindon et al., 2006). Metabonomics is often used when examining urine or other complex body fluids for changes in metabolic composition. In contrast, metabolomics is more often used when examining a tissue or organ, as the metabolites are somewhat less complex primary or secondary metabolic processes. Both approaches are sometimes used in the same study. The advantages of each approach create an especially valuable screening tool. Databases can be built around these data, against which metabonomic patterns can be compared, and presumptive identifications of unknown exposures can be made. If metabolomic work has been done on the key metabolic product peaks, this information can open avenues for research to reveal more complete mechanisms of toxicity and potential biochemical points for developing effective CWA countermeasures.

The techniques currently used in metabolomics and metabonomics give researchers the ability to make an unbiased assessment of biochemical status at the cellular, tissue, or organism level. MS and NMR are the primary technologies of choice with which to explore the metabolome (Choi et al., 2001). Both technologies have made recent advances in sensitivity and data manipulation and storage.

Mass spectrometry is the most common technique because of its high accuracy and sensitivity. When examining a typical metabolomic reservoir, such as blood plasma or urine, there is relatively little upfront sample preparation. In a complex tissue such as liver or brain, dissection, microdissection, homogenization, and differential centrifugation are often used to further define the sample. Another common sample separation method applied before MS analysis is liquid chromatography (LC) or gas chromatography (GC) (Choi et al., 2001). Both techniques partition the sample as it enters the MS instrument, increasing the temporal resolution of the data. Mass spectrometry can measure biochemical changes in the nanomolar to femtomolar range. Simple quadrapoles, triple-quadrapoles, ions traps, TOF, Fourier Transform (FT), or hybrid systems have all been used. The flavor of MS chosen depends on the appropriate combination of accuracy, robustness, and sensitivity needed for the particular application (Choi et al., 2001). Regardless of type of MS, one of the main bottlenecks lies in the handling and analysis of the massive volumes of data generated. There are many ongoing efforts to develop new tools to resolve this problem.

NMR, and specifically, 1H magic angle spinning NMR, has been used for years (Nicholson et al., 1999). Its main advantages lie in the fact that NMR does not require any upfront chromatographic separation, nor does it destroy the samples during analysis. It has the ability to measure

molecular compartmentalization and dynamic biochemical changes in real time. Thus, it is not surprising that its data output is more information dense than MS. Although it can be more difficult to define the individual peaks in NMR, NMR is slightly better than MS at identifying and defining very dense patterns of peaks (Nicholson et al., 1999). For these reasons, NMR is routinely used to define relative concentrations of hundreds of compounds in complex, undefined mixtures.

The biochemical reactions at the tissue and organism levels induced by CWA, or any xenobiotic stimuli, are very complex and often not linear. To create biochemical order, the cell compartmentalizes these reactions. This is necessary in any living system to ensure robustness and redundancy in the network. Crude tissue homogenates created during any transcriptomic, proteomic, or metabolomic examination usually disrupts this order. Thus, it is very difficult to sort out what levels of individual biochemicals are ''normal.'' Therefore, it is much more informative and valuable to think about defining the function of key networks, rather than simply defining a list of distinct biochemicals (Glassbrook et al., 2000; Marcotte, 2001). Metabolomics is an important adjunct to the more linear way of thinking in transcriptomics and proteomics. However, to date, there is no automated way to interpret complex metabolomic data and create clear pathways (sometimes termed the ''interactome'') from metabolomic data. Manual interpretation of metabolomic data is still the norm. The automated tool is a critical development toward which many bioinformatics and ''omics'' researchers are working. One of the current goals is the development of in silico models of metabolism into which metabolomic, transcriptomic, and proteomic data can be fed (Loew and Schaff, 2001; Stefanovski et al., 2003; Borodina and Nielsen, 2005). As these types of models become increasingly sophisticated, and being able to receive input from a wider variety of platforms, these tools will become invaluable to many types of researchers. The ability to illustrate and test interactively changing pathway diagrams will help to build a more comprehensive understanding of both the molecular and biochemical pathway level effects of any xenobiotic exposure or other environmental changes. Overall, the integration of the data from all three approaches, transcriptomic, proteomic, and metabolomic, is synergistic and will continue to accelerate the development of a complete understanding of the molecular and biochemical toxicity of CWA.

V. RESULTS

A. Microarray Analysis of Sulfur Mustard Exposure Reveals Potential Therapeutic Targets Confirmed by Protein Analysis

Wide-ranging molecular, cellular, and tissue injuries have been described in a number of organ systems after exposure to SM (Papirmeister et al., 1991). The pulmonary system in particular is severely affected by SM. The development of pulmonary injury over the first 24 h postexposure involves multifocal, petechial hemorrhages on the pleural surface of the lung; lesions of the trachea, bronchi, and larger bronchioles; a progressive depletion of the bronchiolar-associated lymphoid tissue with necrosis of the lymphoid cells; necrosis and sloughing of the tracheal and bronchial epithelia followed by the formation of fibrinous pseudomembranes within the bronchi; and pulmonary edema with occasional alveolar hemorrhage (Anderson et al., 1996). Treatment of SM-induced pulmonary injury is primarily supportive in nature, requiring medical management that creates a heavy logistical burden (Papirmeister et al., 1991). In an effort to develop effective SM therapeutics, microarray analysis of SM-exposed rat lung tissue was used to elucidate the molecular mechanism of SM toxicity and to identify potential therapeutic targets (Dillman et al., 2005). These studies monitored changes in gene expression across a significant portion of the rat genome (~16K genes) and revealed temporal and dose–response effects of SM exposure. One of the most prominent molecular effects of SM exposure is DNA damage due to cross-linking at the N-7 of guanine (Papirmeister et al., 1991). Based on microarray analysis, Dillman et al. (2005) reported that there is a p53-mediated response following SM exposure. p53 is a protein transcription factor involved in the cellular response to DNA damage (Vogelstein and Kinzler, 2004). p53 up-regulates genes involved in cell cycle regulation and DNA

repair following DNA damage. The conclusion that there is a p53 response following SM exposure was based on the observation that many of the most significantly altered genes in SM-exposed rat lung are p53-responsive genes involved in cell cycle regulation, including cyclin G (Okamoto and Beach, 1994), p21 (el-Deiry et al., 1993), mdm2 (Barak et al., 1993), and transducer of ErbB2 (Tob) (Matsuda et al., 2001) (Figure 6.1). Many other p53-responsive genes, including Eph2A (Dohn et al., 2001), IEX1 (Schafer et al., 1998), Bax (Miyashita and Reed, 1995), snk (Burns et al., 2003), ATF3 (Zhang et al., 2002), and metallothionein (Fan and Cherian, 2002) were altered following SM exposure. The significant modulation of p53-responsive genes is depicted in Figure 6.2. The modulation of p53-responsive genes observed by microarray analysis correlates with past observations and suggests that DNA damage is the most likely source of the initial injury. Interestingly, it has been

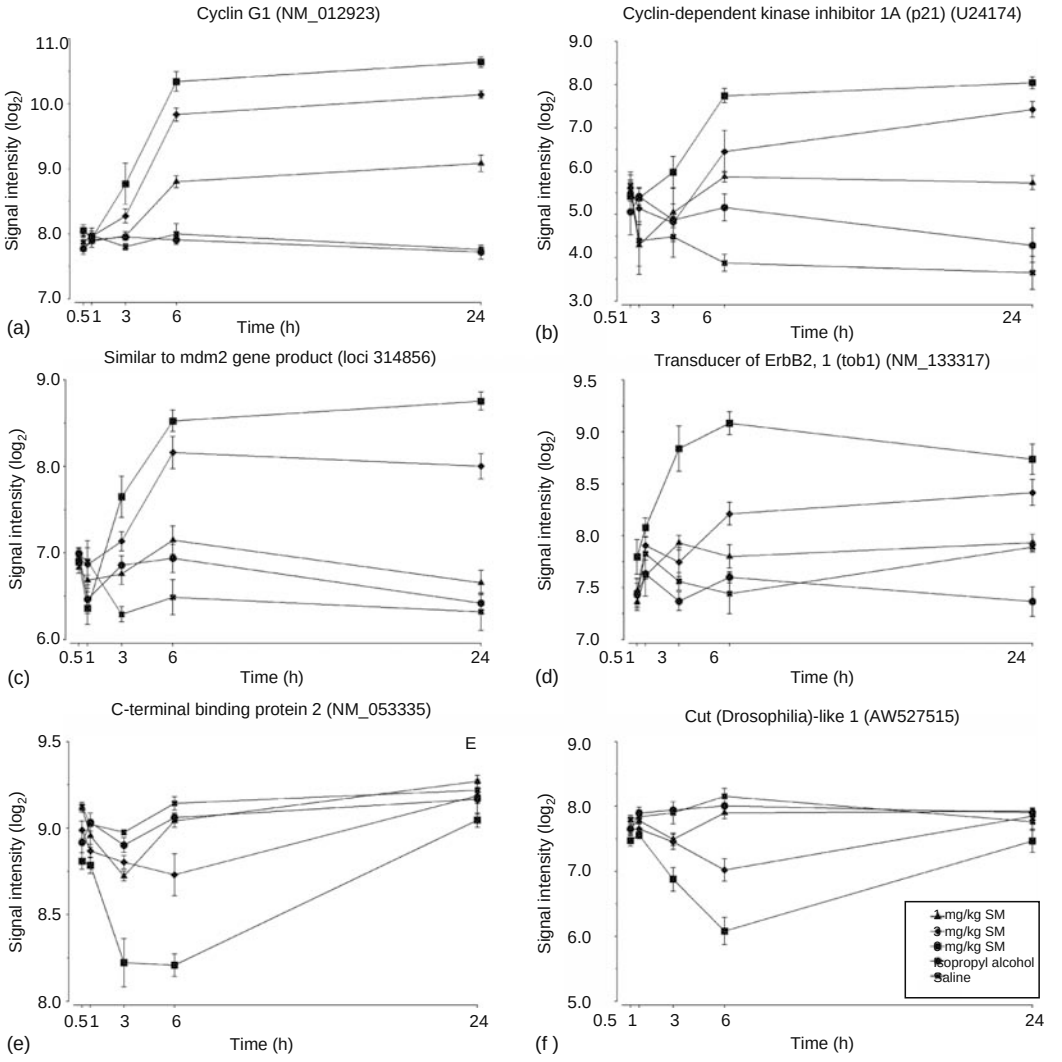

FIGURE 6.1 Expression profiles of cell cycle regulatory genes modulated by SM in rat lung. A two-way plot of $\log_2$ expression level versus time for each treatment or SM dose was generated. (▲) 1 mg/kg SM; (◆) 3 mg/kg SM; (■) 6 mg/kg SM; (●) isopropyl alcohol; (×) Saline. Error bars represent standard deviation. (Reprinted from Dillman, J.F., C.S. Phillips, L.M. Dorsch, M.D. Croxton, A.I. Hege, A.J. Sylvester, T.S. Moran, and A.M. Sciuto., *Chem Res Toxicol*, 18:28–34, 2005. With permission.)

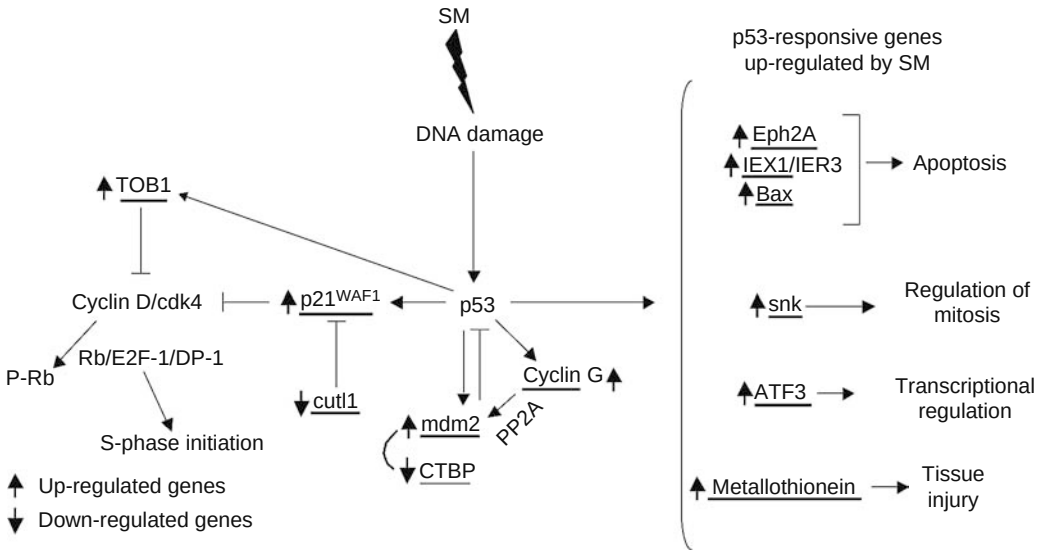

FIGURE 6.2 Gene expression data from SM-exposed rat lung reveal a potential p53 response. Microarray data were used to identify a critical pathway in the molecular response to SM exposure in the lung. (Reprinted from Dillman, J.F., C.S. Phillips, L.M. Dorsch, M.D. Croxton, A.I. Hege, A.J. Sylvester, T.S. Moran, and A.M. Sciuto., *Chem Res Toxicol*, 18:28–34, 2005. With permission.)

shown that there is a high incidence of lung cancer in former mustard gas workers and there are p53 mutations in many of these cancers (Manning et al., 1981; Tokuoka et al., 1986; Easton et al., 1988; Nishimoto et al., 1988; Takeshima et al., 1994).

Although these microarray data strongly suggest a p53 response following SM exposure in the lung, they do not directly show activation of p53 protein. A p53 response is typically characterized by increased phosphorylation of the p53 protein after a DNA-damaging event (Rosenthal et al., 1998). Thus, monitoring phosphorylation of p53 can serve as a rapid indicator of a p53 response. Phosphorylated p53 accumulates in normal human epidermal keratinocytes (NHEK) within 15–30 min after exposure to SM in a concentration-dependent manner (Figure 6.3; Minsavage and Dillman, 2007). These data suggest that exposure of human keratinocytes to SM can initiate signaling involved in directing the initial events of the p53 response. Altogether, these studies show that microarray analyses can identify mechanisms of toxicity, provide a rationale for selection of potential therapeutic targets for drug development, and identify potential biomarkers of toxicity.

B. Microarray Analysis of Phosgene Exposure Reveals Mechanisms of Toxicity that Correlate with Biochemical Data

Phosgene is a toxic industrial chemical that has been used in the past as a CWA. It is a potential threat to civilian and military personnel, particularly in urban environments where industrial areas are located in close proximity to residential areas. The primary route of exposure is via inhalation, with the pulmonary system being the most prominent target of toxicity. Sciuto et al. (2005) used oligonucleotide microarrays to assay global changes in lung gene expression following phosgene inhalation in mice. Gene expression data from phosgene-exposed mouse lungs were analyzed by principal component analysis (PCA) (Figure 6.4). PCA reduces the complexity of high-dimensional data and simplifies the task of identifying patterns and sources of variability in a large data set. The samples (three biological replicates for each time point and exposure condition) are represented by

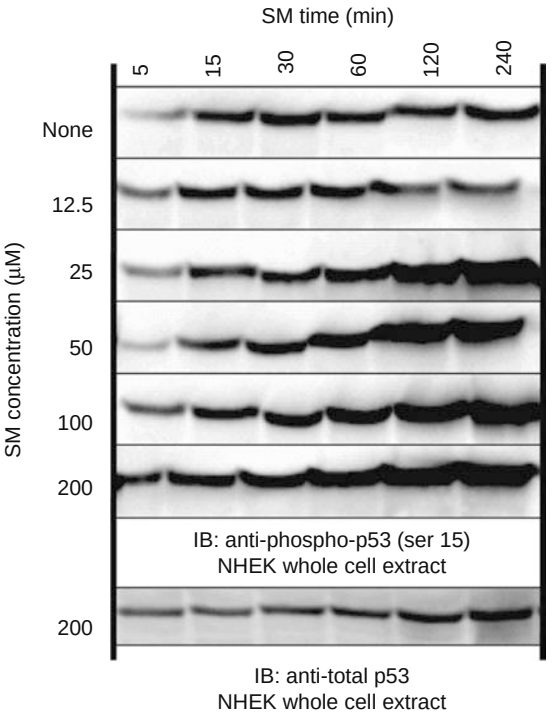

FIGURE 6.3 SM induces phosphorylation of p53. Normal human epidermal keratinocytes were exposed to 12.5, 25, 50, 100, or 200 μM SM for 5, 15, 30, 60, 120, or 240 min. Whole cell extracts were isolated, proteins fractionated by SDS–PAGE, and immunoblotted (IB) using antibodies that specifically recognize phosphorylated p53 (*top panels*). Total p53 was also analyzed. (Reprinted from Minsavage, G.D., Dillman III, J.F., *J. Pharmacol. Exp. Ther*., 321, 202, 2007. With permission.)

the points in the three-dimensional plot. The distance between any pair of points is related to the similarity between the two observations in high-dimensional space. Samples that are near each other in the plot are similar in a large number of variables (i.e., expression level of individual genes). Conversely, samples that are far apart in the plot are different in a large number of variables. In this data set one of the major sources of data variability is exposure condition (air or phosgene). There is a clear separation of the air-exposed samples as compared with the phosgene-exposed samples regardless of time (Figure 6.4). The time interval following phosgene exposure (0.5–72 h) is also a major source of data variability (Sciuto et al., 2005). This information was used to design an analysis of variance (ANOVA) using exposure, time, and sample as factors. The genes were ranked by p value based on this three-way mixed model ANOVA. Figure 6.5 reports the most significantly changed genes over time and dose. One of the most significantly altered genes is glutamate cysteine ligase (GCL) catalytic subunit, the rate-limiting enzyme in the synthesis of glutathione. The temporal profile of GCL gene expression in mice is shown in Figure 6.6. The expression level of GCL begins to significantly increase after 0.5 h and is higher than levels in the 8 h time-point-matched air-exposed mice. From 24 to 72 h, GCL gene expression trends toward the levels observed in controls, but remains elevated.

The microarray results for GCL described here were cross-validated with biochemical data from a previous study (Sciuto et al., 2003). The gene expression of GCL in phosgene-exposed lung tissue was compared with glutathione levels measured in bronchoalveolar lavage fluid (BALF) from phosgene-exposed lung tissue. The data are plotted as statistical means from both analytical

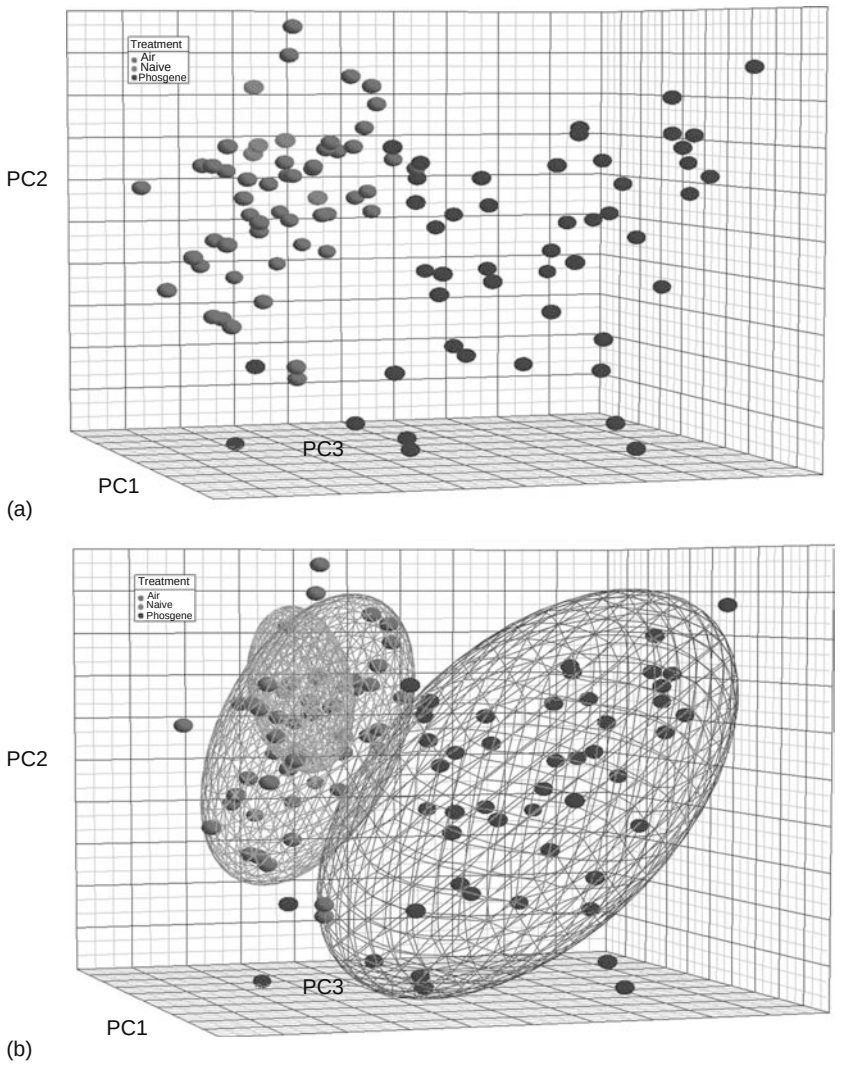

FIGURE 6.4 Principal component analysis (PCA) of gene expression data from phosgene-exposed mouse lung. Gene expression values were transformed by the addition of a constant followed by the $\log_2$ and were visualized by PCA (a, b). Point color corresponds to treatment group (red, air; green, naïve; blue, phosgene). The axes correspond to principal component 1 (PC1, *x*-axis), PC2 (*y*-axis), and PC3 (*z*-axis). The PCA in (b) is the same as (a) and shows ellipsoids representing a space two standard deviations from the mean of the data points. (Reprinted from Sciuto, A.M., C.S. Phillips, L.D. Orzolek, A.I. Hege, T.S. Moran, and J.F. Dillman., *Chem Res Toxicol*, 18:1654–1660, 2005. With permission.)

methods over the course of 72 h (Figure 6.7). GCL itself was not measured in the previous biochemical studies (Sciuto et al., 2003), but because it is the rate-limiting enzyme in GSH synthesis (Tian et al., 1997), it should serve as an appropriate indicator of GSH formation. It is clear that phosgene exposure caused an increase in the expression levels of GCL that matches the increased levels determined for BALF GSH from the previous study (Sciuto et al., 2003).

Taken together, these results demonstrate that gene expression data are an important component in understanding the mechanism of toxicity following a chemical exposure and can elucidate specific toxic effects of chemical exposure.

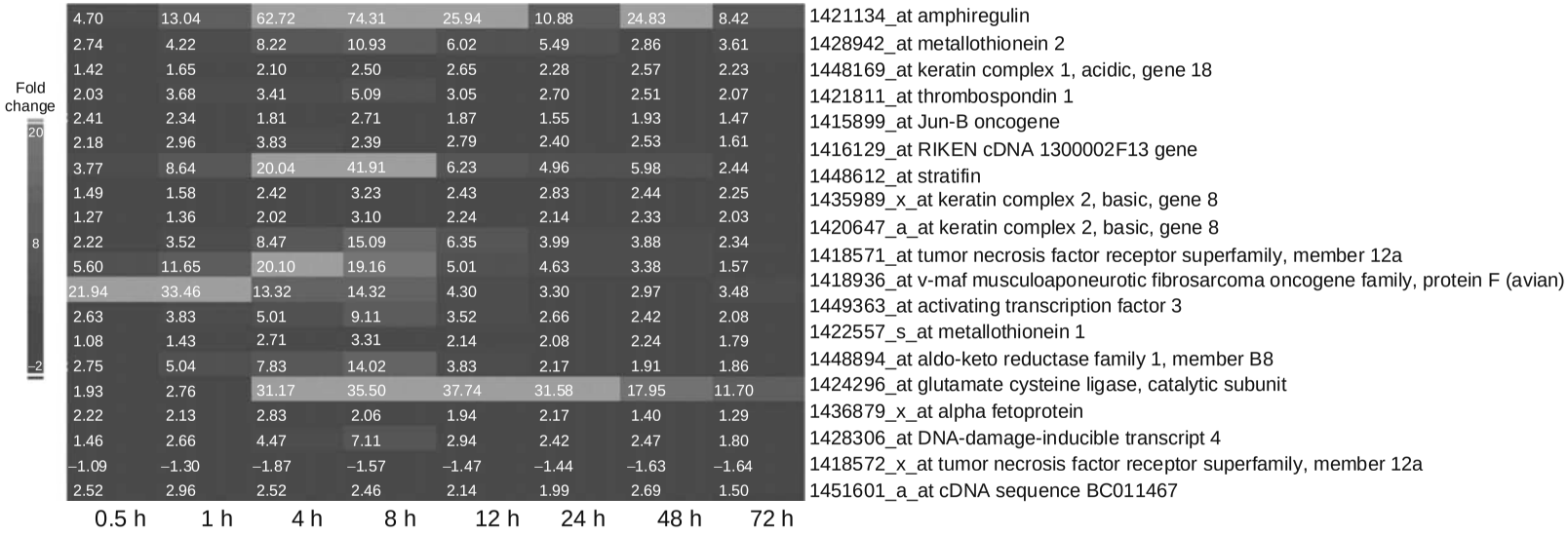

FIGURE 6.5 Heat map revealing gene expression profiles following phosgene exposure. The expression profiles of the most significantly changed genes (20 most significantly changed genes based on Bonferroni-corrected *p*-value) were visualized using a heat map. The fold changes in gene expression were calculated from the antilog$_2$ intensity data as: fold change = (signal intensity after phosgene exposure)/(signal intensity after air exposure). A fold change of 20-fold was used as the maximum value in the color scale (left of map) to show greater resolution in the heat map. Genes that show a change in expression greater than 20-fold are colored in orange. The time points are indicated below the map. (Reprinted from Sciuto, A.M., C.S. Phillips, L.D. Orzolek, A.I. Hege, T.S. Moran, and J.F. Dillman., *Chem Res Toxicol*, 18:1654–1660, 2005. With permission.)

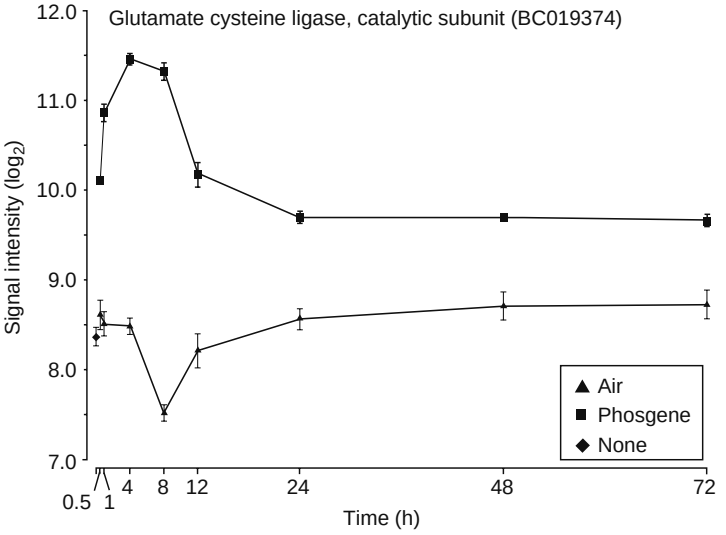

FIGURE 6.6 Glutamate cysteine ligase (GCL) subunit gene expression in mice exposed to phosgene. The $\log_2$ signal intensity represents gene expression level. Sample sizes were three mice per time point for air or phosgene and replicate chips were probed. Data are expressed as mean $\pm$ SEM. There was a significant exposure treatment by time interaction at a level of $p < 2.8 \times 10^{-22}$ (three-way ANOVA). (Reprinted from Sciuto, A.M., C.S. Phillips, L.D. Orzolek, A.I. Hege, T.S. Moran, and J.F. Dillman., *Chem Res Toxicol*, 18:1654–1660, 2005. With permission.)

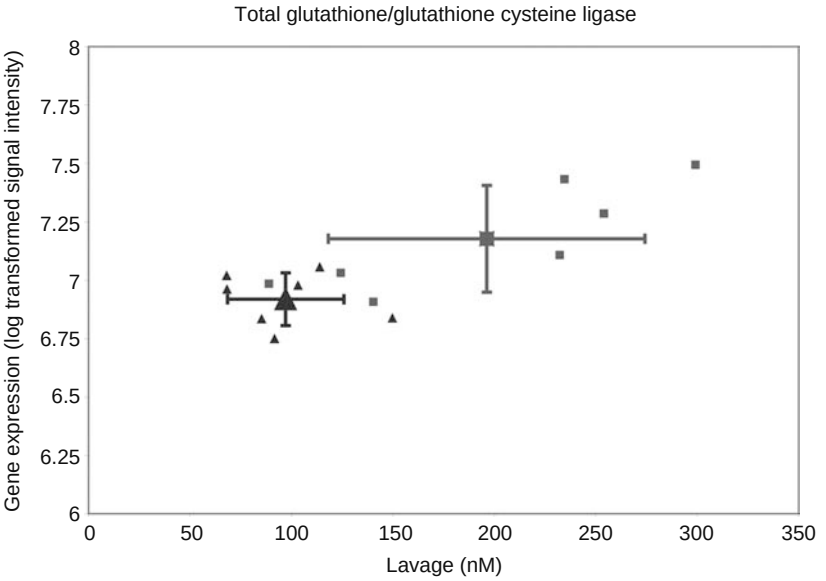

FIGURE 6.7 Correlation between gene expression and biochemical analyses. The correlation between microarray gene expression data from phosgene-exposed mouse lung tissue in this study and biochemical data from phosgene-exposed mouse lung BALF generated in our earlier study (Sciuto et al., 2003) is shown. An average of the gene expression data (ordinate, $\log_2$ signal intensity) plotted against the average of the BALF data (abscissa) for total glutathione/glutathione cysteine ligase for air (▲) and phosgene exposed (■). The smaller ▲ and ■ represent the individual values from individual experiments for each gas exposure treatment. (Reprinted from Sciuto, A.M., C.S. Phillips, L.D. Orzolek, A.I. Hege, T.S. Moran, and J.F. Dillman., *Chem Res Toxicol*, 18:1654–1660, 2005. With permission.)

TABLE 6.1
Summary of Perturbed Components: By Dose and Tissue/Biofluid

Tissue	Dose	Total	$p < 0.05$	$p < 0.01$
Plasma	Low	975	64	12
Plasma	High	1034	70	32
Liver	Low	1763	71	13
Liver	High	1763	160	50

C. Metabolomic Investigation of Liver and Blood Plasma Following Low-Level Exposure to VX Vapor

The metabolomic profiles of plasma and liver of 109 adult (6–8 weeks old) male Sprague-Dawley rats exposed by whole body inhalation to VX vapor were determined using liquid chromatography coupled with mass spectrometry (LC/MS). Of these animals, 39 were air exposed, 35 were exposed to 0.0007 mg/m^3 VX vapor for 240 min (low dose, no significant miosis), and 40 were exposed to 0.0018 mg/m^3 VX vapor for 240 min (high dose, significant miosis). Briefly, the LC/MS data were then mined for perturbed components using parametric and nonparametric statistical methods. Unsupervised analyses were also conducted using hierarchical clustering to identify groups of perturbed metabolic components in the liver and in the plasma (Table 6.1).

As would be expected given a typical dose–response scenario, the livers from the higher-dosed animals exhibited greater perturbation in metabolites than the lower-dosed ones. This could be because of the greater concentration of toxicant in the liver, which can trigger involvement of more metabolic processes as the concentration increases to ameliorate the greater toxicity. The plasma was also a fruitful reservoir of potentially informative markers of exposure. The metabolites with the largest degree of change in the plasma are shown in Table 6.2. From this pilot study, work is ongoing to verify these findings by repetition in other CWA exposure studies.

VI. CONCLUSIONS

Advances in science often come as a result of advances in technology, and this has been especially true in the fields of genomics, proteomics, and metabolomics. The development of microarrays, advancements in MS and NMR instrumentation, and computing power to analyze large multivariate data sets have provided scientists with unprecedented ability to measure biological response at the

TABLE 6.2
Plasma Components with Largest Change in Response

5-Hydroxyxanthotoxin	2-Naphthylamine
Poly-L-glutamate	4-Oxoproline
Pyridoxal	5-Methyl-2-deoxyxytidine
Uridine	DNA
L-Citrulline	Glycocholate
L-Tryptophan	*N*(pai)-Methyl-L-histidine
L-Arginine	Sulfate
Creatine	Uridine
L-Serine	
L-Glutamine	
L-Methionine	
L-Valine	

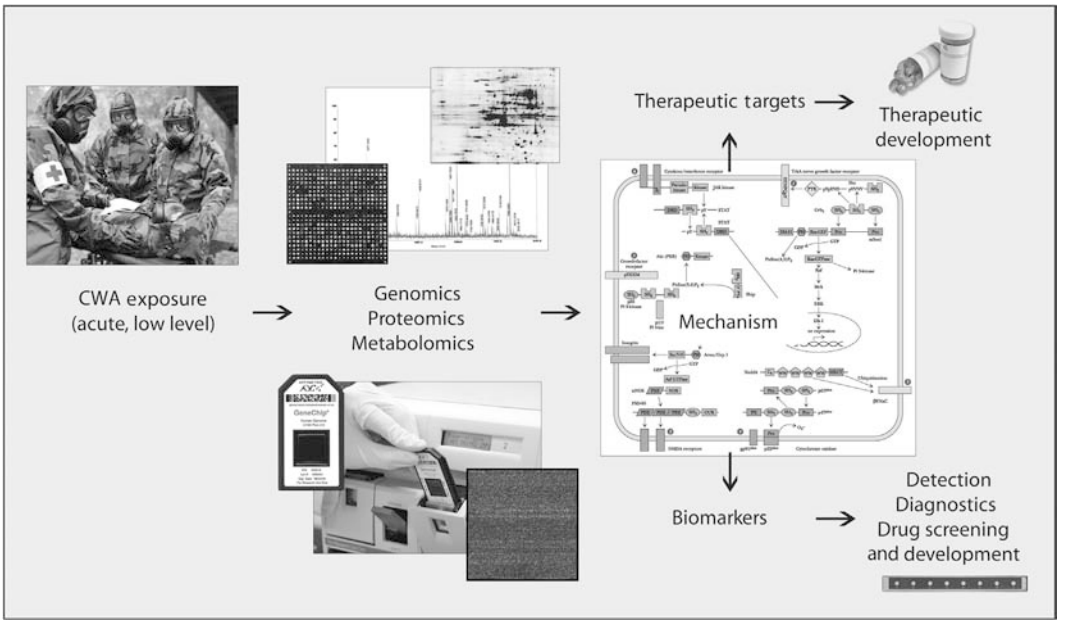

FIGURE 6.8 Applications of genomic, proteomic, and metabolomic approaches to force protection against chemical warfare agents.

whole cell or whole tissue level. This has expanded the capacity for identifying genes and proteins involved in disease, pathways involved in cellular response to toxicants, and biomarkers of effect, exposure, or efficacy (Figure 6.8).

A. Biomarker Discovery

Biomarker discovery has been an accelerating field of research since the advent of genomics, proteomics, and metabolomics. Despite the seemingly recent emergence of biomarker discovery as an important component of medical research, biomarkers have been used clinically for decades. Cholinesterase activity is a prototypical biomarker used in both civilian and military settings to determine exposure to organophosphate pesticides or nerve agents. In the military, cholinesterase testing has been used routinely for force health protection by testing soldiers before deployment to an area where CWAs may be used (TB Med 590, 2001). In addition, cholinesterase testing is used for occupational screening of agent workers. Given the ease of the assays used, the specificity of cholinesterase inhibition by nerve agents, and the link between cholinesterase activity and exposure, cholinesterase has proven to be a useful biomarker of exposure to nerve agent. However, the tests lack the specificity necessary to unequivocally identify the agent responsible for cholinesterase inhibition. To identify the specific agent responsible for cholinesterase inhibition, an analytical chemistry technique is required (e.g., CG/MS). This requires additional sample, time, and expertise that may not be readily available in a battlefield scenario or terrorist attack. In addition, identifying exposure to and effects of low levels of CWA has been problematic. Genomic, proteomic, and metabolic technologies provide a new way to uncover novel biomarkers for rapid and accurate force health protection and monitoring.

B. Mechanisms of Toxicity and Therapeutics

Understanding the mechanism of toxicity of a CWA is critical for the rational development of CWA therapeutics. As described, genomics, proteomics, and metabolomics approaches are an important

component for understanding mechanisms of toxicity. Identifying the key genes and proteins involved in the cellular response to a toxicant uncovers not only the mechanism of toxicity itself, but also identifies potential therapeutic targets in the pathways that are activated by the toxicant, as well as potential biomarkers of exposure. The information derived from these global approaches can also lead to the design of drug screening assays and to the identification of candidate biomarkers for development of detection strategies and diagnostics.

C. Advanced Development of Therapeutics (FDA Approval)

One important area where genomics, proteomics, and metabolomics can play a critical role is in the advanced development of CWA therapeutics. Since traditional clinical trials are not an option for the testing of CWA therapeutics, we must rely solely on animal data to determine efficacy. (New Drug and Biological Drug Products; Evidence Needed to Demonstrate Effectiveness of New Drugs when Human Efficacy Studies are not Ethical or feasible. 2002). The FDA has recognized this challenge and has developed the "Animal Rule" (www.fda.gov/CBER/rules/humeffic.htm). Under this rule, FDA can rely on animal studies to provide substantial evidence of the effectiveness of therapeutics when (1) there is a reasonably well-understood pathophysiological mechanism for the toxicity, (2) the effect is demonstrated in more than one animal species expected to react with a response predictive for humans, (3) the animal study end point is clearly related to the desired benefit in humans, and (4) the data or information on the pharmacokinetics and pharmacodynamics of the product or other relevant data or information in animals and humans are sufficiently well understood to allow selection of an effective dose in humans, and it is therefore reasonable to expect the effectiveness of the product in animals to be a reliable indicator of its effectiveness in humans (quoted from www.fda.gov/CBER/rules/humeffic.htm). Genomics and proteomics technologies and approaches can play an important role by helping understand the pathophysiological mechanism for the toxicity (see (1) above). This will also aid in understanding the molecular mechanism of efficacy of the countermeasure. Genomics and proteomics technologies may also be used to extrapolate CWA exposure data from animals to humans (Dillman and Phillips, 2005). Recently, the FDA has encouraged the submission of genomic data as part of the application for a new drug (Guidance for Industry: Pharmacogenomic Data Submissions, 2005). They have proposed broad guidelines to govern the use of this data in the regulatory process. Though this guidance is not specifically addressed to the Animal Rule, it is likely to become an important component of new drug applications that relies on the Animal Rule for approval.

D. Use of Animals in Research (Reduction, Refinement, and Replacement)

The use of genomics and proteomics approaches allows researchers to obtain more data from a single animal experiment than was previously possible. Thousands of response variables can be measured from a single animal sample (gene expression profiles, protein expression, metabolic profile). This allows data gathering from animal experiments to be much broader in scope, more efficient, and more informative. This contributes to the goal of the "Three Rs" (reduction, refinement, and replacement) in animal research in the DoD.

VII. SUMMARY

The application of genomics, proteomics, and metabolomics has revolutionized research in the life sciences. These approaches hold much promise to accelerate the mission of developing countermeasures against CWA, improving the development of protective material, and enhancing our capabilities for force health protection.

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7 Novel Approaches to Medical Protection against Chemical Warfare Nerve Agents

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CONTENTS

The opinions or assertions contained herein are the private views of the authors and are not to be construed as reflecting the view of the Department of the Army or the Department of Defense.

I. INTRODUCTION

Nerve agents are organophosphorus (OP) compounds that are among the most toxic substances known to mankind (Dacre, 1984). Originally, OPs were developed for use as insecticides (Ballantyne and Marrs, 1992), but their extreme toxicity toward higher vertebrates led to their adoption as weapons of warfare (Maynard and Beswick, 1992). The most likely route of exposure to these agents is through inhalation or by absorption through the skin. All OPs produce their acute toxic effects by irreversibly inhibiting acetylcholinesterase (AChE, E.C. 3.1.1.7), the enzyme that breaks down the neurotransmitter acetylcholine (ACh) (Koelle, 1963; Taylor, 1990). The accumulation of ACh in response to OP exposure causes an overstimulation of cholinergic receptors at the neuromuscular junctions (Stewart, 1959; Stewart and Anderson, 1968; Heffron and Hobbinger, 1979), which can lead to muscle weakness, increased secretions, respiratory depression, seizures, coma, and ultimately death resulting from respiratory or cardiovascular failure or convulsion consequences (de Candole, 1953).

The conventional approach for the treatment of OP intoxication involves efforts to counteract the effects of AChE inhibition, which includes the administration of an anticholinergic drug such as atropine sulfate to antagonize the effects of elevated ACh levels and an oxime such as 2-PAM chloride to reactivate OP-inhibited AChE (Wilson and Ginsburg, 1955; Bajgar et al., 1971; Heath and Meredith, 1992). In addition, an anticonvulsant drug such as diazepam may also be administered to control OP-induced tremors and convulsions. In conjunction with therapy, individuals at high risk for exposure to GD are pretreated with the spontaneously reactivating AChE inhibitor pyridostigmine bromide, which temporarily protects about 30% of peripheral AChE from irreversible inhibition by OP (Gordon et al., 1978; Leadbeater et al., 1985). Although these antidotal regimens are effective in increasing survivability, they suffer from several disadvantages: (1) they do not prevent postexposure incapacitation, convulsions, performance deficits, or in the case of recurring seizures, permanent brain damage (Leadbeater et al., 1985); (2) a greater than marginal improvement of these pharmacological approaches is not possible, because stronger drugs or higher doses are likely to produce unacceptable performance decrements by themselves (Dunn and Sidell, 1989; Castro et al., 1991); (3) the efficacy of this antidotal regimen demands precise timing and sequencing of drug administration at the time of OP exposure, which is very difficult to execute on the battlefield; and (4) some nerve agents, such as GD, present an additional challenge due to the rapid dealkylation of inhibited AChE that is resistant to therapeutic reversal by oxime (Fleisher and Harris, 1965).

The contents of this article describe the progress made in the last 5 years in exploring and developing nerve agent medical countermeasures that provide higher survival rates, avoid or reduce enduring adverse effects to survivors, and significantly lengthen the windows in which prophylaxes or treatments can be effectively administered. Because of the fast inactivation of AChE by OPs and the inability to distinguish between nerve agents, medical treatment must be administered within ~1 min and it must be effective for all OP nerve agents. In this regard, significant advances were made on the development of bioscavengers for human use, and the search for broad-spectrum oximes and reversible cholinesterase inhibitors was initiated.

II. ENZYME-BASED BIOSCAVENGERS

A novel approach to treating OP poisoning is the use of enzymes to sequester these compounds in circulation before they reach their physiological target, the central nervous system (CNS) AChE. Ideally, the scavenger should enjoy a long residence time in circulation, should be biologically innocuous in the absence of nerve agent, and should not present an antigenic challenge to the immune system. For these reasons, a formal program to identify candidate bioscavengers was begun in 1993 and has focused on enzymes of mammalian (specifically human) origin. Candidate bioscavenger proteins, in general, function either by stoichiometrically binding and sequestering

toxic OPs or by catalytically cleaving OPs into nontoxic products. In the former category are naturally occurring human proteins that bind or react with nerve agents, including enzymes such as cholinesterases (ChEs) and carboxylesterases (CaEs). One mole of each of these stoichiometric scavengers has the capacity to bind one mole of nerve agent. Candidate enzymes with catalytic activity against nerve agents include the human organophosphorus acid anhydride hydrolases (OPAHs) (Masson et al., 1998), such as paraoxonase (Hu PON). In general, the use of catalytic scavengers would be advantageous because small amounts of enzyme would be sufficient to detoxify large amounts of nerve agent.

A. Plasma-Derived Human Butyrylcholinesterase

Because the use of ChEs as stoichiometric bioscavengers was most extensively characterized and the goal of research on scavenger molecules was to generate a means to protect humans from the toxic effects of nerve agents, efforts for the past 5 years primarily focused on plasma-derived human butyrylcholinesterase (Hu BChE, EC 3.1.1.8). Hu BChE has several advantages as an exogenously administered prophylactic for human use (Ashani, 2000). First, it reacts rapidly with all OP nerve agents including, soman (GD), sarin (GB), tabun (GA), and VX. Second, it is readily absorbed from sites of injection and remains in human circulation for long periods of time. Third, because the enzyme is from a human source, it is not expected to produce any adverse immunological responses on repeated administration into humans. Fourth, because the enzyme has no known physiological function in the body, it is unlikely to produce any physiological side effect. Because the biochemical mechanism underlying prophylaxis by exogenous Hu BChE was established and tested in several species, including nonhuman primates, this concept should enable a reliable extrapolation of results from animal experiments to human application. A dose of 200 mg of Hu BChE is envisioned as a prophylactic treatment in humans that can protect from exposure of up to $2 \times LD_{50}$ of GD. Recent efforts focused on advancing the use of Hu BChE in humans are described below.

1. Isolation and Purification

The primary requirement to advance Hu BChE as a bioscavenger for human use was to obtain sufficient amounts of purified enzyme for conducting preclinical pharmacological studies. A procedure for the large-scale purification of Hu BChE from Cohn Fraction IV-4 paste, a by-product of human plasma generated during the production of human blood proteins, such as γ-globulin, clotting factors, and others, was developed. This procedure involved resuspension of 80 kg of Cohn fraction IV-4 paste in nine volumes of cold distilled water followed by adjustment of pH of the suspension to 4.90. The suspension was clarified by centrifugation and filtration, and the pH was adjusted to 8.0. The supernatant was filtered and purified by procainamide affinity chromatography followed by anion exchange chromatography. The final product contained ~5 million units corresponding to ~8 g of Hu BChE. The enzyme was filtered through a 0.45 μm filter and stored in 25 mg batches in lyophilized form at -20°C.

The purity of the Hu BChE preparations was followed by specific activity, evidence of a single protein band on SDS-PAGE and HPLC. The specific activity of the purified enzyme was about 700 U/mg measured in 50 mM sodium phosphate buffer at pH 8.0 at 25°C, using 1 mM butyrylthiocholine as the substrate (Grunwald et al., 1997). Active-site titration of purified enzyme with 7-(*O,O*-diethylphosphinyloxy)-1-methylquinolinium methyl sulfate (DEPQ) revealed that 1 mg of enzyme contains 11 nmol of active sites. Each nmol of enzyme contains 64–68 U of activity. The absorbance of a 1 mg/mL solution at 280 nm varies from 1.8 to 1.92 (Lockridge et al., 1979; Grunwald et al., 1997). The enzyme migrated as a single band on a reducing SDS-PAGE, with a subunit molecular weight of 85 kDa as shown in Figure 7.1. The size of the band was reduced to 65 kDa upon treatment with *N*-glycosidase F (Figure 7.1). The intact protein migrated as a single band on native polyacrylamide gel electrophoresis, which could be stained for enzyme activity.

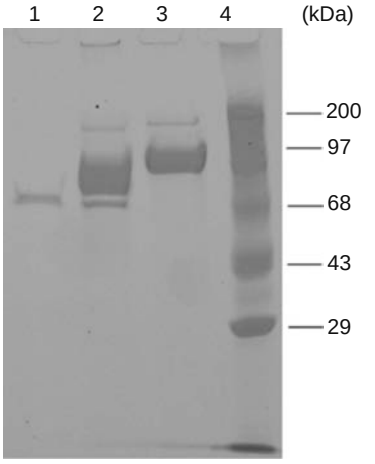

FIGURE 7.1 SDS-PAGE of Hu BChE. 5 U of purified Hu BChE native or following deglycosylation with *N*-glycosidase F was analyzed on a 10% SDS-polyacrylamide gel. Following electrophoresis at 100 V for 75 min, the gel was stained with Bio-Safe Coomassie. Samples are *N*-glycosidase F (lane 1), deglycosylated Hu BChE (lane 2), Native Hu BChE (lane 3), and molecular weight markers (lane 4).

2. Characterization

Hu BChE is present in serum as a soluble, globular tetrameric form, consisting of four identical subunits (Lockridge et al., 1979, 1987). The tetramer has a molecular weight of 340 kDa and it migrates as a single peak on sucrose density gradients, with a sedimentation coefficient ($s_{20,w}$) of 12.44 (Das and Liddell, 1970). The complete amino acid sequence of this enzyme was determined by amino acid as well as nucleotide sequencing (Lockridge et al., 1979; McTiernan et al., 1987). Glu is the residue at the amino terminus and Leu is the amino acid at the carboxyl terminus. Each subunit consists of 575 amino acids, which contain three disulfide bridges. The molecular weight of each subunit is 85 kDa, of which 65 kDa is for the protein and 20 kDa (24%–26%) for the carbohydrate (Haupt et al., 1966; Saxena et al., 1998a). *N*-linked carbohydrate structures are present on Asn at positions 17, 57, 106, 241, 256, 341, 455, 481, and 486 (Lockridge et al., 1987). The site-specific carbohydrate structures for 8 of 9 *N*-linked glycosylation sites were determined (Garcia et al., 2004). The major structures consist of an asialylo, galactosylated bi-antennary oligosaccharide primarily without core fucosylation. Asn 481 and Asn 486 were inseparable with the cleavage strategies employed, which precluded an absolute assignment of carbohydrate structures for these sites. Although all the sites displayed some degree of microheterogeneity, some sites were more heterogeneous; for example, Asn 241 had 25% oligo-mannose structures, whereas Asn 256 and Asn 455 consisted of 11% and 12% tri-galactose, tri-antennary structures, respectively. The x-ray crystal structure of a genetically engineered low glycosylation form of monomeric Hu BChE was recently described (Nicolet et al., 2003).

3. Pharmacokinetics and Toxicity

The bioavailability and circulatory stability of purified Hu BChE, administered by different routes of administration, was examined in mice, guinea pigs, and monkeys. Purified Hu BChE displayed high bioavailability in the circulation of mice, guinea pigs, and nonhuman primates when administered by i.v., i.m., or i.p. injections (Figure 7.2). The enzyme displayed a mean residence time (MRT) of about 48 h in mice, 109–110 h in guinea pigs, and 72–74 h in monkeys (Doctor and Saxena, 2005). This prolonged circulatory stability was previously reported in rats and mice (Raveh et al., 1989, 1993; Ashani et al., 1991), guinea pigs (Allon et al., 1998), and rhesus monkeys

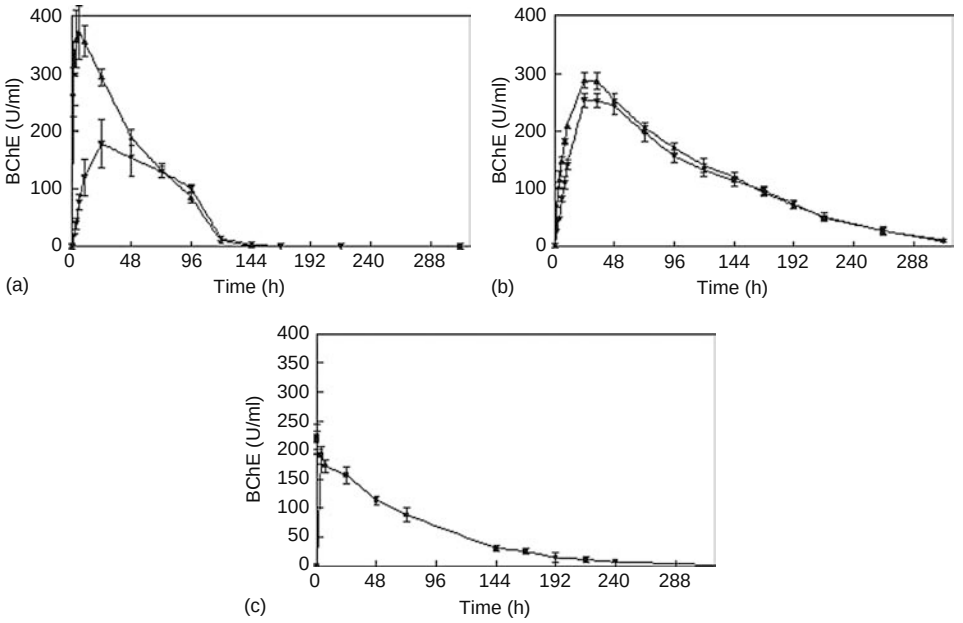

FIGURE 7.2 Time course of Hu BChE in the blood of mice (a), guinea pigs (b), and rhesus monkeys (c) following i.m. (▼), i.p. (▲), or i.v. (■) bolus injections. The dose of enzyme administered was 90 mg/kg for mice, 60 mg/kg for guinea pigs, and 30 mg/kg for monkeys. Data at each time point is an average of six animals for mice and guinea pigs and four for monkeys.

(Raveh et al., 1997). The route of administration affected the time at which the enzyme attained peak activity in circulation, but did not affect the MRT, and an enzyme level between 80% and 100% of peak activity was maintained for a period of ~3–10 h. Also, regardless of the route of administration, 60%–90% of administered enzyme was found in the circulation of animals.

Because the animals were administered large doses of Hu BChE, which resulted in up to 300-fold increases in circulating BChE levels, it was important to demonstrate that Hu BChE was not toxic to these animals. The animals were observed for any abnormal physiological or behavioral signs for 2 weeks; they were euthanized and blood was collected for determining hematology and serum chemistry parameters. A gross necropsy was also performed and a full set of tissues, including brain, heart, lung, liver, intestine, kidney, eye, spleen, and muscle injection sites, were examined for any gross or histological changes. General observation of mice and guinea pigs with circulating levels of Hu BChE as high as 300 U/mL did not reveal any signs of clinical toxicity. Also, results of necropsy together with those from analysis of hematology and serum chemistry parameters did not show any clinical signs of pathology following administration of large doses of Hu BChE (Saxena et al., 2005). Similarly, guinea pigs administered 60 mg/kg of Hu BChE did not show any signs of toxicity as observed by histopathology or hematology or serum chemistry parameters (Sun et al., 2005).

4. Behavioral and Physiological Safety

Since the major use of bioscavengers is prophylactic, it was essential to demonstrate that Hu BChE is devoid of undesirable effects. Thus, several studies evaluated behavioral and physiological effects of equine (Eq) BChE administered alone as well as prior to nerve agent exposure (Broomfield et al., 1991; Genovese and Doctor, 1995; Matzke et al., 1999). Administration of Eq BChE to rats (resulting in circulating BChE levels as high as ~55 U/mL) did not affect acquisition or retention of a passive avoidance task or disrupt performance of a food-maintained

operant behavior task (Genovese and Doctor, 1995). The enzyme was also shown to provide significant protection against performance degradation produced by MEPQ ((7-methylethoxyphosphinyloxy)-1-methylquinolinium iodide), a peripherally active OP compound. Similarly, the safety and efficacy of Eq BChE was also demonstrated in rhesus monkeys using a memory-intensive serial probe recognition (SPR) task, in which subjects were required to recall a list of stimuli. Repeated administration of a commercial preparation of Eq BChE, which produced a 7- to 18-fold increase in circulating BChE level, did not systematically affect performance on the task (Matzke et al., 1999), and rhesus monkeys pretreated with 460–503 nmol of Eq BChE were protected against 2 or $3 \times LD_{50}$ of GD or GB (Broomfield et al., 1991).

Similar studies were conducted to address the safety and efficacy of Hu BChE in mice (Ashani et al., 1991a), rats (Brandeis et al., 1993), guinea pigs (Allon et al., 1998), and rhesus monkeys (Raveh et al., 1997). In all cases, doses of Hu BChE sufficient to protect against OP exposure were devoid of behavioral side effects. More recently, the behavioral safety of large doses of Hu BChE alone, physostigmine alone, and Hu BChE pretreatment followed by physostigmine challenge were evaluated in mice (Clark et al., 2005). Clark et al., 2005 demonstrated that mice that were administered 2000 U of Hu BChE (30 times the dose required to protect humans from $2 \times LD_{50}$ of GD) did not show any significant alteration in acoustic startle or prepulse inhibition behavior. As expected, mice that received physostigmine (0.4 mg/kg) by s.c. injection showed significant decreases in the amplitude of acoustic startle reflex to 50 ms, 120 dB noise pulses. In addition, the time to peak startle reflex and the amount of prepulse inhibition of the acoustic startle reflex were significantly increased. This effect was partially mitigated in mice pretreated with Hu BChE.

The behavioral and physiological safety of large doses of Hu BChE was also examined in rhesus monkeys using an SPR task designed to assess attention and short-term memory (Myers et al., 2003). Concurrent with the cognitive-behavioral assessment, blood was collected at critical points throughout the study and analyzed for BChE activity, production of antiBChE antibody, and gross clinical pathology. Each monkey received 150 mg (105,000 U or 32 mg/kg) of Hu BChE by i.v. injection, 60 min prior to testing on the SPR task. No cognitive-behavioral decrements of any kind were detected in SPR performance tests and no robust or consistent signs of clinical pathology were detected in any of the blood assays during the 5 week observation period. These results provide strong support for the behavioral and physiological safety of Hu BChE in mice and rhesus monkeys.

5. *In Vitro* and *In Vivo* Stability

The thermal stability of purified Hu BChE in lyophilized (1 mg) or liquid form (10 mg/mL in 50 mM sodium phosphate, pH 8.0 + 10% glycerol + 1 mM EDTA) was examined by measuring enzyme activity following storage at 4°C, 25°C, 37°C, or 45°C. Lyophilized samples were resuspended in 1 mL of 50 mM sodium phosphate buffer, pH 8.0 for measuring BChE activity using the Ellman assay (Ellman et al., 1961). Hu BChE preparations were found to be stable when stored in lyophilized form at 4°C, 25°C, 37°C, or 45°C for over 2 years (Saxena et al., 2005). The enzyme was also stable when stored in liquid form at 4°C and 25°C for 1 year.

The effect of storage in lyophilized form at −20°C on *in vivo* circulatory stability of Hu BChE preparations was determined by measuring the pharmacokinetic profile of the enzyme following i.m. administration into Balb/c mice as described above. As shown in Table 7.1, the pharmacokinetic properties of the enzyme were not affected by storage at −20°C for at least 3 years.

6. Efficacy

Due to the limited availability of purified Hu BChE, previous studies examined the efficacy of Hu BChE against 2–3 × LD_{50} of OP nerve agents (Ashani et al., 1991; Brandeis et al., 1993; Raveh et al., 1997). More recently, the efficacy of Hu BChE was evaluated in guinea pigs and cynomolgus monkeys against multiple LD_{50} challenges of nerve agents (Lenz et al., 2005). Guinea pigs were protected against a cumulative of $5 \times LD_{50}$ of either GD or VX and there was a decrease in molar

TABLE 7.1
***In Vivo* Stability of Hu BChE[a] in Mice**

	Time of Storage at −20°C (Months)				
Parameters	**5**	**12**	**19**	**27**	**37**
MRT (h)	50.2 ± 3.2	55.2 ± 0.9	50.5 ± 1.4	49.3 ± 0.8	50.6 ± 2.7
T_{max} (h)	24	24	24	24	24
C_{max} (U/mL)	10.1 ± 0.5	11.6 ± 0.4	17.7 ± 0.6	20.6 ± 0.8	17.7 ± 2.7
AUC	754 ± 44	806 ± 25	1,216 ± 35	1,411 ± 20	1,097 ± 82

[a] The circulatory stability of Hu BChE following storage at different time periods was evaluated in mice by measuring the pharmacokinetic parameters of 100 U of enzyme following i.m. administration.

concentration of circulating Hu BChE activity equivalent to the amount of OP administered at a given time period (Saxena et al., 2004). For example, guinea pigs administered with Hu BChE that was sufficient to provide protection against $8 \times LD_{50}$ of GD attained peak blood BChE levels of ~300 U/mL and the enzyme level was reduced to ~100 U/mL following challenge with $5.5 \times LD_{50}$ of GD. This decrease of ~200 U/mL in blood BChE level corresponds to the amount of enzyme required to neutralize ~5–$5.5 \times LD_{50}$ of GD. With VX challenge, less enzyme was administered since the LD_{50} of VX is lower than that for GD. No signs of OP poisoning were observed in animals that received Hu BChE pretreatment and all animals survived the duration of challenge. Animals were subjected to necropsy, 7 or 14 days following nerve agent challenge and all tissues appeared normal on light microscopic examination. In nonhuman primates, cynomolgus monkeys were protected against a cumulative challenge of $5.5 \times LD_{50}$ of GD. Of the six animals challenged, one died after the final challenge dose of GD (a total dose of $5.5 \times LD_{50}$ within 4 h) and one was euthanized 48 h after the final dose of GD. The four surviving animals displayed no signs of poisoning and exhibited no signs of delayed toxicity as revealed by examinations of blood chemistry and hematology parameters for 14 months (Sun et al., 2005). Table 7.2 summarizes the results of efficacy studies with Hu BChE using i.v. or s.c. challenge of OP nerve agents.

TABLE 7.2
Protection by Hu BChE Against Nerve Agent Poisoning

Test Species	**Nerve Agent**	**Protection (LD_{50})[a]**	**Impairment**	**Recovery**	**Reference**
Mouse	GD	2.6	1 of 11	10 of 11	Ashani et al., 1991
Mouse	GA, GB, GD, VX	1.6–4.9	None	Immediate	Raveh et al., 1993
Rat	GD	1.5	None	Immediate	Brandeis et al., 1993
Guinea pig	GD	5.5	None	Immediate	Lenz et al., 2005
Guinea pig	VX	5.0	None	Immediate	Lenz et al., 2005
Rhesus monkey	GD	3.3	4 of 8	15 min to 2 h	Raveh et al., 1997
Rhesus monkey	VX	2.1	2 of 4	20 min to 2 h	Raveh et al., 1997
Cynomolgus monkey	GD	5.5	1 of 5	4 of 6[b]	Lenz et al., 2005

[a] Values represent multiple of median lethal doses (LD_{50}s) of nerve agent survived after Hu BChE administration.
[b] One animal died after the third dose of GD and one was impaired and euthanized 48 h later. The four surviving animals were held for long-term observations.

The pharmacokinetics, onset time, and severity of toxic manifestations following exposure to OPs depend not only on the animal species but also on the route of entry of OP challenge. Because inhalation is the most likely route of exposure to OPs on the battlefield or in public places, the efficacy of Hu BChE should be evaluated against inhalation exposure to OP vapor. The first such study was described by Allon et al. (1998), who reported that exogenously administered Hu BChE was effective in protecting guinea pigs from inhalation toxicity due to nose only exposure to GD. More recently, Saxena et al. (2006) evaluated the efficacy of Hu BChE against a whole-body exposure to GB vapor. The study was conducted in Göttingen minipigs because the pig offers many similarities in anatomy and physiology to humans. The efficacy of two doses of Hu BChE (3 and 7.5 mg/kg) in protecting male Göttingen minipigs from an exposure of 4.1 mg/m^3 of GB vapor for 60 min was evaluated. This dose of GB vapor was lethal to 99% of untreated exposed pigs (LCt_{99}). Hu BChE was administered by i.m. injection, 24 h prior to whole-body exposure to GB vapor. EEG, ECG, and pupil size were monitored throughout exposure, and blood drawn from a surgically implanted jugular catheter before and during exposure was analyzed for AChE and BChE activities and the amount of GB present in plasma. A dose of 3 mg/kg of Hu BChE resulted in a peak blood BChE activity of $14.8° \pm °2.1$ U/mL, whereas 7.5 mg/kg of Hu BChE resulted in a peak blood BChE activity of $41.2° \pm °0.9$ U/mL. All animals exposed to GB vapor alone or pretreated with 3 mg/kg of Hu BChE died following exposure to GB vapor, whereas animals pretreated with 7.5 mg/kg of Hu BChE survived the GB exposure. The amount of GB bound in plasma of minipigs pretreated with 7.5 mg/kg of Hu BChE was 200-fold higher compared to that in plasma from pigs that did not receive Hu BChE, suggesting that Hu BChE was effective in scavenging GB in blood and in preventing it from reaching other tissues. In addition to increasing survivability, pretreatment with Hu BChE was effective in preventing acute cardiac and neurological toxicity due to exposure to GB vapor. As it is most likely that inhalation will be the human route of exposure, more efficacy studies using this route are needed before one can establish the protective dose of enzyme in man.

7. Immunoreactivity

A critical prerequisite for any potential bioscavenger is a prolonged circulatory residence time and the absence of antienzyme antibodies following repeated administrations of enzyme. It was demonstrated that multiple injections of Eq BChE into rabbits, rats, or rhesus monkeys, resulted in a MRT spanning several days, and the induction of antienzyme antibodies (Genovese et al., 1993; Gentry et al., 1993, 1996). On the other hand, administration of purified macaque (Ma) BChE into macaques of the same species resulted in much longer MRT (225 ± 19 h) compared to that reported for heterologous Hu BChE (33.7 ± 2.9 h). After a smaller second injection of Ma BChE, given 4 weeks later, predicted peak plasma levels of enzyme activity were attained, although the four macaques showed wide variation in the MRT (54–357 h). No antibody response was detected in macaques following either injection of enzyme (Rosenberg et al., 2002).

More recently, the consequences of repeated injections of Hu BChE and plasma-derived mouse (Mo) BChE from CD-1 mice were examined in Balb/c (Sun et al., 2003) and CD-1 (Saxena et al., 2004) mice following two i.m. injections 4 weeks apart. The fate of two heterologous (Hu BChE) and homologous (Mo BChE) injections were followed by measuring blood BChE activity and antiBChE IgG levels. In Balb/c mice, the first injection of Mo BChE cleared slowly with an MRT = 91.8 h compared to an MRT = 56.7 h for the heterologous Hu BChE injection. As expected, the second injection of Hu BChE cleared much faster from the circulation of mice compared to the first injection. Surprisingly, the second injection of Mo BChE also cleared faster (MRT = 61.6 h) than the first injection and did not attain the predicted peak enzyme level. No circulating antiHu BChE IgG was detected following the first Hu BChE injection but, significant levels of antibodies to Hu BChE were detected 2 days following the second Hu BChE injection. Similarly, no circulating antiMo BChE IgG was detected following the first Mo BChE injection. However, antibodies to

Mo BChE, although 100-fold less than the levels observed with Hu BChE, were detected 5 days following the second Mo BChE injection. This could be due to differences in BChEs from the two strains of mice, which was subsequently confirmed by repeating the study in CD-1 mice.

In CD-1 mice, the clearance of homologous Mo BChE activity following the first injection also occurred slowly (MRT = 73 h) compared to the heterologous Hu BChE injection (MRT = 48 h). As expected, the second injection of Hu BChE cleared much faster from the circulation of mice compared to the first injection (MRT = 26 h). The second injection of homologous Mo BChE on the other hand, attained a peak enzyme level that was similar to that observed following the first injection and displayed an MRT of 79 h. As expected, circulating antiHu BChE IgG could be detected 5 days following the first Hu BChE injection, which increased dramatically after the second injection. No significant antibody response was detected following either of the two homologous Mo BChE injections. The observation that the second injection of Mo BChE resulted in a pharmacokinetic profile that was similar to that of the first injection is in agreement with the lack of a humoral response to the injected enzyme.

The observed extended stability of exogenously administered Mo BChE into mice and Ma BChE into macaques suggests that even a single injection of homologous BChE is sufficient to maintain the enzyme at a long-lasting therapeutic level. The results of both studies with two injections of BChE clearly demonstrated the utility of homologous BChE as an effective and safe scavenger, exhibiting high stability and low immunogenicity in recipient animals. With respect to the potential use of Hu BChE in humans, these results are consistent with a reported *in vivo* half-life of 8–11 days and the absence of untoward immunological and physiological side effects following blood transfusions and i.v. injections of partially purified Hu BChE into humans (Jenkins et al., 1967; Stovner and Stadskleiv, 1976; Cascio et al., 1988; Ostergaard et al., 1988).

B. Recombinant Human Butyrylcholinesterase

1. Sources

Hu BChE, which is currently under advanced development, represents a first generation biological scavenger. This material is obtained from outdated human plasma (Cohn Fraction IV-4 paste) and the overall availability is related to the quantity of processed human plasma available at any given time. Sufficient amounts of Cohn Fraction IV-4 paste are generated in the United States by blood processing establishments to produce at least 100,000 doses of the product per year. Although this amount of material may be adequate for use by first responders in case of civilian exposure, deliberate or accidental or limited combat engagement, it is not sufficient to protect the entire population or even the entire military. To identify a more reliable source of Hu BChE, recent research efforts are focused on the development of recombinant Hu BChE (rHu BChE), which is compatible with humans. If successful, such efforts will allow a constant supply of material of reproducible purity and activity. There are a variety of potential sources of rHu BChE, which include transgenic plants (Mor et al., 2001), transgenic animals (Cerasoli et al., 2005a), transfected insect larva (Choudary et al., 1995), or algae (Siripornadulsil et al., 2002). In addition, rHu BChE can be expressed in mammalian cell lines. For example, Altamirano and Lockridge (1999) expressed and purified rHu BChE from Chinese Hamster Ovary (CHO) cells, which was a mixture of monomers, dimers, and tetramers.

Subsequently, predominantly tetrameric rHu BChE was obtained from both CHO and human embryonic kidney epithelial (HEK 293A) cells by coexpressing it with the proline rich attachment domain of the collagen tail (Duysen et al., 2002; Chilukuri et al., 2005a). A major limitation of these cell-based expression systems is the low yield of the recombinant product which makes them impractical for producing therapeutic quantities of the bioscavenger. This problem was recently overcome with the availability of Protexia™, which is rHu BChE expressed in the milk of transgenic goats (Cerasoli et al., 2005a).

2. Characterization

The successful use of recombinant enzymes as OP bioscavengers requires that their biochemical properties be similar to those of the native enzyme, they have a long mean residence time in circulation and be devoid of behavioral side effects and immunological responses. The catalytic properties of rHu BChE expressed in CHO or HEK 293A cells were compared with those of Hu BChE. In general, rHu BChEs were shown to possess similar catalytic parameters (K_m and k_{cat}) to those for Hu BChE for substrates such as butyrylthiocholine and benzoylcholine (Duysen et al., 2002; Chilukuri et al., 2005a). Similarly, the inhibition of Protexia by OP nerve agents and carbamates was found to be comparable to that of Hu BChE (Cerasoli et al., 2005a).

Although the catalytic and inhibitory properties of recombinant enzyme were similar to those of Hu BChE, a major difference was observed when it was injected into mice and rats; rHu BChE was cleared from circulation within 2 min, as compared to Hu BChE which circulated with an MRT of 46–56 h (Duysen et al., 2002; Chilukuri et al., 2005a). This difference in the pharmacokinetic parameters of recombinant and native Hu BChE appears to be due to the combined effect of the molecular weight, and charge- and size-based heterogeneity in glycans (Saxena et al., 1998a). Indeed it was shown that rHu BChE expressed in CHO cells and goat milk consists mainly of monomers and dimers (Cerasoli et al., 2005a; Garcia et al., 2005), whereas Hu BChE is predominantly tetrameric. The tetramerization of rHu BChE improved its MRT from 2 min to 16–18 h (Duysen et al., 2002; Chilukuri et al., 2005a). Glycan analysis revealed that the glycans of rChEs including rHu BChE display a remarkable heterogeneity in size and consist of hybrid and complex bi-antennary, tri-antennary, and tetra-antennary structures. On the other hand, plasma ChEs, including Hu BChE, contain mature glycans that are predominantly of the complex bi-antennary type, confirming that these structures are responsible for the extended MRTs of the enzymes (Saxena et al., 1998a; Garcia et al., 2004). In addition, Protexia has a different glycosylation pattern than that of Hu BChE and contains a carbohydrate moiety which has been demonstrated to be immunogenic in humans (Garcia et al., 2005). These problems with rHu BChE prompted investigations to reduce its immunogenicity and improve its circulatory stability through posttranslational modifications.

3. Efficacy

The short MRT of rHu BChE in animals has precluded the testing of its protective properties against OP agents. Using tetrameric rHu BChE in mice, which has an MRT of 16 h, Duysen et al. (2002) demonstrated that it conferred 50% protection from cocaine toxicity. Because rHu BChE protects against cocaine toxicity by hydrolyzing cocaine, the improvement in its MRT from 2 min to 16 h was sufficient to protect mice. However, the circulatory stability and bioavailability of tetrameric rHu BChE were still not comparable to that for Hu BChE (Table 7.3), which

TABLE 7.3
Pharmacokinetic Parameters of Native and Recombinant Hu BChE in Mice

Parameters	rHu BChE	PEG-rHu BChE	Hu BChE
MRT (h)	18.3 ± 1.7	36.2 ± 2.0	45.7 ± 1.7
C_{max} (U/mL)	1.2 ± 0.2	29.2 ± 3.6	30.3 ± 1.3
AUC (U)	23.1 ± 4.7	1,258 ± 300	1,768 ± 39

[a] The circulatory stabilities of rHu BChE, PEG-rHu BChE, and Hu BChE were determined in mice by measuring the pharmacokinetic parameters of 100 U of enzyme following i.p. administration.

indicated that large quantities of stoichiometric rHu BChE would be needed to demonstrate its efficacy against OP nerve agents. To improve their biological residence time, rHu BChEs produced in goat milk and 293A cells were modified to include polyethylene glycol adducts (PEGylation). PEGylation of therapeutic proteins, namely colony stimulating factor, interleukin-2, tumor necrosis factor, and interleukin-6, was shown to alter their molecular properties and improve their stability, biological half-life, water solubility, and immunologic characteristics (Charles et al., 2000). Indeed it was shown that PEG modification of tetrameric and monomeric rHu BChE (Chilukuri et al., 2005a, 2005b) did not alter their enzymatic properties but greatly enhanced their circulatory residence time and bioavailability in animals (Table 7.3). Similarly, it was reported that PEGylated Protexia had a pharmacokinetic profile very similar to that of the Hu BChE (Cerasoli et al., 2005b), suggesting that differences in the pharmacokinetics between plasma-derived and recombinant enzymes can be addressed using *in vitro* posttranslational modifications. Furthermore, efficacy studies with PEGylated Protexia in guinea pigs yielded results that appear to be similar to those described previously with Hu BChE, that is, complete protection against $5.5 \times LD_{50}$ of GD or VX. These preliminary results suggest that an effective recombinant stoichiometric bioscavenger that is comparable to the native bioscavenger can be developed.

4. Improving Efficacy by Mutagenesis

Studies have demonstrated that it is indeed possible to influence the bioscavenging performance of rHu BChE by site-directed mutagenesis. This technique was used to obtain mutant rHu BChEs which possessed an increased affinity for OPs or were more easily reactivated by oximes or possessed a reduced rate of aging. For example, a mutant rHu BChE in which Asp at position 70 located at the peripheral anionic site was mutated to Gly, demonstrated a threefold and eightfold lower rate of aging for paraoxon- and DFP-inhibited mutant BChE, respectively, compared to wild-type enzyme (Masson et al., 1997). Similarly, the mutation of Glu197 to Asp or Gln and Trp82 to Ala resulted in reductions in the rate constants for inactivation with three stereoisomers of GD (Saxena et al., 1998b). The GD-inhibited Glu197Gln and Trp82Ala rHu BChEs also had slower rates of reactivation by 2-PAM as well as lower rates of aging compared to wild-type enzyme.

Further attempts were made to design mutations in rHu BChE that would impart OP-hydrolyzing activity to this enzyme. The rationale for the design of these mutations was based on the fact that OP inhibitors are in reality hemisubstrates for these enzymes. Their acylation reaction with enzyme is similar to that of normal substrates, but the subsequent reaction, equivalent to deacylation of the active-site serine, cannot be affected because the amino acid group responsible for dephosphylation is not in the appropriate position (Jarv, 1984; Millard et al., 1995). The perceived solution to this problem was to insert a second catalytic center into the active site specifically to carry out the dephosphylation step of the reaction (Millard et al., 1995). Applying this rationale, a mutant rHu BChE was created in which Gly at position 117 was mutated to His (Lockridge et al., 1997). This mutant enzyme not only hydrolyzed butyrylthiocholine, benzoylcholine, and *O*-nitrophenyl butyrate but also acquired the ability to hydrolyze OPs including echothiophate, paraoxon, GB, and VX. Wild-type rHu BChE was irreversibly inactivated by echothiophate and paraoxon whereas the Gly117His rHu BChE regained 100% activity within 2–3 min following reaction with these OPs. Recently, it was shown that transgenic mice expressing Gly117His rHu BChE were protected from toxicity due to echothiophate whereas wild-type mice showed severe cholinergic signs of toxicity and died following exposure to the same amount of OP (Wang, 2004). To affect the hydrolysis of rapidly aging nerve agents such as GD it was necessary to inhibit the aging reaction by replacing the carboxyl group (Glu197) adjacent to the active site Ser with an amide group (Millard et al., 1998). Unfortunately, these mutants have catalytic activities that are too slow for practical use, and thus the search for a catalytic bioscavenger continues.

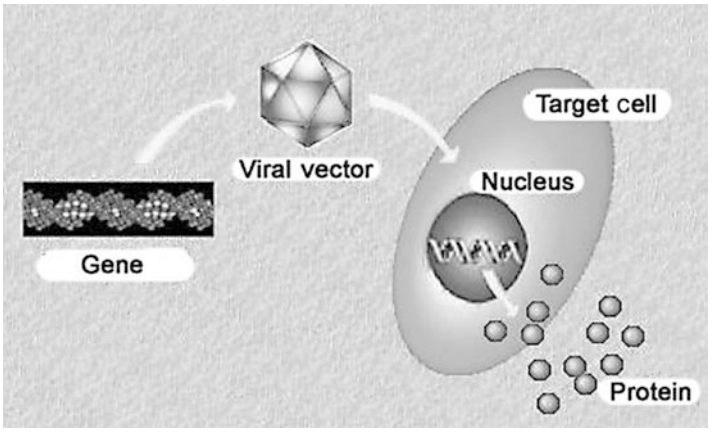

FIGURE 7.3 Concept of gene therapy.

5. Gene Therapy

An alternate approach to introducing Hu BChE in circulation is via gene therapy. Gene therapy involves the introduction of genetic material such as the gene for Hu BChE into cells for therapeutic purposes. Today, the hope is that gene therapy will be the ultimate method of protein delivery in which the delivered gene enters the body's cells and turns them into small factories to produce the therapeutic protein for prolonged periods (Figure 7.3). Technologies for gene introduction have advanced significantly over the past 30 years since the introduction of DEAE-dextran and calcium phosphate coprecipitation methods. Gene-delivery techniques can be categorized into viral and nonviral methods. Viral methods use genetically engineered retroviruses, adenoviruses, adeno-associated viruses (AAV), and other viruses that have been optimized for facilitated gene transfer procedures. Nonviral methods make use of cationic lipids, polymers, targeting proteins, as well as mechanical methods such as electroporation. Both methods have advantages and disadvantages, strengths and weaknesses, making them rapidly advancing fields of biotechnology with great promise for treating inherited and acquired diseases.

So far, only viral methods to introduce genes for stoichiometric and catalytic bioscavengers have been attempted. Recombinant adenoviruses containing genes for Hu PON1 when administered into mice 4 days prior to pesticide challenge, boosted PON1 levels in blood and significantly prevented inactivation of brain AChE (Cowan et al., 2001). Recently, Li et al. (2006) showed that introduction of AAV containing Hu AChE gene into AChE knockout mice, resulted in the expression of AChE in liver, lung, and heart for several months and protected mice from DFP toxicity. The gene therapy approach was also tested using a double mutant Ala328Trp/Tyr332Ala rHu BChE with enhanced cocaine hydrolase activity for protection against cocaine toxicity in rats. Adenoviral vectors containing this mutant enzyme when administered into rats shortened the half-life of cocaine and blunted cardiovascular effects (Gao et al., 2004). These preliminary studies suggest that gene therapy can be a method of choice for introducing catalytic and stoichiometric bioscavengers *in vivo*. However, this method cannot be applied in its present form because of the toxicity and immunogenicity associated with it, as reported in recent clinical trials against cancer and enzyme deficiency diseases.

C. Catalytic Bioscavengers

1. Enhancing Activity of Stoichiometric Bioscavengers

The stoichiometric scavengers are extremely efficient in providing protection against all OP nerve agents. However, due to their high molecular weight, relatively large amounts of enzymes are

required to neutralize smaller amounts of nerve agents. Caranto et al. (1994) demonstrated that the catalytic activity of OP-inhibited AChE could be rapidly and continuously restored in the presence of an appropriate oxime. The improved detoxification of OP compounds by AChE in combination with an oxime was also demonstrated *in vivo*. For example, the therapeutic addition of HI-6 following pretreatment of mice with FBS (fetal bovine serum) AChE endowed pseudocatalytic properties to this stoichiometric scavenger, such that the ratio of GB neutralized per FBS AChE molecule increased from 1:1 to ~65:1. Unfortunately, recent studies indicate that none of the existing oximes were efficient in reactivating OP-inhibited Hu BChE (Saxena et al., 2000; Luo et al., 2004). Therefore, efforts were expanded to identify a catalytic enzyme capable of hydrolyzing OP nerve agents. The advantages of such a molecule would be twofold: (1) smaller quantities of the same high molecular weight catalytic bioscavenger would potentially produce the same or greater extent of protection and (2) because it would not be inactivated in the process of detoxifying the nerve agent, it would be available to provide protection against prolonged exposures to OPs.

2. Sources of OP Hydrolases

Enzymes from a variety of sources were examined for their ability to degrade OPs: (1) bacterial OPAH or phosphotriesterase from *Pseudomonas diminuta* or *Flavobacterium* sp. exhibited hydrolytic activity toward various nerve agents (Dumas et al., 1990), (2) organophosphorus acid anhydrolase or OPAA from *Alteromonas haloplanktis* was shown to hydrolyze GD, DFP, and GF (DeFrank et al., 1993), (3) squid DFPase from *Loligo vulgaris* hydrolyzed DFP and other OPs (Scharff et al., 2001), and (4) PON1 from human and other mammalian plasma (Gan et al., 1991; Masson et al., 1998; Tuovinen et al., 1999; Josse et al., 1999, 2001). Some of these enzymes were also shown to function as catalytic bioscavengers *in vivo*. For example, recombinant OPAH from *P. diminuta* was shown to protect mice against behavioral side effects and lethality due to GD (Broomfield et al., 1993). Similarly, pretreatment alone with OPAH purified from *Pseudomonas* sp. was shown to protect mice from lethality due to paraoxon, diethylfluorophosphate, and GA (Ashani et al., 1991b; Raveh et al., 1992). Most of these enzymes possess short circulation times *in vivo* and they have neither the ability to hydrolyze all known toxic OPs nor the high turnover required to dispose off the OPs from blood in one circulation time. In addition, these bacterial enzymes are likely to initiate potent immune responses in humans; therefore, they are not suitable for repeated use in humans.

Hu PON1, on the other hand, has the potential for providing protection without the complication of inducing an immune response. PON 1 is a member of a multienzyme family (PON1, PON2, and PON3) that possess 60%–70% DNA identity. They are calcium-dependent enzymes that hydrolyze a broad variety of esters and lactones (Billecke et al., 2000; Jakubowski, 2000; Aharoni et al., 2004). PONs are also able to hydrolyze various OPs, including several nerve agents (Davis et al., 1996), although at much lower rates. PON1 is involved in drug metabolism and is used in drug inactivation. PON1 and PON3 appear to be associated with HDL. *In vitro* assays show that PON1 inhibits the oxidation of lipids in low-density lipoprotein, thereby reducing the level of oxidized lipids involved in the initiation and progression of atherosclerosis (Aviram et al., 1998; Biggadike et al., 2000; Lusis, 2000; Ahmed et al., 2001). The level of PON1 in blood and its catalytic efficiency appear to have a major impact on the individual's susceptibility to pollutants, OPs, and atherosclerosis (Lusis, 2000). However, this enzyme does not possess the desired catalytic activity at a rate that is fast enough for use as a catalytic bioscavenger. Since the agent must be cleared from the bloodstream within one circulation time (Broomfield et al., 1991), a functional catalytic scavenger must have both a lower K_m and a high k_{cat}. Therefore, Hu PON1 is currently being subjected to mutation in efforts to generate faster catalytic anti-nerve agent enzymes. Since OPs are ''accidental'' substrates for PON (Masson et al., 1998), it is likely that improvement in activity can be realized through protein engineering.

3. Evolving Mammalian Paraoxonases

The two major difficulties in designing appropriate site-directed mutations in Hu PON1 were the lack of knowledge on the residues at the active site and its three-dimensional structure. Based both on site-directed mutations of amino acids believed to be at or near the active site of Hu PON1 and on limited sequence homology with squid DFPase, Josse et al. (1999, 2001) proposed that the enzyme had the shape of a sixfold beta propeller. Subsequently, Yeung et al. (2004, 2005) used this homology-built model to conduct site-directed mutagenesis studies to identify and "map" amino acid residues critical for binding and hydrolysis of substrate. The x-ray crystal structure of a mouse–rat–rabbit–human chimera of PON1 obtained through gene shuffling experiments and expressed in *Escherichia coli* (Aharoni et al., 2004) was obtained and shown to be a sixfold beta propeller (Harel et al., 2004). The successful expression of a functionally active PON1 in a bacterial expression system paved the way for designing a recombinant version of a naturally occurring mammalian enzyme that is capable of being developed as a catalytic biological scavenger to protect against nerve agent poisoning.

More recently, Amitai et al. (2006) assessed the ability of various OP hydrolases obtained by directed evolution and expressed in *E. coli* to catalytically scavenge toxic OP nerve agents. Nine directly evolved PON1 variants were tested for detoxification of GF, GD, and other toxic OPs. GF detoxification was 10-fold faster with PON1 mutants Val346Ala and Leu69Val compared to wild-type enzyme. Val346Ala mutant also exhibited four-, seven, and ninefold faster rates of hydrolysis for DFP, cloropyrophos-oxon, and GD, respectively. This study identified three key positions in PON1 structure that affect OP hydrolysis, Leu69, Val346, and His115 and several amino acid replacements that significantly enhance the hydrolysis of toxic OPs. It also indicated that PON1 was less active toward the more toxic optical isomers of GF, GB, and *O*-isopropyl-*O*-(*p*-nitrophenyl) methyl phosphonate. Therefore, variants of PON1 evolved in *E. coli*, although much improved over the wild-type enzyme, do not possess the desired catalytic activity at a rate that is fast enough for use as nerve agent antidotes. Also, the effect of multiple catalytic activities against other substrates (e.g., lactones, fatty acids) on the health of the individual receiving this enzyme will have to be properly evaluated. In addition, the enzyme derived from *E. coli* will certainly produce an immune reaction in humans and will have poor retention time. Therefore, research efforts should continue toward creating such an enzyme by specific mutation of Hu PON1 and searching for other appropriate catalytic enzymes from a human source.

D. Extrapolation of Animal Data to Humans

The exogenous administration of FBS AChE (Ashani et al., 1991a; Maxwell et al., 1992), Eq BChE (Broomfield et al., 1991), and Hu BChE (Ashani et al., 1991a; Raveh et al., 1993, 1997; Saxena et al., 2006) were successfully used as a prophylactic measure in mice, rats, guinea pigs, minipigs, and nonhuman promates to protect against several OPs. The animals were pretreated with one of these enzymes delivered by either i.v. or i.m. injection and challenged with multiple doses of OPs. The animals not only survived the multiple lethal doses of OP exposure but did not show any postexposure toxicity usually observed after multiple drug treatment. The examination of enzyme activity data from these investigations demonstrated that in all cases the decrease in molar concentration of circulating enzyme was proportional to the molar concentration of the given OP challenge. This was true until most of the enzyme (>75%–90%) was inhibited by the administered OP, regardless of the routes of administration of the enzyme or OP. Thus, the protection conferred after sequential exposure to OP could be predicted in all species of animals.

The efficacy of ChEs as antidote is dependent on their rate of OP detoxification and their availability in blood circulation. Protection with ChEs is therefore independent of the test species as long as sequestration takes place in circulation before the OP reaches the physiological target sites (Ashani et al., 1998). The *ex vivo* titration of AChE and BChE with various OPs in the fresh human and animal blood provided the amounts of these enzymes available for sequestration of OPs. In addition, Ashani and Pistinner (2004) spiked human blood with Hu BChE and titrated it with

GB, GD, and VX to determine the *in vitro* mass balance of these OPs. They developed a mathematically based toxicokinetic model for the estimation of the upper limit of Hu BChE dose required for protection against OP toxicity. The model addressed the relationship between the Hu BChE dose needed to maintain 30% of residual red blood cell (RBC) AChE activity and other parameters (level and duration of OP exposure, bimolecular rate constants of inhibition of Hu AChE and Hu BChE by OPs, and time elapsed from enzyme administration). They validated the Hu BChE dose by *in vitro* experiments and data from published human studies (Ashani et al., 1998; Ashani and Pistinner, 2004). The proposed model suggested that the upper limit doses of 134, 115, and 249 mg/kg of Hu BChE were sufficient to protect RBC AChE above 30% following a challenge with $1 \times LD_{50}$ of VX, GD, and GB, respectively.

Allon et al. (1998) observed that in guinea pigs pretreated with Hu BChE and exposed to GD vapor, only one-fourth of the enzyme was used for the sequestration of $2 \times LD_{50}$ of GD. Recently, Saxena et al. (2006) observed a similar sequestration of GB vapor in the Göttingen minipig. These results indicate that ~200 mg of Hu BChE may be sufficient to protect humans against an exposure of $2 \times LD_{50}$ or more of GD.

III. OXIME-BASED THERAPY

A. *In Vitro* Evaluation of Oxime Reactivation

As mentioned earlier, conventional oxime-based therapy for nerve agent poisoning consists of a muscarinic antagonist such as atropine, an anticonvulsant such as diazepam, and an oxime reactivator such as 2-PAM. The effectiveness of this antidotal regimen is highly dependent on the oxime, which is responsible for the reactivation of OP-inhibited AChE. The reactivation of AChE activity restores the normal function of the cholinergic system and reverses the chain reaction that follows nerve agent poisoning. The *in vivo* screenings of oximes are conducted in animal models and results from are extrapolated to humans. Since the *in vivo* efficacy of an oxime depends on its pharmacokinetic and pharmacological properties, the potency of a nerve agent in inhibiting different AChEs, and the presence of endogenous bioscavengers, the results of *in vivo* screening are highly dependent on the animal model used. To allow a reasonable animal to human extrapolation of experimental results, *in vitro* comparisons of reactivation of nerve agent inhibited animal and human AChEs by oxime are conducted. The advantages of this method are several-fold. First, kinetic measurements of oxime reactivation yield multiple parameters, such as oxime affinity and oxime reactivity, that can be used to design better oximes. Second, the interaction of an oxime with the pharmacological target (i.e., AChE) is not influenced by its pharmacokinetics and can be more precisely analyzed. Third, AChE reactivation is a more sensitive measure of oxime effectiveness than *in vivo* survival because the background signal (i.e., AChE activity) for reactivation is much lower than the background signal (i.e., agent LD_{50}) for *in vivo* efficacy in animals. Although this simplified *in vitro* evaluation does not consider other factors that affect *in vivo* antidotal efficacy, several investigations have demonstrated that there is a reasonable correlation between *in vitro* and *in vivo* data, and the efficacy of an oxime primarily depends on its ability to reactivate nerve agent inhibited AChE (Oldiges and Schoene, 1970; Schoene and Oldiges, 1973; Schoene, 1980). If the *in vitro* oxime reactivation of nerve agent inhibited animal AChE is similar to that of human AChE, it is likely that the results of *in vivo* animal study will reliably extrapolate to humans.

Due to the high degree of homology between the amino acid sequences of different AChEs and the similarity in their active sites, the enzymes generally show similarity in their interaction with substrates and inhibitors. However, the reactivation of nerve agent inhibited AChE by oxime is more sensitive to subtle changes in the conformation of the enzyme. In the early 1980s, it was shown that although the reactivation of nerve agent inhibited human erythrocyte AChE by different oximes was similar to rat and bovine AChEs, the reactivation of GD-inhibited electric eel AChE by HI-6 was markedly slower than that of Hu AChE (de Jong and Wolring, 1984). Therefore, the

TABLE 7.4
Second-Order Reactivation Rate Constants (mM^{-1} min^{-1}) of Nerve Agent-Inhibited Human and Guinea Pig Erythrocyte AChEs by Different Oximes

	Hu AChE			Guinea Pig AChE		
Oxime	**GB**	**VX**	**GF**	**GB**	**VX**	**GF**
Obidoxime	29.98	32.63	0.42	14.18	12.51	0.06
2-PAM	9.06	7.67	0.06	2.70	3.34	0.03
HI-6	13.5	20.98	27.55	0.33	0.14	0.19
HLo-7	35.08	63.16	92.75	3.91	3.63	0.63

Source: Data from Worek, F. et al., *Biochem. Pharmacol.*, 68, 2237, 2004.

authors concluded that electric eel AChE was not a suitable model for conducting *in vitro* oxime evaluations. However, studies using different mammalian AChEs or AChEs from various tissues of the same species have shown similar behavior in oxime reactivation of nerve agent inhibited AChE. For example, AChEs purified from human brain and muscle did not show significant differences in the reactivation of nerve agent inhibited enzyme by HI-6, obidoxime, and 2-PAM (Puu et al., 1986). Similarly, de Jong and Wolring (1985) reported that GD-inhibited AChEs from three different mouse tissues displayed very similar reactivation and aging properties.

However, two recent studies revealed significant species-related differences between the reactivation of nerve agent inhibited guinea pig and Hu AChEs. Using erythrocyte ghosts, Worek et al. (2002) showed that the reactivation rates of GB-, VX-, and GF-inhibited Hu AChE were always faster than that of guinea pig AChE, and in some cases the rate constants were well over 100-fold for Hu AChE compared with those for guinea pig AChE (Table 7.4). More recently, studies with purified recombinant Hu AChE and guinea pig erythrocyte AChE demonstrated that reactivation rates of human enzyme inhibited by nerve agents containing bulky side chains such as GD, GF, and VR by HI-6 and HLo-7, were 100- to 400-fold faster than those for guinea pig enzyme. This species-related difference was significantly reduced when monopyridinium oxime 2-PAM was used as a reactivator (Table 7.5). The results of these two studies suggest that

TABLE 7.5
Second-Order Reactivation Rate Constants (M^{-1} min^{-1}) of Nerve Agent-Inhibited AChEs by Oximes

AChE	**Oxime**	**GD**	**GF**	**VR**
Guinea pig[a]	2-PAM	17	7	17
	HI-6	51	133	81
	HLo-7	139	195	243
Rhesus monkey	2-PAM	55	26	130
	HI-6	19,610	26,843	64,406
	HLo-7	ND[b]	23,767	98,814
Human[a]	2-PAM	51	28	252
	HI-6	21,506	29,700	19,580
	HLo-7	26,180	85,060	43,800

Source: Data for guinea pig and human AChEs from Luo et al. (2006a).

[a] k_R values were determined at 25°C in 50 mM sodium phosphate buffer, pH 8.0.
[b] ND Not determined

guinea pig is not a suitable model for the *in vivo* evaluation of oximes. However, because the oxime-induced reactivation of nerve agent inhibited Hu AChE is faster than guinea pig AChE, if an oxime is effective in guinea pigs, it is likely to be effective in humans.

Another valuable animal model that is used for evaluating nerve agent toxicity and oxime efficacy are nonhuman primates such as monkeys. However, no direct comparison was made on the oxime reactivation of nerve agent inhibited monkey and Hu AChEs. Reactivation studies using rhesus monkey erythrocyte ghost AChE showed much smaller species-related differences between the oxime reactivation of nerve agent inhibited monkey and Hu AChEs than those found between guinea pig and Hu AChEs. The second-order rate constants for the reactivation of monkey AChEs by all three oximes were within four-fold of that of Hu AChE (Table 7.5). These results suggest that monkeys are a suitable animal model for evaluating the efficacy of oximes against nerve agent poisoning.

B. *In Vivo* Evaluation of Oximes

Many of the oximes that are currently being evaluated as candidates for clinical use against nerve agents were originally identified by *in vivo* screening in rodents. For example, Hagedorn et al. (1978) performed *in vivo* screening of hundreds of oximes against nerve agents to identify compounds, such as HI-6 and HLo7, that were worthy of advanced research and development. However, in recent years *in vivo* screening has not identified oximes that are further improvements on these oximes (Aas, 2003).

There are several issues that probably contribute to the declining effectiveness of *in vivo* screening as a method to identify improved oxime candidates. First, animals such as mice are rather insensitive to small improvements in oxime efficacy because they have high baseline LD_{50} values for nerve agents against which small increases in agent LD_{50} values resulting from oxime therapy are difficult to demonstrate. High LD_{50} values for nerve agents in mice are due to high levels of CaE in blood that produces LD_{50} values that are 10-fold higher than those in nonhuman primates, which do not contain this endogenous scavenger in their blood (Maxwell and Brecht, 1991). Second, the *in vivo* efficacy of an oxime is a result of the combined effect of itsz pharmacological effect and pharmacokinetics. An oxime that produced an improvement in pharmacological effect but had poor pharmacokinetics would probably not be identified by *in vivo* screening. Third, the only pharmacological effect that is evaluated by *in vivo* screening is an increase in survival whether it is due to increased affinity for a pharmacological target or increased reactivity with that target. As a result of these issues, the initial screening of oximes to identify improvements has been shifting to *in vitro* screening of the ability of oximes to reactivate nerve agent inhibited AChE (Kuca et al., 2005).

Although the aforementioned problems with *in vivo* screening methodology have in recent years slowed the identification of new oximes with improved efficacy against nerve agents, other advances have improved the potential for licensure of improved oximes once they are identified. Since nerve agents cannot be administered to humans due to ethical prohibitions, drugs used to treat nerve agent poisoning are approved by the U.S. Food and Drug Administration (FDA) under a regulatory mechanism known as the ''animal rule.'' Two critical requirements of the FDA animal rule are the demonstration of drug efficacy in multiple animal species and the identification of the mechanism of action of the proposed drug to allow extrapolation of drug efficacy from animals to humans.

A recent advance in the identification of the mechanism of action of oximes has been the demonstration of a high correlation between *in vitro* oxime reactivation of nerve agent inhibited AChE and *in vivo* efficacy (Maxwell et al., 2006). The *in vivo* efficacy of 2-PAM and obidoxime against several nerve agents in guinea pigs was expressed as protective ratios (i.e., agent LD_{50} in oxime-treated group/agent LD_{50} in control group). *In vitro* oxime reactivation of AChE inhibited by these nerve agents was expressed as reactivation rate constants. Because variation in atropine dose can have a significant effect on *in vivo* oxime efficacy, the dose of atropine used in these animals was

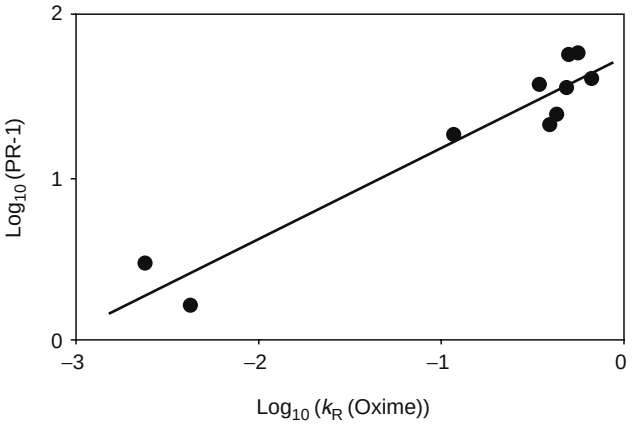

FIGURE 7.4 Regression analysis of in vitro oxime reactivation and in vivo oxime efficacy in guinea pigs. (Data from Maxwell, D.M. et al., *Arch. Toxicol.*, 80, 756, 2006.)

held constant but the dose of oxime was varied. The variation in the dose of oxime was normalized by using the product of the oxime reactivation rate constant (k_R) and the dose of oxime administered to guinea pigs. A regression analysis of the relationship between the *in vivo* protective ratio (PR) of oximes at different doses and their *in vitro* (k_R) is shown in Figure 7.4. Efficacy of oximes against VX, GB, and GF was expressed as PR-1 because a PR of 1 denotes an absence of oxime protection, and oxime reactivation was expressed as k_R(Oxime). Regression analysis showed that PR-1 was proportional to (k_R(Oxime))0.56 with $r^2 = 0.91$. This evaluation demonstrated that *in vitro* oxime reactivation explained 91% of the 23-fold variation in the *in vivo* efficacy of oximes against nerve agents, which provides a strong argument to regulatory agencies that the mechanism of oxime efficacy is understood well enough to satisfy the requirements of the animal rule.

Another recent advance that has strengthened the ability of developers to gain approval of oximes by regulatory agencies is the development of a kinetic method to estimate *in vivo* efficacy of oximes, providing that the necessary *in vitro* oxime reactivation kinetic parameters have been determined (Worek et al., 2005). This method is able, for example, to use the kinetic parameters for *in vitro* oxime reactivation of Hu AChE to calculate the concentration of oxime that is necessary to produce 20% reactivation of inhibited AChE in 5 min in humans. It also allows the theoretical comparison of repetitive versus single injections of oximes to design an optimal drug regimen for nerve agent casualties. This kinetic method was validated with data from pesticide-poisoned patients and simulations were also performed for intravenous and percutaneous nerve agent exposure and intramuscular oxime treatment using published data. This method of estimating the concentration of oxime necessary to produce *in vivo* efficacy in humans is a significant advance over the previous method of estimating human doses of oximes from doses determined in animal experiments (Sundwall, 1961; Sidell, 1997) because the kinetic parameters for reactivation of animal and Hu AChEs have been found to differ significantly (Worek et al., 2002).

C. Design and Development of the Next Generation of Oximes

Since the 1970s, the U.S. Army has fielded the monopyridinium oxime 2-PAM as an antidote for treating nerve agent poisoning. However, the efficacy of 2-PAM is very limited against poisoning by some nerve agents such as GD and GF (Boskovic et al., 1984; de Jong and Kosen, 1985; Lundy et al., 1992; Luo and Liang, 1997; Worek et al., 1998). For this reason, numerous studies in the last several decades have focused on developing improved oximes. The ideal next generation oxime should be able to effectively reactivate AChE inhibited by nerve agents of diverse structures. The

design and development of a broad-spectrum oxime, however, requires a better understanding of the mechanism of oxime-induced reactivation of AChE.

The first study with 2-PAM in the 1950s showed that the interaction of the quaternary pyridinium structure with the anionic site at the active site of AChE was responsible for its increased reactivation potency compared with hydroxylamine. More potent bis-pyridinium oximes such as TMB4 and obidoxime were studied and obidoxime was fielded by most European countries (Moore et al., 1995). Obidoxime is somewhat better than 2-PAM in treating poisoning by these nerve agents, but this 4-position oxime forms a stable phosphoryl oxime that inhibits reactivated AChE, which may limit its use in the treatment of nerve agent and OP pesticide poisoning (Luo et al., 1999; Herkenhoff et al., 2004; Kiderlen et al., 2005).

In the 1970s and 1980s, new promising Hagerdorn oximes HI-6 and HLo-7 were synthesized and shown to be the most potent reactivators of GD-inhibited AChE (Hagedorn et al., 1978; de Jong et al., 1989). Despite the rapid aging (dealkylation) of GD-inhibited AChE (de Jong and Wolring, 1984; Talbot et al., 1988; Shafferman et al., 1996; Worek et al., 2004), HI-6 was very effective in protecting GD-poisoned monkeys (Lipp and Dola, 1980; Clement and Erhardt, 1994). In addition, these two oximes are potent reactivators of AChE inhibited by other nerve agents that contain bulky side chains, such as GF and VR. Other bis-pyridinium oximes including obidoxime are significantly less potent reactivators of GF- and VR-inhibited AChE (de Jong et al., 1989; Worek et al., 2002; Luo et al., 2006a). However, a significant limitation for the use these two H-series oximes on the battlefield is their poor stability in aqueous solution (Eyer et al., 1986, 1989). To overcome this problem, countries, like Canada, have fielded an auto-injector containing the powder form of HI-6 (Moore et al., 1995). Another drawback of HI-6 as a nerve agent antidote is its lack of reactivity for GA-inhibited AChE (de Jong et al., 1989). HLo-7 is superior to HI-6 in reactivating GA-inhibited AChE, but the potency is much less than that for AChE inhibited by other nerve agents such as GB, GD, GF, VX, and VR (Eyer et al., 1992; Luo et al., 2006a).

Although it has been 30 years since the synthesis of the first H-series oxime HI-6, the mechanism for its potency in reactivating GD-inhibited AChE is still unknown. Also, GA-inhibited AChE is resistant to reactivation by HI-6, but can be reactivated by 4-position oximes including obidoxime, TMB4, HLo-7, and MMB4. Recent site-directed mutagenesis studies showed that the superior reactivation by bis-pyridinium oximes is due to the interaction of the second pyridinium ring with the peripheral anionic site of AChE which facilitates attack by the oxime group (Ashani et al., 1995; Luo et al., 2006a). Mutagenesis studies further indicated that the high reactivation potencies of HI-6 and HLo-7 depend not only on the 4-carboxylamide pyridinium structure, but also on the ether oxygen of the linker.

To avoid problems due to the poor stability of HI-6 and HLo-7, the U.S. Army has down selected another oxime candidate, MMB4, to replace the current oxime 2-PAM in the antidotal regimen. MMB4 is a bis-pyridinium bis-oxime with the oxime group at 4-position and a single methylene group linking the two pyridinium rings. MMB4 was shown to be efficacious in protecting animals against GD poisoning (Koplovitz and Stewart, 1992). Its ability to reactivate AChE inhibited by different nerve agents including GB, GD, GF, VX, VR, and GA was demonstrated both *in vitro* and *in vivo* (Shih et al., 2004; Luo et al., 2006a). However, with the exception of guinea pig AChE, the reactivation potency of MMB4 for nerve agent inhibited animal and Hu AChEs is significantly lower than that for HI-6 and HLo-7 (Luo et al., 2006a, 2006b).

Recently, scientists in the Czech Republic have been actively involved in the development of new oximes as nerve agent antidotes (Kuca et al., 2006). Their most notable effort has been the synthesis of several new oxime candidates for the reactivation of GA-inhibited AChE including K027, K048, K074, and K075. These compounds are comparable or superior to TMB4, in either reactivating GA-inhibited AChE *in vitro* or protecting animals from GA poisoning (Cabal et al., 2005; Kuca et al., 2005; Cali et al., 2006). They are bis-pyridinium 4-oximes, similar to TMB4, the best previously known reactivator for GA-inhibited AChE. However, these promising oximes are significantly less effective than HI-6 in reactivating AChE inhibited by other nerve agents such as

GB, GD, or GF (Kuca et al., 2004, 2006; Kuca et al., 2005). On the other hand, HI-6, as well as its analog ICD-585, failed to reactivate GA-inhibited AChE (Kuca et al., 2004; Luo et al., 2006b), demonstrating the uniqueness of AChE–GA conjugate. This was confirmed by the recent x-ray crystallographic study of GA-inhibited mouse AChE conjugate, which revealed a unique repositioning of two amino acids (Phe338 and His447) in the active center of the enzyme conjugate (Ekstrom et al., 2006a).

x-Ray crystallography was also used to study the interaction of oximes with AChE. The x-ray crystal structures of Mo AChE with different oximes revealed differences in the binding of oximes both at the active and the peripheral anionic site of the enzyme, which may be responsible for the observed differences in the ability of these oximes to reactivate GA–AChE conjugates (Ekstrom et al., 2006b). Further studies on the interactions between different oximes and the amino acid residues at the active and peripheral anionic site of AChE using site-directed mutagenesis and x-ray crystallographic techniques should be continued to uncover important information on the reactivation of nerve agent inhibited AChE by oximes and aid the design and development of the next generation of oximes.

IV. CENTRALLY ACTING PRETREATMENT DRUGS

Another approach used to protect against OP toxicity is to pretreat with low, nonlethal doses of reversible AChE inhibitors. These agents act by transiently binding to a fraction of AChE and protecting it from inhibition by OP nerve agents. Indeed it was shown that pretreatment with pyridostigmine, a quaternary carbamate inhibitor of both AChE and BChE in conjunction with post-exposure therapy with oxime and atropine, increased survivability of guinea pigs (Leadbeater et al., 1985) as well as nonhuman primates (Dirnhuber et al., 1979) from multiple LD_{50}s of GD. Therefore, pyridostigmine was given to many soldiers during the Persian Gulf War (Keller, 2006) and subsequently approved by the U.S. FDA for use by military personnel who are under threat of exposure to GD. However, because pydridostigmine does not cross the blood–brain barrier, it does not prevent CNS impairment. Therefore, the use of reversible inhibitors of AChE that are capable of crossing the blood–brain barrier and potentially protecting against the CNS effects of OP nerve agents were considered (Leadbeater et al., 1985).

Pretreatment with the tertiary carbamate physostigmine was shown to protect mice and guinea pigs from the incapacitating effect of OP exposure (Deshpande et al., 1986; Lim et al., 1988, 1991; Anderson et al., 1991; Harris et al., 1991; Miller et al., 1993; Meshulam et al., 1995; Lallement et al., 2001a). Because physostigmine readily enters the brain, it was shown to afford protection to both peripheral and central AChEs. However, AChE inhibition in the CNS by physostigmine raised concerns of unwanted centrally mediated side effects. These side effects could be partly compensated through the combinatory use of physostigmine with trihexyphenidyl (Artane) or scopolamine, a centrally acting antimuscarinic drug, to counteract the accumulation of brain ACh due to AChE inhibition in the CNS (Lim et al., 1988; Meshulam et al., 1995; Philippens et al., 2000). Thus, pretreatment with physostigmine and scopolamine or physostigmine and trihexyphenidyl not only provided complete protection to nonhuman primates from lethality of GD, but also prevented convulsions and loss of consciousness (von Bredow et al., 1991). In addition, centrally active physostigmine was more effective than pydridostigmine in protecting from toxicity due to GD, GB, and other OP compounds (Leadbeater et al., 1985; Solana et al., 1990; Miller et al., 1993). Although physostigmine appears to be an effective pretreatment for OP nerve agent toxicity, there are several problems with its use: (1) it has a short half-life *in vivo* and (2) it has severe side effects, including nausea, vomiting, and diarrhea (Coehlo and Birks, 2001).

Over the past decade, several reversible centrally acting AChE inhibitors including donepezil, rivastigmine, metrifonate, galanthamine, and huperzine A (HUP) were developed for the treatment of Alzheimer's disease. These inhibitors have great potential as pretreatments and treatments to counteract the toxicity of OP nerve agents. The efficacy of various pretreatments such as

pyridostigmine, physostigmine, physostigmine + scopolamine, and HUP were compared against GD toxicity (Lim et al., 1998; Lallement et al., 2001b). Surprisingly, the measurement of peripheral and central ChE activities indicated that HUP but not physostigmine inhibited central AChE. These pretreatments did not increase tolerance to cumulative GD poisoning, but delayed death from 30 min to 8–24 h after the last GD injection. Subsequently, HUP was demonstrated to protect mice, monkeys (Grunwald et al., 1994), and guinea pigs (Lallement et al., 1997) against nerve agent toxicity by pretreatment of animals prior to challenge with GD. A protective ratio of 2.0 was obtained 6 h after pretreatment of monkeys with HUP, with no postchallenge supporting therapy. In guinea pigs, pretreatment with HUP was shown to prevent seizures and neuropathological damage to the hippocampus following exposure to GD. These studies highlighted the superiority of HUP as an antidote against nerve agent toxicity compared to pyridostigmine and physostigmine in as much as the duration of protection conferred following a single dose administration of the prophylactic drug.

Recently, Janowsky et al. (2005) showed that pretreatment with donepezil significantly attenuated the hyperthermia, hypoactivity, and diarrhea in rats that were exposed to DFP. Similarly, Albuquerque et al., (2006) demonstrated that another centrally acting AChE reversible inhibitor, galanthamine, was far more effective than pyridostigmine when combined with atropine and less toxic than HUP when administered before or soon after acute exposure to lethal doses of GD, GB, and paraoxon. Taken together, these studies suggest that these and other CNS acting reversible AChE inhibitors can be developed as drugs for treating OP nerve agent toxicity.

V. SUMMARY

Over the last 5 years, unprecedented progress was made in the development of an enzyme bioscavenger to protect against chemical warfare nerve agents. The main objective of developing an enzyme bioscavenger was to sequester the nerve agent before it reached its physiological target, the CNS AChE. This has to be accomplished within one circulation time and Hu BChE has been proven to perform this task. Hu BChE will be advanced to phase 1a clinical trials in the very near future. It is an ideal pretreatment that is: (1) safe because it is derived from a human source and (2) efficacious because its effectiveness was demonstrated in a variety of animal species against almost all OPs. Its stockpiling is not an issue because it was shown to be stable in lyophilized form for more than 3 years.

Many alternate approaches were attempted to acquire sufficient quantities of the enzyme. Recombinant Hu BChE was produced in the milk of transgenic goats, plants, algae, and mammalian cell lines. Of these, material derived from transgenic goat milk, namely Protexia has advanced more rapidly. However, further studies need to be done with this material for making it safe for human use. The presence of potentially immunoreactive glycans on this molecule needs to be thoroughly addressed. Also the lower MRT of Protexia compared to Hu BChE will have to be addressed before it can be used as an efficient bioscavenger. In this regard, PEGylation of select lysines on the surface of rHu BChE appears to increase its MRT although this chemical modification may not eliminate its immunoreactivity.

Another alternative is the search or development of a catalytic scavenger. Several OP hydrolases were identified that have the potential to catalytically hydrolyze OPs. Most of them are of bacterial source, and thus are not suitable for human use. Also, efforts were extended to explore the utility of Hu PON1 as a scavenger that can hydrolyze OPs at a rate that is sufficient to prevent them from reaching the CNS. With the application of gene shuffling technology, much progress was made in this regard; however, the search for the right enzyme that has a broad specificity to hydrolyze OPs in humans will have to be continued.

A promising advance was made with the application of gene therapy for introducing catalytic and stoichiometric bioscavengers *in vivo*. It was shown that recombinant adenoviruses containing genes for Hu PON1 when administered into mice boosted PON1 levels in blood. Similarly, the

introduction of AAV containing Hu AChE gene into AChE knockout mice resulted in the expression of AChE in liver, lung, and heart for several months and protected mice from DFP toxicity. The gene therapy approach was also tested with a double mutant Ala328Trp/Tyr332Ala rHu BChE with enhanced cocaine hydrolase activity for protection against cocaine toxicity in rats. These preliminary studies suggest that viral vectors can be used for the delivery of bioscavengers *in vivo*. However, this method is not applicable in its present form because of the toxicity and immunogenicity associated with these vectors.

In addition to investigations on bioscavengers as pretreatment drugs, efforts were also focused on developing broad-spectrum oximes for the treatment of OP nerve agent poisoning. Recent efforts at developing new and improved oximes employed *in vitro* evaluation more often than *in vivo* evaluation. Although this simplified *in vitro* evaluation does not consider other factors that affect *in vivo* antidotal efficacy, there is a reasonable correlation between *in vitro* and *in vivo* data, and the efficacy of an oxime depends primarily on its ability to reactivate nerve agent inhibited AChE. If the *in vitro* oxime reactivation of nerve agent inhibited animal AChE is similar to that of Hu AChE, it is likely that the results of *in vivo* animal study will reliably extrapolate to humans. A comparative *in vitro* study using guinea pig, monkey, and Hu AChEs, however, indicated that there were significant differences in the reactivation of guinea pig and Hu AChEs. These remarkable species variations were not found between monkey and Hu AChE, indicating that more reliable results on oxime efficacy will be obtained from monkeys than guinea pigs.

Recent efforts in developing broad-spectrum oxime have not yet resulted in the identification of an oxime that can reactivate AChE inhibited by all nerve agents at reasonable rates. For example, several bis-pyridinium 4-oximes that were found to be highly effective for the reactivation of GA-inhibited AChE were ineffective in reactivating GD-inhibited AChE. To further our understanding on the interaction between AChE, OPs, and oximes, x-ray crystallographic and site-directed mutagenesis studies were initiated. These studies revealed the crucial role of peripheral anionic site of AChE in interacting with the second pyridinium structure of HI-6 and HLo-7, which is responsible for the enhanced potency of these oximes. Further studies on the mechanism of oxime reactivation may pave the way for the discovery of more efficient and broad-spectrum next-generation oximes for the treatment of nerve agent poisoning.

It was also shown that the efficacy of postexposure therapy for OP toxicity could be enhanced by pretreating with low, nonlethal doses of reversible AChE inhibitors. These agents act by transiently binding to a fraction of AChE and protecting it from inhibition by OP nerve agents. Pyridostigmine, a quaternary carbamate, was the first inhibitor approved by the USFDA for use by military personnel who are under threat of exposure to GD. However, because pydridostigmine does not cross the blood–brain barrier, it does not prevent CNS impairment. Therefore, some of the centrally acting AChE inhibitors are being tested as replacements for pyridostigmine. Prominent among the ones that were tested recently are physostigmine, galanthamine, HUP, and donepezil. Although results with galanthamine are encouraging, much more needs to be done before any of these inhibitors can be considered as effective pretreatments.

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8 Nerve Agent Bioscavengers: Progress in Development of a New Mode of Protection against Organophosphorus Exposure

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CONTENTS

Although treatment for intoxication by organophosphorus (OP) poisons exists, the treatment regimen suffers from certain deficiencies. To overcome these disadvantages, the concept of using a bioscavenger has emerged as a new approach to reduce the in vivo toxicity of chemical warfare nerve agents. Bioscavengers fall into two broad categories, stoichiometric, that is, proteins that bind and detoxify a poison in some fixed molecular ratio, and catalytic, that is, proteins that can cause the breakdown of a molecule of a poison, regenerate, and then repeat the process until all of the poison molecules have been destroyed. To be an improvement over current treatment, a biological scavenger should have minimal or no behavioral or physiological side effects, should provide protection against exposure to as much as five LD_{50}s of one or more nerve agents, and should reduce or eliminate any behavioral or physiological side effects normally associated with the currently fielded therapy. Studies with equine or human butyrylcholinesterase or fetal bovine serum acetylcholinesterase showed that none of these scavengers elicited behavioral side effects

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when administered to rats or monkeys. These three scavenger enzymes as well as carboxylesterase were each capable of providing protection against 2–16 LD_{50}s of GD, GB, or VX depending on the scavenger and the test species (rat, mouse, rabbit, guinea pig, or rhesus monkey). When behavioral testing was performed on animals, that is, mouse, rat, or rhesus monkey, pretreated with a bioscavenger, following administration of up to 5 LD_{50}s of GD or VX, either no, or only very minor and transient deficits were reported. These results were in stark contrast to the prolonged, up to 14 days, behavioral incapacitation experienced by animals that were pretreated with pyridostigmine and received the same dose of nerve agent followed by the standard atropine and oxime therapy with or without diazepam. Although several challenges still remain to be met before bioscavengers could augment or replace the current therapeutic regimes for nerve agent intoxication, the results to date offer impressive evidence for the value of this approach as the next generation of pharmaceuticals to afford protection against nerve agent poisoning with a virtual absence of behavioral side effects.

I. INTRODUCTION

Organophosphorus anticholinesterases (OPs) are among the most toxic substances that have been identified (Dacre, 1984). Originally, OPs were developed for use as insecticides (Ballantyne and Marrs, 1992), but their extreme toxicity toward higher vertebrates has led to their adoption as weapons of warfare (Maynard and Beswick, 1992). The OPs most commonly used as chemical weapons (referred to as nerve agents), which the U.S. military forces might expect to encounter, are tabun (GA), sarin (GB), soman (GD), cyclosarin (GF), VX, and VX-like compounds such as the Russian V-agent, VR. However, OP pesticides can also be regarded as a potential threat in a terrorist context. The molecular weights of OP nerve agents range from 140 to 267 Da, and under standard conditions they are all liquids that differ in their degrees of volatility (Somani et al., 1992). They have median lethal dose (LD_{50}) values in mammals, including estimates for humans, in the μg/kg dose range for all routes of exposure except dermal, where LD_{50} doses are in the mg/kg range (Maynard and Beswick, 1992). OPs produce their acute toxic effects by irreversibly inhibiting the enzyme acetylcholinesterase (AChE, E.C. 3.1.1.7) (Koelle, 1963; Taylor, 2001b). This inhibition leads to an increase in the concentration of acetylcholine in the cholinergic synapses of both the peripheral and central nervous systems. Because numerous lines of evidence indicate that the sister enzyme butyrylcholinesterase (BuChE, E.C. 3.1.1.8) plays a functional role in the cholinergic system (Mesulam et al., 2002; Duysen et al., 2007), inhibition of BuChE could also contribute to the increase in concentration of acetylcholine in the cholinergic synapses, including those in the central nervous system. The physiological consequences of elevated acetylcholine include alterations in the function of the respiratory center (de Candole et al., 1953; Stewart, 1959; Stewart and Anderson, 1968; Brimblecombe, 1977) and overstimulation at neuromuscular junctions (Bajgar et al., 1971; Heffron and Hobbiger, 1979; Chabrier and Jacob, 1980). A sufficiently high level of acetylcholine or a sufficiently rapid increase in acetylcholine concentration precipitates a cholinergic crisis, resulting in dimming of vision, headache, shortness of breath, muscle weakness, and seizures. In the extreme, organophosphorus intoxication can be a life-threatening event, with death usually resulting from respiratory failure. This is often accompanied by secondary cardiovascular components, including hypotension, cardiac slowing, and arrhythmias (Taylor, 2001a, 2001b). The time lapse between exposure (except dermal) and significant inhibition of synaptic AChE is very short (one or two circulation times), so therapy must be administered rapidly upon exposure or immediately after onset of symptoms to be effective.

This suggests that a prophylactic approach based on the reduction of the concentration of OP toxicant in the blood before it can reach its site of action (synaptic endplates) should be particularly attractive; potentially incapacitating or even fatal exposures could be mitigated to mild symptoms such as salivation or shortness of breath and lower level exposures could be rendered inconsequential.

II. CURRENT THERAPY FOR NERVE AGENT EXPOSURE

The conventional approach adopted by the United States and its NATO allies for treatment of OP intoxication seeks to counteract the effects of AChE inhibition and to reactivate the enzyme. Cholinolytic drugs such as atropine are administered at the onset of signs of OP intoxication to antagonize the effects of the elevated acetylcholine levels that result from the inhibition of AChE (Heath and Meredith, 1992). Additionally, an oxime nucleophile is given, which reacts with the inhibited ''phosphylated' enzyme to displace the phosphyl group and restore normal activity (Heath and Meredith, 1992). In the United States, the oxime of choice for treatment of nerve agent poisoning is the chloride salt of 2-PAM, usually referred to as 2-PAM-Cl. In France, the methylsulfate salt of 2-PAM is recommended and is registered as Contrathion. In the United Kingdom, the sulfate salt of 2-PAM, usually referred to as P2S, is the oxime of choice, although bis-pyridinium oximes may be more effective depending on the particular organophosphorus agent (Bismuth et al., 1992). Anticonvulsant drugs such as diazepam are also administered to control OP-induced tremors, convulsions, and seizures. In conjunction with therapy, individuals at high risk for exposure to the nerve agent GD are pretreated with the spontaneously reactivating AChE inhibitor pyridostigmine bromide (PB), which when used at recommended dosages (which produce less than 30% inhibition of plasma AChE) temporarily masks the active site of some AChE molecules, and thus protects a fraction of the enzyme from irreversible inhibition by the OP agent (Gordon et al., 1978).

These treatment regimens, with minor variations, have been the standard in the United States and in NATO countries for many years; although beneficial, they suffer from a number of disadvantages. Although current approaches can be effective in preventing lethality, they do not prevent performance deficits, behavioral incapacitation, loss of consciousness, or the potential for permanent brain damage, all of which can result from acute OP toxicity (Leadbeater et al., 1985). Furthermore, it appears that greater than marginal improvement of these pharmacological approaches will be difficult because stronger drugs or higher doses of currently fielded drugs are likely to produce unacceptable performance decrements by themselves (Dunn and Sidell, 1989; Castro et al., 1992).

Several nerve agents, including cyclosarin, sarin, and in particular soman, present an additional therapeutic challenge in that after they inhibit AChE, they undergo a second reaction in which the phosphonyl group attached to the inhibited enzyme is dealkylated. This process, known as aging, results in a phosphylated AChE that is refractory to either spontaneous or oxime-mediated reactivation (Fleisher and Harris, 1965). The rate of aging depends on the chemical structure of the alkoxy chain being dealkylated; the bulkiest branched chains promote very rapid aging, for example, the halftime of aging of soman-phosphonylated human AChE is 2 min at 37°C, whereas that of the sarin-phosphonylated enzyme is about 4 h (Harris et al., 1966). The ineffectiveness of therapeutically administered oxime as a treatment for exposure to some nerve agents explains the continued research efforts aimed at alternative approaches to protection (Dunn and Sidell, 1989). One approach in particular has focused on preventing the critical enzyme AChE from becoming inhibited in the first place.

III. NERVE AGENT BIOSCAVENGERS: AN ALTERNATIVE TO CONVENTIONAL APPROACHES

Although successful to a degree, current treatments for acute nerve agent poisoning always result in the victim suffering a toxic insult that subsequently must be therapeutically managed. To avoid the onset of a toxic insult, recent efforts have focused on identifying proteins that can act as biological scavengers of organophosphorus compounds and can remain stable in circulation for long periods of time. The concept of using a protein that can react with a nerve agent, either on a one to one basis (stoichiometrically) or with the capability to breakdown the nerve agent (catalytically) to protect against the toxic effects of those compounds is not new. As early as 1956 it was shown that injection

of exogenous paraoxonase (EC. 3.1.8.1) could protect rats against several times the LD_{50} of paraoxon (Main, 1956). This approach avoids the side effects and the requirement for rapid administration associated with current antidotes (Erdmann et al., 1965; Wiezorek et al., 1968; Sidell and Groff, 1970; Vojvodic, 1970; Wenger, 1979; Clement, 1982; Huff, 1986; Dunn and Sidell, 1989; McDonough, 2002) by prophylactically inactivating (through sequestration or hydrolysis) anticholinesterase agents before they can react with the target AChE. The time frame for this inactivation to occur before endogenous AChE is affected is quite narrow [estimated to be approximately 2 min in humans (Talbot et al., 1988)], so for situations involving acute exposure, the scavenger function must be very rapid, irreversible, and specific. Ideally, the scavenger would enjoy a long residence time in the bloodstream, would be biologically innocuous in the absence of nerve agent, and would not present an antigenic challenge to the immune system. For these reasons, a formal program to identify candidate bioscavengers was started in 1993 by the U.S. Army Medical Research Materiel Command, and it has focused on enzymes of mammalian (particularly human) origin.

Candidate bioscavenger proteins, in general, function either by stoichiometrically binding and sequestering the anticholinesterase or by catalytically cleaving the OP substrate into biologically inert products. In the former category are naturally occurring human proteins that bind or react with nerve agents, including enzymes such as cholinesterases (ChEs) and carboxylesterases (CaEs). Each of these stoichiometric scavengers has the capacity to bind one molecule of nerve agent per molecule of protein scavenger. Although this approach has been proven to be effective in laboratory animals, it has the disadvantage that the extent of protection is directly proportional to the concentration of unreacted, active scavenger in the bloodstream at the time of nerve agent exposure. Because the molecular weight of the protein scavengers listed above is in the range of 60–80,000 Da and the molecular weight of the nerve agents is about 180 Da, the concentration mass ratio of scavenger to nerve agent is ~500:1. Thus, a high concentration of scavenger protein in circulation is necessary to protect against exposure to multiples of an LD_{50} dose of nerve agent, although lower concentrations would be sufficient to prevent inactivation of synaptic AChE after a low-dose OP exposure. It might be possible to mitigate the need for large amounts of scavenger by also administering, either prophylactically or immediately after exposure, a currently fielded oxime. Oxime treatment might allow the continual reactivation of the bioscavenger in vivo, in effect converting the stoichiometric scavenger into a pseudocatalytic one.

Candidate enzymes with bona fide catalytic activity against nerve agents include the human organophosphorus acid anhydride hydrolases (OPAHs) (Masson et al., 1998), such as paraoxonase 1 (PON1) (Yeung et al., 2004, 2005). The ability to engineer site-specific amino acid mutations into naturally occurring scavenger enzymes can allow investigators to alter the binding and catalytic activities of these enzymes. In general, the use of scavengers with catalytic activity would be advantageous because small amounts of enzyme, meaning lower concentrations in circulation, would be sufficient to detoxify large amounts of nerve agent (as in an acute exposure).

By nearly all criteria, the use of biological scavengers, either stoichiometric or catalytic, as a prophylactic approach to providing protection against an exposure to a nerve agent offers numerous advantages over conventional treatments. One way to evaluate the potential benefits of a bioscavenger enzyme is to consider the extent to which its presence would reduce the concentration of an OP in vivo. Indeed, the halftime for reaction of a nerve agent with a biological scavenger in vivo can be calculated using some very conservative assumptions. Based on toxicity estimates in humans, the expected concentration of a nerve agent in the blood at an LD_{50} dose would be about 8×10^{-7} M (Lenz et al., 1997). The bimolecular rate constant for reaction of soman with AChE is $\sim 9 \times 10^{7}$ M^{-1} min^{-1} (Hanke and Overton, 1991; Ordentlich et al., 1993). If a scavenger such as BuChE were present in blood at a concentration of 1 mg/mL (1×10^{-5} M), then the rate constant for reaction of scavenger with toxicant would be pseudo first order and the $t_{1/2}$ for the reduction of toxicant would be ~3–7×10^{-4} min. Under those conditions, which assume perfect mixing and that all of the scavenger and all of the toxicant remain in the bloodstream, the concentration of toxicant would be

reduced to 1/1000th of its initial concentration within 10 half-lives ($2–4 \times 10^{-3}$ min). Where actual measurements have been made of the rate of reduction of concentration of soman in animals (guinea pigs), it was found that, in the absence of an exogenous scavenger, the concentration of a 2 LD_{50} dose of soman in circulation was reduced by 1000-fold in about 1.5 min (Langenberg et al., 1997). These results support the contention that if a bioscavenger were present in circulation at a sufficiently high concentration at the time of exposure, the reduction in toxicant concentration to a physiologically insignificant level (with no measurable inhibition of synaptic AChE) would be very rapid, and it would certainly occur in less than one circulation time at most concentrations of OP that might be encountered in a battlefield or civilian exposure setting. The need to administer, repetitively, a host of pharmacologically active drugs with a short duration of action at a precise time following exposure is all but eliminated if a scavenger is used. The potential for military personnel to require use mission oriented protective posture (MOPP) gear is greatly reduced. Finally, with the appropriate scavengers, such an approach could afford protection against all of the current threat agents, including those that induce rapid aging of AChE and are refractory to treatment by the current atropine and oxime treatment regime.

IV. STOICHIOMETRIC SCAVENGERS AND THE PROTECTION THEY OFFER

Several different enzymes that react with OPs but do not catalyze their hydrolysis have been tested over the last 15 years for their ability to provide protection against nerve agent poisoning. Wolfe et al. (1987) first reported the use of exogenously administered AChE as a bioscavenger (Table 8.1). In that study, fetal bovine serum acetylcholinesterase (FBS-AChE) was administered to mice 20 h before a multiple LD_{50} challenge of VX was administered. Complete protection was afforded against a 2 LD_{50} dose of VX (100% survival of exposed animals), whereas moderate protection (80% survival of exposed animals) was observed after a challenge of three times the LD_{50}. No protection was observed against higher multiple LD_{50} challenges of VX. When animals pretreated with FBS-AChE were exposed to soman, little protection was afforded, probably due to the fact that high levels of CaE in mouse plasma raise the LD_{50} of soman, but not VX, much higher than it is in other mammals (see below). Because protection is calculated as a multiple of the normal LD_{50}, the scavenger concentration used was not high enough to produce a statistically significant difference. However, FBS-AChE pretreatment in conjunction with postexposure atropine and 2-PAM treatment

TABLE 8.1
Protection from Organophosphorus Intoxication by the Bioscavenger FBS-AChE

Bioscavenger	Test Species	Nerve Agent	Protection (LD_{50})[a]	Serum $t_{1/2}$[b]	References
FBS-AChE	Rhesus monkey	GD	2–5	30–40 h	Maxwell et al. (1992), Wolfe et al. (1992)
FBS-AChE	Mouse	GD	2 (w/Atropine + 2-PAM)	40–50 h	Wolfe et al. (1987)
FBS-AChE	Mouse	GD	2 (after CBDP treatment)	~24 h	Doctor et al. (1991)
FBS-AChE	Mouse	GD	2–8	24–26 h	Ashani et al. (1991), Maxwell et al. (1991, 1993)
FBS-AChE	Mouse	MEPQ	4	~24 h	Doctor et al. (1991), Maxwell et al. (1991)
FBS-AChE	Mouse	VX	2–3.6	~24–50 h	Wolfe et al. (1987), Doctor et al. (1991), Maxwell et al. (1991)

[a] Values represent multiples of median lethal doses (LD_{50}s) of nerve agent survived after FBS-AChE administration.
[b] Half-life of administered FBS-AChE in blood circulation.

protected mice from exposure to 2 LD_{50}s of soman. The authors reported that animals displayed no detectable side effects in response to administration of FBS-AChE.

Maxwell et al. (1993) carried out a similar set of experiments using rhesus monkeys pretreated with the scavenger FBS-AChE. When monkeys pretreated with FBS-AChE were challenged with either 1.5 or 2.5 LD_{50}s of soman, there was protection (Table 8.1) with no decrements in performance as compared with animals treated with FBS-AChE alone when assessed by a serial probe recognition (SPR, discussed in Section VI) task. The animals were also monitored for the generation of an antibody response against the administered FBS-AChE, but none was detected. The authors cautioned, however, that whenever a foreign protein is administered to an animal, the potential for an antibody-mediated immune response must be assessed on a case-by-case basis. Maxwell et al. (1993) also compared the relative protection against soman afforded to CaE-inhibited (CDBP treated) mice by three different treatments: pyridostigmine pretreatment with atropine therapy postexposure, postexposure oxime (HI-6), and atropine therapy, or FBS-AChE pretreatment alone. The authors concluded that the FBS-AChE pretreatment offered superior protection against both soman toxicity (survival after 8–10 LD_{50} doses) and behavioral incapacitation. The results of these and other studies using FBS-AChE are summarized in Table 8.1.

Broomfield et al. (1991) reported that equine-BuChE (eq-BuChE) afforded complete protection against a 2 LD_{50} challenge dose of soman in rhesus monkeys (Table 8.2) with no supporting therapy and against 3–4 LD_{50} doses when atropine was administered (postexposure). Protection against a single LD_{50} dose of sarin was also demonstrated. There were no fatalities in any of these cases (Table 8.2). Furthermore, when animals were assessed for behavioral deficits, again using an SPR task, they all returned to baseline performance within 9 h after soman exposure (vide infra) (Castro et al., 1992).

In a related study, Wolfe et al. (1992) assessed the ability of pretreatment with either FBS-AChE or eq-BuChE to protect rhesus monkeys against multiple LD_{50} doses of soman (Tables 8.1 and 8.2). Survival and the ability to perform the primate equilibrium platform (PEP) behavioral task were the variables assessed. The animals that received FBS-AChE as a pretreatment were protected against a cumulative exposure of 5 LD_{50}s of soman and showed no decrement in the PEP task. Two of the four monkeys that received purified eq-BuChE did show some transient decrement in PEP task performance when the cumulative dose of soman exceeded 4 LD_{50}s. All of the experimental animals were observed for an additional 6 weeks, and none displayed any residual or delayed performance decrements, suggesting that no residual adverse effects were present. These results were reviewed and expanded upon by Doctor et al. (1993) in studies where mice pretreated with FBS-AChE were also administered the oxime HI-6 immediately postexposure to sarin. In theory, the oxime will continuously regenerate the inhibited scavenger enzyme in vivo; this approach is predicted to increase the amount of sarin that could be scavenged by a given amount of AChE, endowing this stoichiometric scavenger with pseudocatalytic properties. The therapeutic addition of HI-6 after pretreatment with FBS-AChE was found to enhance the efficacy of the scavenger enzyme against sarin in vivo, increasing the ratio of neutralized organophosphorus compound per FBS-AChE

TABLE 8.2
Protection from Organophosphorus Intoxication by the Bioscavenger eq-BuChE

Bioscavenger	Test Species	Nerve Agent	Protection (LD_{50})[a]	Serum $t_{1/2}$[b]	References
eq-BuChE	Rhesus monkey	GB	1	620 h	Broomfield et al. (1991)
eq-BuChE	Rhesus monkey	GD	2 (4 w/atropine)	620 h	Broomfield et al. (1991)
eq-BuChE	Rhesus monkey	GD	5	30–40 h	Wolfe et al. (1992)

[a] Values represent multiples of median lethal doses (LD_{50}s) of nerve agent survived after eq-BuChE administration.
[b] Half-life of administered eq-BuChE in blood circulation.

TABLE 8.3
Protection from Organophosphorus Intoxication by Endogenous Plasma CaE

Bioscavenger	Test Species	Nerve Agent	Protection (LD_{50})[a]	References
CaE[b]	Mouse	GD	16	Maxwell et al. (1987)
CaE	Guinea pig	GD	3.5	Maxwell et al. (1987)
CaE	Rabbit	GD	3	Maxwell et al. (1987)
CaE	Rat	GD	8–9	Maxwell et al. (1987), Ember (1991)
CaE	Rat	GB	8	Maxwell (1992)
CaE	Rat	GA	4–5	Maxwell (1992)
CaE	Rat	VX	1	Maxwell (1992)
CaE	Rat	Paraoxon	2	Maxwell (1992)

[a] Values represent multiples of median lethal doses (LD_{50}s) of nerve agent survived due to the presence of CaE. Because CaE is an endogenous plasma protein in these species, the protection it offers was measured by comparing LD_{50} values in untreated and CBDP-treated animals; 2 mg/kg CBDP completely abolishes endogenous plasma CaE activity (Maxwell, 1992).

[b] For each species, the activity of the host's endogenous CaE was tested.

molecule from 1:1 (in the presence of AChE alone) to ~65:1. To date, similar in vivo studies using BuChE with oximes have not been reported, although preliminary in vitro data suggest that currently fielded oximes do not reactivate OP-inhibited BuChE rapidly enough to make this enzyme pseudocatalytic.

CaE is another enzyme with the potential to be a good antiorganophosphorus scavenger molecule (results summarized in Table 8.3) (Maxwell et al., 1991). Although AChE and BuChE were found to be more efficient scavengers for soman in mice than CaE (i.e., they have higher bimolecular rate constants), the latter enzyme was capable of affording equal protection on a molar basis. CaEs (EC 3.1.1.1) catalyze the hydrolysis of a wide variety of aliphatic and aromatic esters and amides (Augustinsson, 1958; Satoh, 1987). Catalysis occurs by a two-step process in which the substrate acylates the active site serine of CaE, which subsequently deacylates by the addition of water (Aldridge and Reiner, 1972). CaE can be distinguished from AChE and BuChE by the fact that AChE and BuChE react with positively charged carboxylesters, such as acetylcholine and butyrylcholine, and are readily inhibited by carbamates, whereas CaE does not react with positively charged substrates and is inhibited by carbamates only at high concentrations (Aldridge and Reiner, 1972). These differences in substrate specificity also extend to the reaction of CaE with OP compounds. Positively charged OP compounds, such as VX, react poorly with CaE whereas neutral OP compounds, such as soman, sarin, and paraoxon, react rapidly. Dephosphylation of the active site phosphorylated serine of CaE is a very slow process compared to deacylation (Satoh and Hosokawa, 1998), and therefore CaE has usually been considered to be a stoichiometric detoxification mechanism for OP compounds.

CaE is 60 kDa enzyme that is found in many mammalian tissues—lung, liver, kidney, brain, intestine, muscle, and gonads—usually as a microsomal enzyme. In some species, CaE is also found in high concentrations in plasma; plasma CaE is synthesized in the liver and secreted into the circulation via the Golgi apparatus of hepatocytes (Miller et al., 1999). Secretion of CaE appears to be controlled by the presence or absence of a retention signal at the carboxy terminal of the enzyme (Figure 8.1). CaE that is retained in the liver has a highly conserved carboxy-terminal tetrapeptide sequence (HXEL in single letter amino acid code, where X represents any amino acid), whereas the secretory form of CaE has a disrupted version of this retention signal in which the terminal leucine residue is replaced by either histidine–lysine or histidine–threonine (Miller et al., 1999). Mammalian species that have high levels of secretory CaE in their plasma require much larger doses of OP

Biochemical basis for CaE cellular trafficking

Enzyme	COOH-terminal residues	Reference
Intracellular CaEs		
Rabbit Es-1	..TEHIEL	Korza and Ozols, 1988
Rabbit Es-2	..QKHTEL	Ozols, 1989
Hamster AT51p	..GKHSEL	Sone et al., 1994
Human CaE-1	..TEHIEL	Shibata et al., 1993
Human CaE-2	..ERHTEL	Schwer et al., 1997
Pig CaE	..IKHAEL	Matsushima et al., 1991
Rat Es-10(pI6.1)	..WKHVEL	Robbi et al., 1990
Rat Es-B	..PHHNEL	Yan et al., 1994
Mouse Es-X	..REHVEL	Ellinghaus et al., 1998
Mouse Es-22	..TEHTEL	Ovnic et al., 1991a
Consensus	..HXEL	
Secreted CaEs		
Mouse Es-1	..TEHTE*HK*	Ovnic et al., 1991b
Rat Es-1	..TEHTE*HT*	Long et al., 1988; Robbi and Beaufay, 1992; Murakami et al., 1993

FIGURE 8.1 The carboxy-terminal amino acid residues of carboxylesterase enzymes from disparate species are aligned to show the conserved ''HXEL'' motif found among intracellular enzymes (shown in bold letters), and the disrupted versions of this retention motif found in the mouse and rat secreted carboxylesterase isoenzymes (alterations to the motif shown in italics). The capacity of the carboxy-terminal HXEL motif to act as an endoplasmic reticulum retention signal has been directly demonstrated. (From Medda and Proia, 1992.)

compounds to produce toxicity than do species with low levels of plasma CaE (Maxwell et al., 1987). For example, the LD_{50} dose for soman in rats is 10-fold larger than the LD_{50} in nonhuman primates, which correlates with the differences in the plasma concentrations of CaE found in these species (Figure 8.2). Studies comparing the levels of CaE in the plasma of rats, guinea pigs, nonhuman primates, and humans as determined by the extent of binding of a radiolabelled organophosphorus compound, diisopropylfluorophosphate (DFP), clearly demonstrate the potential protective effect of this enzyme (Patel et al., 2005). Notably, human plasma has recently been shown to be devoid of CaE (Li et al., 2005; Satoh and Hosokawa, 2006). Although a human CaE gene has been cloned and expressed (Miller et al., 1999), there is no commercial source of highly purified CaE for use in in vivo testing of protective efficacy. Therefore, the primary evidence demonstrating the effectiveness of CaE as a stoichiometric scavenger against OPs, especially sarin and soman, has been by comparison of OP LD_{50}s in animals with high endogenous plasma levels of CaE to OP

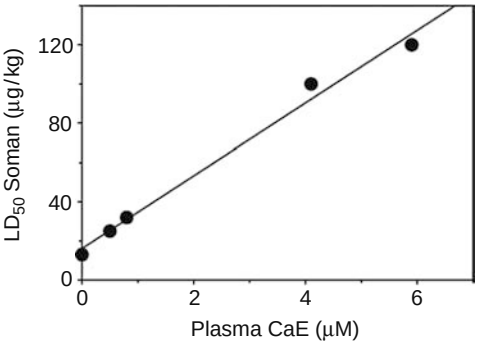

FIGURE 8.2 Effect of plasma CaE concentration on soman LD_{50} (administered subcutaneously) in different species. Data points (from lower left to upper right of graph) for species were monkey, rabbit, guinea pig, rat, and mouse. (Data taken from Maxwell, D.M. et al., *Cholinesterases: Structure, Function, Mechanism, Genetics and Cell Biology*, J. Massoulie et al., eds., American Chemical Society, Washington DC, 1991.)

LD_{50} levels in animals of the same species whose plasma CaE has been chemically inhibited (Maxwell et al., 1987). For example, inhibition of plasma CaE prior to the LD_{50} determination of soman in rats reduces its LD_{50} by approximately eightfold (Table 8.3) strongly suggesting that circulating CaE is an effective endogenous bioscavenger against OP compounds.

Recent investigations of the reactivation of OP-inhibited CaE have suggested that it may be possible to increase its potential as an OP scavenger by exploiting its turnover of OP compounds. Maxwell et al. (1994) observed that OP-inhibited CaE does not undergo the aging process that prevents oxime reactivation of OP-inhibited ChEs, whereas Jokanovic et al. (1996) found that OP-inhibited CaE from plasma underwent spontaneous reactivation with a halftime of 1–2 h. Comparisons of the amino acid sequences of CaE, AChE, and BuChE are informative with regard to the critical amino acid residues required for occurrence of aging versus spontaneous reactivation. Of the seven conserved amino acid residues that have been identified by theoretical studies and confirmed by site-directed mutagenesis to be important for aging of OP-inhibited AChE and BuChE, only two are conserved in CaE (Maxwell et al., 1998). Conversely, a highly conserved histidine found in CaE from six mammalian species and two insect species, but not in mammalian ChEs, correlates with the higher level of spontaneous reactivation of OP-inhibited CaE compared with OP-inhibited ChE (Maxwell et al., 1994). Interestingly, introduction of a histidine into BuChE at a position nearly identical to the position of the conserved histidine of CaE, that is, in the oxyanion hole, produces spontaneous reactivation of OP-inhibited BuChE (Millard et al., 1995; Lockridge et al., 1997; Millard et al., 1998). A more detailed discussion of the relative merits of FBS-AChE, eq-BuChE, and plasma CaE as scavengers, which describes the extent of protection they offer against a variety of nerve agents, both in vitro and in vivo in mice, was presented by Doctor et al. (1991). The authors note that some of the in vivo differences seen in sensitivity and protection may be due to variations in the circulatory pharmacodynamics of the different organophosphorus compounds, such that those inhibitors that distribute more slowly from circulation are more readily scavenged. This concept provided early support for the feasibility of using scavengers to protect against exposures to nerve agent.

Ultimately, the goal of research on scavenger molecules is to generate a means to protect humans from the toxic effects of nerve agents. In an effort to minimize any physiological, immunological, or psychological side effects of scavenger use in humans, research efforts have focused primarily on the use of human BuChE (Hu-BuChE), with additional work on the model systems of either intracellular human CaE or FBS-AChE, which does not induce an immune response in rhesus monkeys (Maxwell et al., 1992). In a series of studies, Ashani et al. examined the scavenger properties of FBS-AChE and particularly plasma-derived Hu-BuChE in mice, rats, and rhesus monkeys with respect to several different nerve agents as well as other organophosphorus compounds (Table 8.4) (Ashani et al., 1991; Raveh et al., 1993, 1997). They found that

TABLE 8.4
Protection from Organophosphorus Intoxication by Exogenous Hu-BuChE

Bioscavenger	Test Species	Nerve Agent	Protection (LD_{50})[a]	Serum $t_{1/2}$[b]	References
Hu-BuChE	Rhesus monkey	GD	2	~30 h	Raveh et al. (1997)
Hu-BuChE	Rhesus monkey	VX	1.5	~30 h	Raveh et al. (1997)
Hu-BuChE	Rat	GD	2–3	46 h	Raveh et al. (1993)
Hu-BuChE	Rat	VX	2	46 h	Raveh et al. (1993)
Hu-BuChE	Mouse	GD	2.1	21 h	Raveh et al. (1997)
Hu-BuChE	Mouse	GB	1.6	21 h	Raveh et al. (1997)
Hu-BuChE	Mouse	GA	1.8	21 h	Raveh et al. (1997)
Hu-BuChE	Mouse	VX	4.9	21 h	Raveh et al. (1997)

[a] Values represent multiples of median lethal doses (LD_{50}s) of nerve agent survived after Hu-BuChE administration.
[b] Half-life of administered Hu-BuChE in blood circulation.

TABLE 8.5
Protection by Hu-BuChE against Nerve Agent Poisoning

Species	Toxin	Dose (LD_{50})	Impairment	Recovery
Guinea pig	GD	5.5	None	Immediate
Guinea pig	VX	5.5	None	Immediate
Cynomolgus	GD	5.5	1/5	4/6[a]

Source: All data are from Lenz, D.E., et al., *Chem. Biol. Interact.*, 157–158, 205, 2005.

[a] One animal died after the third dose of GD and one was impaired and later euthanized after 48 h. Remaining four animals were normal, survived and they were held for long-term observations.

following administration of exogenous ChE, there was a linear correlation between the concentration of ChE in the blood and the level of protection against organophosphorus poisoning. Furthermore, the extent of protection granted to mice was sufficient to counteract multiple LD_{50} doses of soman. When the protective effect of pretreatment with Hu-BuChE was compared in mice and rats, it was found that in both species the same linear correlation existed between blood concentration of Hu-BuChE and protection against soman, sarin, or VX (Table 8.5). They further noted that to be effective, a scavenger had to be present before exposure to the organophosphorus compound, because (as discussed above) the nerve agent had to be scavenged within one blood circulation time period (Raveh et al., 1993). In the final paper in this series, the authors report similar protection results against a 3.3 LD_{50} dose of soman or a 2.1 LD_{50} dose of VX in rhesus monkeys (Raveh et al., 1997). They also report considerable protection against soman-induced behavioral deficits in a spatial discrimination task. Those efforts have recently been expanded upon by Lenz et al. (2005), Saxena et al. (2005), and Doctor and Saxena (2005). Lenz et al. determined the pharmacokinetics and efficacy of Hu-BuChE in guinea pigs and cynomolgus monkeys against multiple LD_{50} challenges of nerve agents. The halftime for elimination of the plasma-derived Hu-BuChE was about 70–75 h for guinea pigs or cynomolgus monkeys, respectively. Guinea pigs were protected against a cumulative 5.5 LD_{50} of either soman or VX. At necropsy, 7 or 14 days after surviving the nerve agent challenge, all tissues appeared normal upon light microscopic examination. No signs of poisoning were observed in the experimental animals during the efficacy studies. Cynomolgus monkeys were protected against a cumulative challenge of 5.5 LD_{50} of soman as well. Of six animals challenged, one died after the final challenge dose of soman (total dose of 5.5 LD_{50}s administered within 4 h) and one was euthanized 48 h after the final dose of soman. The remaining animals displayed no immediate signs of poisoning and exhibited no lasting effects of poisoning as assessed by blood chemistry examinations and long-term (>20 month) observations. The efficacy results are summarized in Table 8.5. These studies were complimented by the work of Saxena et al. (2005) who measured the in vitro stability of the plasma-derived Hu-BuChE. They found that the lyophilized form of the enzyme had a shelf life of over 24 months at 4, 25, or 37°C. In addition, the material, when reconstituted, showed no alteration in its in vivo properties with respect to the pharmacokinetics in mice or rhesus monkeys. When plasma-derived Hu-BuChE was administered to mice or rhesus monkeys at a dose in 10-fold excess of that needed for protection against a 5 LD_{50} challenge of soman, no deficits on a variety of behavioral tasks were observed. The overall conclusion from these studies was that plasma-derived Hu-BuChE was both safe and efficacious as a biological scavenger. Based on these data, the U.S. Army transitioned the plasma-derived Hu-BuChE to advanced development in October 2004. This product was awarded Investigational New Drug (IND) status by the Food and Drug Administration (FDA) in October of 2006 and is currently being produced by Baxter Pharmaceuticals under good manufacturing practice for additional testing pursuant to a New Drug Application (NDA) submission.

Plasma-derived Hu-BuChE represents a first-generation biological scavenger. This material is obtained from outdated blood and so the overall availability is dependent on the quantity of outdated blood available at any given time. Because this supply is dependent on the extent of donor participation and the need for blood under other circumstances such as natural disasters or unforeseen medical emergencies, basic research efforts are being focused on the development of Hu-BuChE from a recombinant source. If successful, such efforts would allow for a constant supply of material of reproducible purity and activity without affecting the supply of whole blood, collected for other purposes. There are a variety of potential sources of human recombinant protein, to include material from transgenic plants (Mor et al., 2001; Fletcher et al., 2004), transgenic animals (Cerasoli et al., 2005a), transgenic larval sources (Welch et al., 2006), or algae capable of expressing human proteins (Siritunga and Sayre, 2003; Xiong and Sayre, 2004). Reported attempts to express functional Hu-BuChE in bacteria or yeast have failed (Masson et al., 1992; Nachon et al., 2003; Patel, personal communication). To date, recombinant Hu-BuChE (rHuBuChE) enzymes purified from transgenic plants (Mor et al., 2001; Fletcher et al., 2004) and from the milk of transgenic goats (Cerasoli et al., 2005a) have been the most extensively studied (Cerasoli et al., 2005a; Garcia et al., 2005). Studies with rHuBuChE from the milk of transgenic goats have yielded efficacy results in guinea pigs that are very similar to those described previously (vide supra) with plasma-derived material, that is, complete protection against 5.5 LD_{50}s of GD or VX. In its native form, rHuBuChE from transgenic goat milk is primarily either a dimer or a monomer, in contrast to the plasma-derived material which is a tetramer. Additionally, the goat milk derived rHu-BuChE has a glycosylation pattern different from that of the plasma material (Garcia et al., 2005). As a result, goat milk derived rHuBuChE has a different pharmacokinetic profile, with a shorter circulatory half-life. To enhance its biological residence time, the efficacy studies described were carried out with rHuBuChE that had been modified by attachment of several molecules of polyethylene glycol (PEGylated). The PEGylated material had a pharmacokinetic profile very similar to that of plasma-derived BuChE, suggesting that any differences in pharmacokinetics of a recombinant product can be addressed through modification of the protein product (Cerasoli et al., 2005b). The rHuBuChE from goat milk did not evoke any behavioral side effects of its own in guinea pigs, and in addition to survival, animals protected were devoid of any of the typical postpoisoning side effects such as the delayed recovery of function seen when traditional therapy is used. These preliminary results suggest that some form of a recombinant stoichiometric bioscavenger can be developed, thereby providing sufficient material to protect the entire U.S. fighting force.

V. CATALYTIC BIOSCAVENGERS

Although stoichiometric scavengers are able to afford good protection as long as they are present in high levels in the blood stream, they are molecules of high-molecular weight (vide supra); a comparatively large quantity is required to neutralize a small amount of nerve agent. A catalytic scavenger, even having the same high-molecular weight, could be administered in smaller quantities and could produce the same or greater degree of protection. It would also have the advantage of not being consumed in the process of detoxifying the nerve agent, so it would be available to protect against multiple exposures of either high or low dose. Some of these potential bioscavenger proteins along with parameters of their catalytic activities are summarized in Table 8.6 through Table 8.8. As discussed above, in conjunction with an oxime such as HI-6, AChEs that have not undergone aging can be continually reactivated to function pseudocatalytically, eliminating substantially more molar equivalents of organophosphorus compounds than would be predicted based on binding alone. BuChEs are reactivated by currently fielded oximes substantially more slowly, raising questions about their suitability for use in a pseudocatalytic application. Some enzymes, such as the OPAH from *Pseudomonas diminuta* (Serdar and Gibson, 1985), the prolidase from *Alteromonas haloplanktis* (Cheng et al., 1997), the DFPase from *Loligo vulgaris* (Hartleib and Ruterjans, 2001), or HuPON1

TABLE 8.6
Kinetic Properties of Naturally Occurring Catalytic Bioscavengers

Bioscavenger	Source Species	Substrate Specificity	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	References
Phosphotriesterase	*P. diminuta*	GD	36/500	15/7.3	Broomfield (1992), Masson et al. (1998)
Phosphotriesterase	*P. diminuta*	GB	700	N.D.[a]	Dumas et al. (1990)
Phosphotriesterase	*P. diminuta*	Paraoxon	50	3200	Dumas et al. (1990)
Phosphotriesterase	*P. diminuta*	DFP	100	64	Dumas et al. (1990)
Bimolecular Rate Constant (k_{cat}/K_m [M^{-1} min^{-1}])					
Q191/R191[b]					
PON1	Human	GD		$2.8 \times 10^6/2.1 \times 10^6$	Masson et al. (1998)
PON1	Human	GB		$9.1 \times 10^5/6.8 \times 10^4$	Masson et al. (1998)
PON1	Human	DFP		3.7×10^4/N.D.	Masson et al. (1998)
PON1	Human	Paraoxon		$6.8 \times 10^5/2.4 \times 10^6$	Masson et al. (1998)

[a] Not determined.

[b] Two most commonly occurring natural allelic variants of PON1 (Q191 and R191) have been identified. The activity of each form is shown.

TABLE 8.7
Kinetic Properties of Catalytic Mutated BuChE Bioscavengers

Bioscavenger	Substrate	Specificity Spontaneous Reactivation Rate Constant ($\times 10^3$ min^{-1})[a]	References
Wild-type Hu-BuChE	GB	<0.05	Lockridge et al. (1997), Millard et al. (1998)
Wild-type Hu-BuChE	VX	<0.05	Lockridge et al. (1997), Millard et al. (1998)
Wild-type Hu-BuChE	GD	<0.05	Lockridge et al. (1997), Millard et al. (1998)
G117H BuChE[b]	GB	5	Lockridge et al. (1997), Millard et al. (1998)
G117H BuChE	VX	7	Lockridge et al. (1997), Millard et al. (1998)
G117H BuChE	GD	<0.05	Lockridge et al. (1997), Millard et al. (1998)
G117H E197Q BuChE[c]	GB	62	Lockridge et al. (1997), Millard et al. (1998)
G117H E197Q BuChE	VX	78	Lockridge et al. (1997), Millard et al. (1998)
G117H E197Q BuChE	GD (P_SC_R)[d]	6	Lockridge et al. (1997), Millard et al. (1998)
G117H E197Q BuChE	GD (P_RC_R)[d]	6	Lockridge et al. (1997), Millard et al. (1998)
G117H E197Q BuChE GD	(P_SC_S)[d]	77	Lockridge et al. (1997), Millard et al. (1998)
G117H E197Q BuChE	GD (P_RC_S)[d]	128	Lockridge et al. (1997), Millard et al. (1998)

[a] The rate-limiting step in the hydrolysis of organophosphate nerve agents by mutated Hu-BuChEs is the enzyme reactivation step (Lockridge et al., 1997; Millard et al., 1998).

[b] A version of BuChE in which the glycine at amino acid residue 117 has been replaced by histidine.

[c] A double mutant of BuChE containing both histidine (rather than glycine) at amino acid residue 117 and glutamine in place of glutamic acid at residue 197.

[d] The reactivity with each of the four stereoisomers of GD was determined independently.

TABLE 8.8
Kinetic Properties of Mouse-Derived Catalytic Antibody Bioscavengers

Bioscavenger	Substrate Specificity	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	References
Antibody IIA12-ID10	GD, others?	330	25	Brimfield et al. (1993)
Antibody DB-108Q	GD, others?	110	16	Yli-Kauhaluoma et al. (1999)
Antibody DB-108P	GD, others?	100	53	Yli-Kauhaluoma et al. (1999)

(Gan et al., 1991; Masson et al., 1998; Josse et al., 1999a, 1999c, 2001, 2002; Tuovinen et al., 1999; Yeung et al., 2004, 2005) have intrinsic catalytic antiorganophosphorus activity. The *P. diminuta* enzyme has been shown to afford protection against soman lethality in mice and to protect against behavioral side effects (Table 8.6) (Broomfield, 1992). However, because this bacterially derived enzyme has no known mammalian homologs, it will likely be a potent initiator of immune responses and is therefore unlikely to be appropriate for use as a prophylactic scavenger in humans. However, a recent study suggested that encapsulation of bacterially derived OP-hydrolyzing enzymes in liposomes may extend their stability; this approach was shown to protect rats from multiple LD_{50}s of OP pesticides (Petrikovics et al., 2004). Bacterial enzymes conceivably could also be used for skin protection as active components of topical skin protectants (TSPs) or covalently bound to the cornified layer of epidermis (Parsa and Green, 2001). Detoxification of OPs does not need to proceed through hydrolysis; inactivation of these compounds can also be achieved through enzymatic oxidation of their alkyl chains. In particular, breakdown of VX by horseradish peroxidase (Amitai et al., 2002) or by *Caldariomyces fumago* chloroperoxidase (Amitai et al., 2003) could be used in polyfunctional active TSP and for skin decontamination. Additionally, the *P. diminuta* OPAH could be used as a one-time pretreatment either in addition to or in place of conventional therapy, because in the short-term this enzyme is highly effective against GD, GB, and VX, and alone, it induces no known behavioral effects.

The human PON1 (HuPON1) enzyme has been identified as having a similar potential for affording protection (Table 8.6), but without the complication of inducing an immune response; the human immune system should recognize HuPON1 as ''self'' and thus remain quiescent. The HuPON1 enzyme belongs to a family of plasma paraoxonases consisting of PON1, PON2, and PON3 (Primo-Parmo et al., 1996; La Du et al., 1999; Draganov et al., 2000; Furlong et al., 2002; Teiber et al., 2003; Aharoni et al., 2004; Harel et al., 2004b; van Himbergen et al., 2006). The three PON genes are located adjacent to each other at bands q21–q22 on chromosome 7 (Furlong et al., 2002). Of the three PON enzymes, PON1 is the most studied and best understood (Clendenning et al., 1996; Furlong et al., 2000a, 2000b). It was initially identified by Abraham Mazur (1946) when he reported the discovery of an OP-hydrolyzing enzyme in animal tissue (van Himbergen et al., 2006). Although the enzyme was named after its capacity to hydrolyze paraoxon, it was later discovered that it actually exhibits a broad spectrum of activities (Aldridge, 1953a, 1953b). Among the arrays of possible substrates of PON1 are aryl-esters, lactones, phospholipids, and organophosphates (Li et al., 1995, 2000; Davies et al., 1996; Furlong et al., 1998, 2000a, 2000b; Josse et al., 1999a, 1999c, 2002; Billecke et al., 2000; Broomfield et al., 2000; Jakubowski et al., 2001; Rodrigo et al., 2001; Teiber et al., 2003; Lacinski et al., 2004; Yeung et al., 2004; Khersonsky and Tawfik, 2005; Gaidukov et al., 2006). The enzyme is synthesized in the liver and bound to high-density lipoprotein (HDL) and possibly phosphate binding protein (PBP) before it is secreted into circulation (Blatter et al., 1993; Sorenson et al., 1999; Harel et al., 2004b; James and Deakin, 2004; Gaidukov et al., 2006; Morales et al., 2006; Rosenblat et al., 2006). The concentration of PON1 in human plasma is ~50 mg/L, but can vary by as much as 13-fold from one individual to another (Rochu et al., 2007). Of note, the level of PON1 in the circulation of small nonprimate mammals has been inversely correlated with susceptibility to OP intoxication (Li et al., 1993; Furlong et al., 1998, 2000b; Costa et al., 1999, 2003b, 2005). More

importantly, the exogenous administration of purified rabbit PON1 has been shown to protect against OP toxicity in both rats and mice (Li et al., 1995), whereas PON1 knockout mice are more sensitive to OP pesticides than wild-type mice (Shih et al., 1998).

Several polymorphisms of HuPON1 are known to exist, where the most prominent is the Q192R allozyme, which can have a substantial impact on PON1 activities (Furlong et al., 1998, 2000b, 2002; Billecke et al., 2000; Li et al., 2000; Costa et al., 2003a; Yeung et al., 2004; Gaidukov et al., 2006). Although the in vivo substrate of HuPON1 is presently unknown, there is substantial evidence that it may act as a lactonase in vivo (Jakubowski et al., 2001; Teiber et al., 2003; Lacinski et al., 2004; Khersonsky and Tawfik, 2005, 2006). HuPON1 also appears to have antiatherosclerotic properties, protecting low-density lipoprotein (LDL) from oxidation (Aviram et al., 1998; Shih et al., 1998; Brites et al., 2004; van Himbergen et al., 2006). The mature enzyme retains its N-terminus signal peptide and contains 354 amino acid residues. Depending on its glycosylation state, the molecular weight of HuPON1 can range from 38 to 45 kDa (Hassett et al., 1991; Clendenning et al., 1996; Furlong et al., 2000a). Although the exact structure and catalytic mechanism of the HuPON1 enzyme are unknown, results from analyses of a gene-shuffled, bacterially expressed PON1 variant suggest that HuPON1 is a sixfold beta-propeller protein and that catalysis is mediated by a unique His–His dyad (Harel et al., 2004b). It has been established that the PON1 enzyme is a serum OP hydrolase with an absolute requirement for two Ca^{2+} ions (Sorenson et al., 1995; Kuo and La Du, 1998). One calcium is required to maintain the overall structural integrity of the protein, while the other is essential for enzymatic activities (Sorenson et al., 1995; Kuo and La Du, 1998; Josse et al., 1999c; Jakubowski, 2000). Additionally, through chemical modification and site-directed mutagenesis studies, a number of amino acid residues have also been identified as essential for activity (Doorn et al., 1999; Josse et al., 1999a, 1999b, 1999c, 2001, 2002; Aharoni et al., 2004; Harel et al., 2004b; Yeung et al., 2004, 2005; Amitai et al., 2006; Khersonsky and Tawfik, 2006; Rosenblat et al., 2006).

A preliminary study showed that in animals not pretreated with CBDP to inhibit plasma CaE (Maxwell et al., 1987) a dose of 1 mg/mouse of HuPON1 administered in the vein tail 5 min prior to subcutaneous injection of soman does not provide protection against $\geq$1 LD_{50} of this OP; however, a mild protective effect in terms of seizure rate and incapacitation was observed for lower doses of soman (Lallement et al., unpublished). Because the effects of plasma CaE were not reduced (Maxwell et al., 1987, 1998), these results suggest that protection against multiple LD_{50}s of nerve agents could be achieved by using either higher doses of wild-type HuPON1 (5–10 mg/mouse) or mutants of the enzyme with enhanced catalytic activity toward OPs. In addition, the effects would be expected to be more pronounced in a species with little or no plasma CaE.

Although the enzymes discussed above possess the desired catalytic activity, none of them is enzymatically fast enough for use as a nerve agent pretreatment. Because the organophosphorus anticholinesterases have been in the environment for only a little over 50 years, it is not likely that any of the enzymes identified as OPAHs have as their primary function the destruction of OPs. In fact, an OPAH from an *Alteromonas* species has been identified as a prolidase, a dipeptidase that cleaves the penultimate proline from the carboxyl end of a peptide (Cheng et al., 1997). Recently, it was suggested that HuPON1 is a homocysteine thiolactone hydrolase and can protect against protein N-homocysteinylation (Jakubowski, 2000; Jakubowski et al., 2001; Lacinski et al., 2004; Khersonsky and Tawfik, 2005, 2006). Because these enzymes are not likely to have evolved to efficiently hydrolyze OP compounds, it is probable that through mutagenesis, substantial improvements in their catalytic efficiencies can be obtained. To be functional, a catalytic scavenger must have an enzymatic efficiency (the ratio k_{cat}/K_m) as high as possible; none of the catalytic anti-OP enzymes identified to date have a sufficiently low K_M (a measure of the strength of binding of a substrate to the enzyme) or sufficiently high k_{cat} (turnover number) to clear OPs from the bloodstream within the 1–2 min before they can reach critical targets (Broomfield et al., 1991). Therefore, efforts to enhance the catalytic efficiency of human enzymes through introduction of specific amino acid substitutions were initiated. Obvious candidates for such attempts include members of the ChE family (including CaE) and the paraoxonases, which already possess the

desired activity but at insufficient levels. The rationale for the design of mutations in the ChE family was based on the fact that for these enzymes, the organophosphorus inhibitors are in reality hemisubstrates; their initial reaction with the enzyme is similar to that of normal substrates. However, the subsequent reaction, equivalent to deacylation of the active site serine, is blocked because of the geometry of the active site. The amino acid group responsible for deacylation is not in an appropriate position to effect dephosphorylation (Jarv, 1984; Broomfield et al., 1995).

The perceived solution to this problem was to insert a second catalytic center into the active site specifically to carry out the dephosphylation step of the reaction (Broomfield et al., 1995). Applying this rationale, the human wild-type BuChE has been mutated in the oxyanion hole (Figure 8.3 and Table 8.7) to express a mutant enzyme, G117H, with the ability to catalyze the hydrolysis of sarin, DFP, paraoxon, VX, and other nonaging nerve agents (Broomfield et al., 1995; Lockridge et al., 1997). Aging and reactivation are parallel first-order reactions in phosphylated enzymes. In the reactivation reaction, the phosphoryl group is removed from the active site serine residue (S198), restoring activity, whereas in the aging reaction one of the alkyl groups is removed from the phosphoryl group, rendering the group nonreactivatable. To affect the hydrolysis of rapidly aging nerve agents such as soman it is necessary to inhibit the aging reaction so that reactivation is faster. This was accomplished by replacing the carboxyl group (Glutamic acid, E197) adjacent to the active site serine with an amide (Glutamine, Figure 8.4) (Lockridge et al., 1997). Unfortunately, these mutants have catalytic activities that are too slow for practical use (Table 8.7), though they might have applications as self-reactivating stoichiometric scavengers that would be useful for protection against chronic, low-level or percutaneous exposures. As an alternative to such protein engineering, it is possible through careful design and synthesis of transition state analogs to immunize mice and

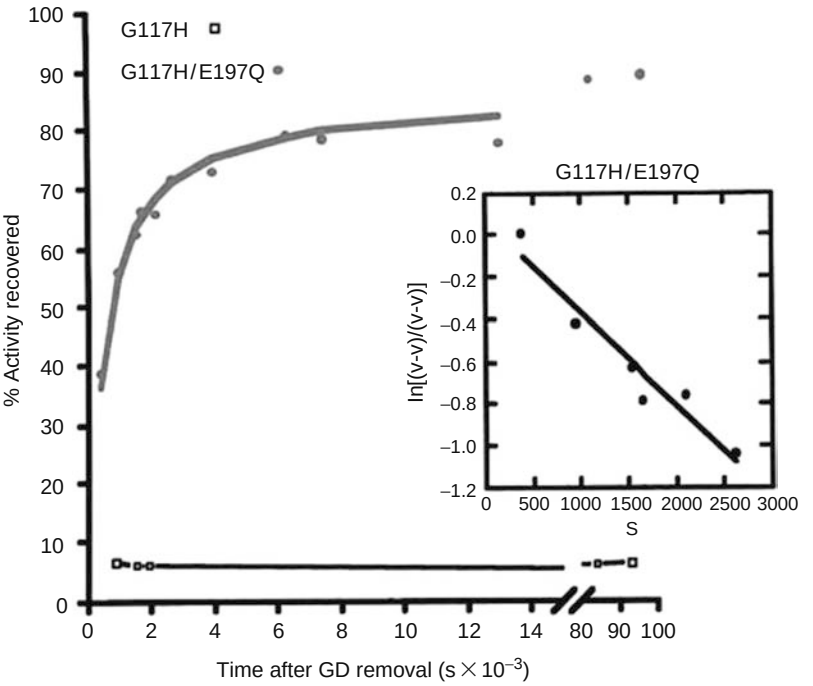

FIGURE 8.3 Comparative reactivation kinetics of soman-inhibited human butyrylcholinesterase single mutant G117H (□) and double mutant G117H/E197Q (●). Note that the recovery rate of the double mutant is very fast (with reaction rates of 77,000 and 128,000/min for the P_SC_S and P_SC_R isomers of soman, respectively), whereas the single mutant does not recover measurably. The insert shows that reactivation of the double mutant with soman can be treated as a first-order reaction for at least 2.5×10^3 s. (Data are from Millard, C.B. et al., *Biochemistry*, 37, 237, 1998.)

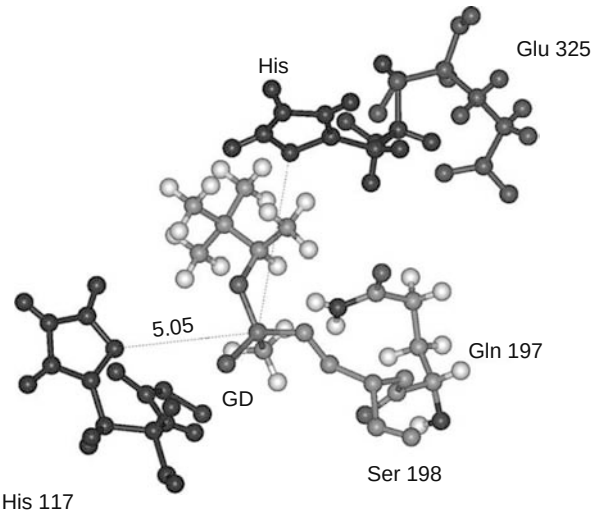

FIGURE 8.4 A ball-and-stick computer model of the active site of the double mutant of butyrylcholinesterase G117H/E197Q. In addition to the His 117 and Gln 197, the active site triad amino acid residues of His 438, Ser 198, and Glu 325 are also depicted with soman at the active site. The distances between the phosphorus atom of soman and His 117 is 5.05 Å and distance between the phosphorus atom of soman and the active site His 438 is 5.94 Å. (Data are from Millard et al., 1998.)

elicit antibodies with catalytic activities against substrates. This approach has been used to create catalytic anti-soman antibodies, albeit with catalytic efficiencies too low to be useful as in vivo scavengers (Table 8.8).

In addition to BuChE, AChE, human CaE, and PON1 are currently being subjected to mutation in efforts to generate additional, faster catalytic anti-nerve agent enzymes. For example, multiple mutations to the active site of an AChE enzyme were recently reported to result in an OP hydrolyase, albeit with slower activity than the G117H mutant of Hu-BuChE (Poyot et al., 2006). It is important to note that in the case of HuPON1, the desired catalytic activity is already present at low levels in the native enzyme. Because OPs are ‘‘accidental’’ substrates for PON1 [see above (Masson et al., 1998; Tuovinen et al., 1999)] it is likely that improvement in activity can be realized through protein engineering. Two of the major difficulties in designing appropriate site-directed mutations of PON1, the lack of knowledge of the residues at the active site and of the enzyme’s three-dimensional structure, were recently overcome by the work of Josse et al. (1999a, 1999b, 1999c, 2001), Aharoni et al. (2004), Harel et al. (2004a,b), and Yeung et al. (2004, 2005). Josse postulated, based on site-directed mutations of amino acids predicted to be at or near the active site of PON1 and by structural homology modeling with squid DFPase, that HuPON1 is a sixfold beta-propeller protein. Harel et al. and Aharoni et al., using a mouse–rat–rabbit–human chimeric gene obtained through gene shuffling and expressed in bacteria, confirmed the postulated structure through x-ray crystallographic studies. Yeung et al. (2004, 2005) have subsequently created site-directed mutants of HuPON1 to identify and ‘‘map’’ amino acids critical for substrate binding and catalytic activity. Other studies have revealed a degree of stereospecificity in the hydolysis of soman by native HuPON1 (Amitai et al., 2006; Yeung et al., 2007). These findings all support the goal to design a recombinant version of PON1 with enhanced anti-OP catalytic activity. Because PON1 is a naturally occurring plasma enzyme produced in the liver, an alternative approach would be to enhance the endogenous enzyme biosynthesis by inducing increased activity of its gene promoter. Recent results on increased expression levels of PON1 by HuH7 hepatoma cells upon action of fibrates and dietary polyphenols are promising in this regard (Gouedard et al., 2003, 2004).

Although most of the potential catalytic scavengers described here have not yet been tested for efficacy in animal models, they are indicative of the types of drugs that may soon be available for use in animals, including humans. Because mutations of BuChE, CaE, and PON1 involve changes of internal amino acid residues in human proteins, the expectation is that these proteins would induce minimal immunological or behavioral side effects.

VI. BEHAVIORAL EFFECTS

Because overt signs, symptoms, or physiological responses may not accompany many low-level exposures to OPs, behavioral toxicological measures may be chosen to detect any toxic changes wrought by exposure. Under such conditions, it is important to ensure that biological scavengers, represented either as elevated levels of naturally occurring proteins or as mutant forms thereof, do not elicit behavioral effects of their own after administration. Unique behavioral effects might also result after pretreatment with a biological scavenger followed by exposure to a nerve agent; it is of great interest to compare the extent of behavioral side effects that ensue from pretreatment with scavenger alone or followed by nerve agent exposure versus those induced by exposure to nerve agent followed by conventional therapy. The discussion here will be limited only to the side effects, if any, resulting from administration of scavengers alone. The other topics, including the ability of scavengers to ameliorate behavioral side effects following nerve agent exposure and the advantages of scavengers versus conventional therapy are discussed in detail elsewhere (Lenz and Cerasoli, 2002).

VII. BEHAVIORAL EFFECTS OF SCAVENGERS ALONE

Most studies that have examined the behavioral effects of biological scavengers have done so by comparing a behavior before scavenger administration, after scavenger administration, and then after exposure to nerve agent (see Lenz and Cerasoli, 2002). There are, however, several studies that have examined the behavioral effects of the biological scavengers themselves in the absence of ChE inhibitors. In a study by Genovese and Doctor (1995), rats were trained to perform three behavioral paradigms: a passive avoidance task, a motor activity, and a scheduled–controlled behavior (Table 8.9). The performance of animals before and after administration of purified eq-BuChE at a dose that would be expected to provide protection against an exposure of several LD_{50}s of an OP compound was assessed. They determined the pharmacokinetic profile of eq-BuChE in rats and then examined the behavior of the animals in the passive avoidance task when the levels of administered eq-BuChE were maximal. Subsequently, the animals were tested after enzyme levels had started to diminish, to enhance the opportunity of detecting any behavioral effects. During the activity tests, individually housed animals were allowed to habituate. Enzyme was given such that maximum levels would be present in circulation about 1 h before the beginning of dark cycle. Motor activity was then monitored for 10 days. As a final test, the effects of excess enzyme were examined in rats trained to perform a variable interval 56 s (VI56 s) schedule of food reinforcement. Previously, cholinergic compounds had been shown to disrupt performance of this task. Animals were observed for 10 days to ensure that any prolonged or delayed effects would be noted. In all cases for all test paradigms, the authors report that eq-BuChE did not disrupt performance of any of the learned tasks, did not upset the circadian cycle of light/dark activity and had no effect on motor activity. They noted that these outcomes were in contrast to those observed when the standard cholinolytic, atropine, was administered. Finally, they evaluated the protective effects of the levels of enzyme given to the rats in the behavioral studies against MEPQ, a peripherally active organophosphorus compound. Although the level of protection observed was lower than the theoretical prediction, the authors suggested that the simultaneous administration of scavenger and MEPQ might have reduced the efficacy of the administered eq-BuChE.

In a separate study, also using eq-BuChE, rhesus monkeys were trained to perform an SPR task (Matzke et al., 1999). Using a six-object list, the monkeys were tested for same–different

TABLE 8.9
Extent of Behavioral Deficits Following Bioscavenger Administration or Conventional Therapy

Protection	Species	Behavioral Tests	Impairment[a]	Recovery Time[b]	References
Atropine	Rat	Passive avoidance, VI56 s schedule	Total	>1 week	Genovese and Doctor (1995)
eq-BuChE	Rat	Passive avoidance, motor activity, VI56 s schedule	None	Immediate	Genovese and Doctor (1995)
BuChE	Rat	Morris water maze	None	Immediate	Brandeis et al. (1993)
Pyridostigmine	Rhesus monkey	Primate equilibrium platform (PEP)	Substantial	N.D.[c]	Blick et al. (1994)
eq-BuChE	Rhesus monkey	Serial probe recognition (SPR)	None	Immediate	Matzke et al. (1999)
eq-BuChE	Rhesus monkey	Observation, SPR	Subtle SPR defect	~6 days	Castro et al. (1994)
eq-BuChE	Rhesus monkey	Observation, SPR	None	Immediate	Matzke et al. (1999)
BuChE	Rhesus monkey	Spatial discrimination	Minor (1/4 had errors)	≥1 day	Raveh et al. (1997)

[a] Behavioral impairment relative to untreated animals.
[b] Time elapsed before performance returns to pretreatment levels.
[c] Not determined.

discrimination and delayed same–different discrimination. Once the animals became proficient at the task (80% correct for three successive sessions on three consecutive days) they received eq-BuChE in a dose similar to that reported by Broomfield et al. as sufficient to afford protection against 2 or 3 LD_{50}s of soman (vide supra) (Fidder et al., 1994). The authors reported that repeated administration of commercially prepared eq-BuChE had no effect on the behavior of the monkeys as measured by the SPR studies (Table 8.9). Given the lack of behavioral effects and the relatively long in vivo half-life of the eq-BuChE, they concluded that this biological scavenger was potentially more effective than current chemotherapeutic treatments for organophosphorus intoxication. Other studies in rodents or monkeys using human BuChE also showed virtually no behavioral effects following administration of this enzyme (Brandeis et al., 1993; Raveh et al., 1997; Matzke et al., 1999). In particular, it has recently been reported that administration of high doses (90 mg/kg, i.e., a dose 30-fold higher than that necessary for protection of 2 LD_{50} of soman in humans) of BuChE to mice induced no toxic behavioral effects (Saxena, 2002).

VIII. SUMMARY

Organophosphorus nerve agents represent a very real threat not only to warfighters in the field but also to the public at large (Ember, 1991). Nerve agents have already been used by terrorist groups against a civilian population and, due to their low cost and relative ease of synthesis, are likely to be used again in the future (Masuda et al., 1995). In addition, many commonly used pesticides and chemical manufacturing by-products can act as anticholinesterases and may be a low-dose exposure threat to workers in a variety of professions. Also, we cannot rule out the possibility of the use of anticholinesterase pesticides against civilians in a terrorist context. Current therapeutic regimens for acute nerve agent exposure are generally effective at preventing fatalities if administered in an appropriate time frame. For acute multi-LD_{50} levels of exposure, pyridostigmine pretreatment

coupled with postexposure administration of an oxime, atropine, and an anticonvulsant does not prevent the substantial behavioral incapacitation or, in some cases, permanent brain damage that can result from organophosphorus poisoning, although it does enhance survival. It is therefore important from both military and domestic security perspectives to develop novel defenses against nerve agents, including the use of bioscavenger molecules that avoid many of the difficulties associated with current treatments. Although the use of nerve agents on the battlefield may be somewhat predictable, their use in a terrorist situation will be, in all probability, an unanticipatable event. The ability to afford long-term protection for first-responders exposed to toxic or incapacitating concentrations of OPs is a notable potential advantage of biological scavengers.

The use of bioscavengers as a defense against OP intoxication has many advantages and few apparent disadvantages. As discussed in detail above, bioscavengers can afford protection against not only mortality, but also most or all of the adverse physiological and behavioral effects of nerve agent exposure. They can be administered prophylactically, precluding the need for immediate postexposure treatment. In addition, the use of bioscavengers has several psychological benefits that are likely to result in a higher degree of user acceptability than exists for conventional therapy. No postexposure autoinjectors are necessary, and protection is afforded with little chance of short- or long-term side effects. Of particular significance is the fact that current candidate bioscavenger proteins are, for the most part, enzymes of human origin. From a scientific standpoint, these proteins are good candidates because they are less likely to be recognized by cells of the immune system and will enjoy prolonged residence times in circulation. From a user point of view, individuals are in essence being protected against nerve agents using a substance that their bodies already produce, rather than being injected with drugs and enzyme inhibitors that alone can produce potent side effects; such a distinction may enhance the comfort and compliance of end users.

There are several challenges that must be met in the future before bioscavengers can augment or replace the current therapeutic regimes for nerve agent intoxication. First, scavenger proteins, either alone or in combination, with a range of specificities that encompasses all known nerve agents, must be defined. It is recognized that the immunogenicity and serum half-life of the scavengers must be determined in humans, and efforts may be required to minimize the former and maximize the latter. Finally, appropriate dosages of scavengers that will, based on animal models, protect against concentrations of nerve agents likely to be encountered under a wide range of scenarios must be determined. Although the majority of the research to date has focused on stoichiometric scavengers, the use of either naturally occurring or genetically engineered enzymes with catalytic activity holds the greatest theoretical promise for the development of a broad specificity prophylactic scavenger. Future efforts are likely to focus on generating, characterizing, and using such enzymes in rodent and nonhuman primate models.

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9 Butyrylcholinesterase and Its Synthetic C-Terminal Peptide Confer In Vitro Suppression of Amyloid Fibril Formation

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CONTENTS

I. INTRODUCTION

The reaction of alcohols and phosphoric acid yields organophosphate (OP) molecules that prevent the breakdown of the neurotransmitter acetylcholine (ACh) through inhibition of the hydrolytic activity of acetylcholinesterase (AChE). OPs were initially aimed to fight insects, but further

developments turned them into chemical weapons (Gallo and Lawryk, 1991; Chambers et al., 2001). Within the central nervous system and in the neuromuscular junctions, AChE is vital for the transmission of cholinergic impulses. A closely similar enzyme, butyrylcholinesterase (BChE) is also capable of hydrolyzing ACh. It has been suggested that human BChE from plasma participates in the endogenous scavenging of naturally occurring drugs (e.g., physostigmine, cocaine), man-made therapeutics (e.g., succinylcholine), as well as anticholinesterase-based pesticides and chemical warfare agents (Raveh et al., 1993, 1997; Jbilo et al., 1994; Schwarz et al., 1995; Sun et al., 2002; Giacobini, 2004), and as such serves as an inherent protector from the short-term damages caused by neurotoxic agents (Glick et al., 2003). Consequently, BChE administration was proposed as a putative prophylactic and therapeutic treatment of OP-poisoned subjects (Doctor and Saxena, 2005). Preclinical studies in various experimental animal models (rats, mice, and monkeys) supported the notion that this treatment may be palliatively successful (Ashani et al., 1991; Doctor and Saxena, 2005). However, the dangers of OP poisoning are not limited to the immediate effects, and it remained unclear whether BChE administration per se would not entail long-term damages. This question became particularly important in view of findings that synaptic acetylcholinesterase (AChE-S) exacerbates the formation of amyloid fibrils, the primary neuropathology characteristic of Alzheimer's disease (AD) (Inestrosa et al., 1996a, 1996b, 2005; Rees et al., 2003, 2005).

AChE and BChE show considerable similarities at the genetic, structural, and enzyme kinetics levels (Chatonnet and Lockridge, 1989; Soreq and Zakut, 1993). In the human brain, BChE is found in neurons and glial cells, as well as in neuritic plaques and tangles in AD patients (Darvesh et al., 2003). Moreover, BChE activity increases in the AD brain, whereas AChE activity decreases progressively (Greig et al., 2002; Giacobini, 2003). This finding raised the question whether BChE, like AChE-S, facilitates the formation of amyloid fibrils. Here, we present findings indicating that BChE does not facilitate—rather, it may attenuate the formation of amyloid fibrils. As such, it is advantageous over AChE-S in that its use would not entail long-term damages; rather, it may possibly serve to neuroprotect treated subjects not only at the short time range, but also in a longer time frame.

Thioflavin T (ThT) fluorescence measurements (Jarrett and Lansbury, 1993) enabled us to reveal that BChE is capable of attenuating the in vitro formation of amyloid fibrils from the AD beta-amyloid peptide, unlike AChE-S, which facilitates such fibril formation. The C-terminal of BChE was identified as responsible for this attenuation function, and a synthetic peptide having the C-terminal sequence of human BChE, BSP41 was found capable of performing a similarly effective attenuation of fibril formation as BChE. AChE-S, in comparison, promoted fibril formation, and its C-terminal peptide, ASP40, failed to affect this process.

Structural modeling and circular dichroism (CD) analyses revealed that both ASP40 and BSP41 peptides form symmetric amphipathic helices. Site-directed mutagenesis showed that an arginine-to-tryptophan substitution in BSP41 is an essential, although insufficient, cause of this intriguing feature of BSP41. Thus, the imperfect amphipathicity of this helix, and the polar properties in the microenvironment of the substituted tryptophan, cooperate to attenuate fibril formation. Together, these findings present neuronal BChE as a potential inherent natural protector from the development of AD neuropathology and increase its value for future therapeutic uses, as it can delay and prevent the long-term neurotoxic consequences of exposure to poisonous warfare agents.

II. BACKGROUND

A. Butyrylcholinesterase Functions as a Natural Bioscavenger

BChE (EC 3.1.1.8), the primary circulating cholinesterase, is found in addition to serum, in liver, lungs, muscles, and heart. BChE presumably functions in lipoprotein metabolism, myelin maintenance, and cellular adhesion (La Du and Lockridge, 1986; Jbilo et al., 1994). In the human brain, the enzyme is found in neurons and glia and was proposed to participate in neurogenesis and neurite

growth (Darvesh and Hopkins, 2003). In AD, BChE's activity increases in the brain (Ballard et al., 2005), where it is found in senile plaques and neurofibrillary tangles (Carson et al., 1991; Guillozet et al., 1997), co-localizing with brain fibrils of amyloid-β peptides (Aβ) (Wright et al., 1993).

In vitro and in vivo studies demonstrate that BChE functions as a natural bioscavenger. Operating like a sponge, it may absorb and degrade OP poisons (e.g., nerve agents) before they cause neurological damage (Grunwald et al., 1997). Of the cholinesterases evaluated so far, human serum BChE (purified from plasma) provided substantial support to this neuroprotection concept in terms of preclinical development. However, human serum proteins are available in limited quantities, and their use may entail health-related dangers. A putative alternative was offered by transgenic goats, producing human BChE in their milk (Cerasoli et al., 2005). Here, we demonstrate that BChE's protective role expands beyond OP poisoning and includes also protection against amyloid neurotoxicity, in contrast with AChE-S, which poses putative long-term risks. Our findings predict a previously nonperceived added value to the therapeutic use of human BChE protein.

B. Definition of Amyloid-β Peptides

The amyloid-β peptide (Aβ) has been identified as a 4 kDa hydrophobic nonglycosylated peptide consisting of 39–43 amino acid residues. It is derived by specific endoproteolytic cleavages from a 700 amino acid residue-long membrane-associated glycoprotein, named ''amyloid precursor protein'' (APP) (Citron et al., 1996; Dickson, 1997). Aβ appears in bundles of amyloid fibrils surrounded by abnormal neurites, and is believed to be the major component of the vascular and plaque filaments in individuals with AD, elderly people, and trisomy 21 carriers (Down's Syndrome) (Head and Lott, 2004).

In vitro studies indicate that synthetic $A\beta_{1-42}$ ($A\beta_{42}$) can form insoluble aggregates and cause neurotoxicity after incubation for several days (Forloni et al., 1996). These $A\beta_{42}$ assemblies may be stained by Congo red and Thioflavin S, similar to those observed in the AD brain (Jarrett and Lansbury, 1993). Both $A\beta_{1-40}$ ($A\beta_{40}$) and $A\beta_{42}$ are formed intracellularly, but exert damaging effects when transported outside of cells. $A\beta_{42}$ is more hydrophobic and toxic than $A\beta_{40}$, and its fibrils clump together to form amyloid plaques (Lue et al., 1999). Although $A\beta_{42}$ is the abundant Aβ form in neuritic plaques, $A\beta_{40}$ is more abundant in cerebrovascular plaques (Lue et al., 1999).

C. Toxic Aβ Protofibrils Precede Plaque Formation

Several studies indicate that the toxicity of Aβ and other amyloidogenic proteins lies not in the insoluble fibrils that accumulate in AD plaques, but rather in the soluble oligomeric intermediates (Hardy and Selkoe, 2002). These soluble oligomers include spherical particles of 2.7–4.2 nm in diameter and curvilinear structures called ''protofibrils'' that appear to represent strings of the spherical particles (Hartley et al., 1999). Recently, it was proposed that a 56 kDa soluble Aβ assembly contributes to the cognitive deficits associated with AD (Lesne et al., 2006). The soluble Aβ oligomers form protein micelles because Aβ is an amphipathic surface-active peptide (Cruz et al., 1997). Intriguingly, Aβ oligomers formation displays critical concentration dependence, and is correlated with the appearance of a hydrophobic environment (Soreghan et al., 1994; Tjernberg et al., 1999; Levine, 2003). The content of soluble Aβ in the human brain is better correlated with the severity of the disease than are plaques (Kuo et al., 1996; Lue et al., 1999; McLean et al., 1999). Thus, soluble oligomers may be more important pathologically than the fibrillar amyloid deposits (Kayed et al., 2003), and agents counteracting their toxicity are continuously sought.

In vitro toxicity studies with synthetic Aβ peptides have shown that Aβ, in an aggregated state (fibril, protofibril, low molecular weight oligomer, or diffusible, nonfibrillar ligand), is toxic to neurons in culture (Pike et al., 1991). $A\beta_{42}$ oligomers that are soluble and highly neurotoxic assemble under conditions that block fibril formation (Chromy et al., 2003). These oligomers

bind to dendritic surfaces in small clusters with ligand-like specificity and are capable of destroying hippocampal neurons at nanomolar concentrations (Standridge, 2006). AD is likely triggered by these soluble, neurotoxic assemblies of Aβ rather than the late stage pathology landmarks of amyloid plaques and tangles (Standridge, 2006), suggesting that both inherent and therapeutic neuroprotective agents would operate by interacting with such assemblies and by masking their neurotoxicity.

III. METHODOLOGY

A. Enzymes

Purified human BChE (from human serum, ~90% pure, as demonstrated by gel electrophoresis, data not shown) and recombinant human AChE-S were from Sigma (Jerusalem, Israel). Enzymatic activity was determined by hydrolysis rates of butyryl- or acetyl-thiocholine, respectively, at 25°C or 4°C, as indicated. Enzyme concentrations were calculated based on the molecular weight of a protein monomer and its known amino acid composition.

B. Peptides

Peptides were synthesized according to the C-terminal sequences of human AChE-S and BChE (SwissProt accession nos. P06276 and P22303, respectively) using a Pioneer or a 433A peptide synthesizer (Applied Biosystems). Purification (>90%) by reverse-phase HPLC was followed by MALDI-TOF mass spectrometry.

C. Kinetics of Aβ Fibril Formation Monitored by Fluorescence Measurements

The fluorescence excitation spectrum of the Thioflavin T (ThT, Sigma, Jerusalem, Israel) shifts from 340 to 450 nm when interacting with β-sheet amyloid structures (Levine, 1993, 1999). Fluorescence signals (excitation, 450 nm; emission, 485 nm) reflect the amount of amyloid fibrils formed.

To follow the kinetics of amyloid fibril formation, a synthetic $A\beta_{40}$ peptide (from Biosource, Camarillo, CA, USA or Sigma, Jerusalem, Israel) was dissolved in 1.6 mM dimethylsulfoxide (DMSO), sonicated (3×5 sec pulses), filtered through 0.22 μm filters, and stored at −70°C. Aliquotes of Aβ were diluted to a final concentration of 162 μM in 20 μL phosphate-buffered saline (PBS) containing 0.02% NaN_3 (fibrillation buffer) with or without cholinesterases or synthetic peptides, using white multiwell plates (96 wells, Nunc, Roskilde, Denmark). Following 20 min pre-incubation at room temperature, 80 μL of 1.25 μM ThT in 50 mM glycine-NaOH, pH 8.5, was added for 6–10 h shaking at 200 rpm, 30°C. Fluorescence was measured at 10–30 min intervals.

Assessment of the fibril formation process involved measuring the lag preceding the onset of fluorescence increase (the nucleation process) and the apparent maximal rate of fluorescence increase (rate of fibril formation) for 300–600 min, depending on the duration of the lag phase. Mean standard error and the kinetic parameters were calculated using Kaleidagraph Software (Reading, PA, USA).

D. Circular Dichroism Measurements

Synthetic peptides, based on the C-terminal sequences of AChE-S and BChE, were dissolved in double distilled water (DDW) to a final concentration of 1×10^{-4} M. Direct CD spectra were recorded at room temperature using a CD Jasco J-810 Spectropolarimeter (Easton, MD, USA) using Hellma 100-QS; 1 mm path length quartz cuvette (Hellma GmbHandCoKg, Germany). Recordings were at 0.5 nm intervals in the spectral range 185–260 nm.

E. Peptide Modeling

Model construction of the analyzed peptides involved the Deep View spdbv 3.7 software (Glaxo Smith Kline, Bredford, UK) followed by distance geometry minimization. Figures were created using the PyMol software (DeLano Scientific LLS, San Carlos, CA, USA). Symmetric tetramers were modeled using CHI suite of macros for CNS 1.1 (Adams et al., 1995). Calculation was carried out in vacuo in initial coordinates of a canonical alpha helix (3.6 res. per turn) and initial cross angle (25°).

IV. OUTCOME AND DISCUSSION

A. Circulation Butyrylcholinesterase Levels Increase with Age

In a U.S. cohort of 472 subjects of Caucasians and African-Americans, the serum levels of both AChE and BChE were found to increase with age by approximately 20% between 20 and 55 years of age (Figure 9.1, Sklan et al. [2004]). Substrate hydrolysis rates per mL serum were predictably 20-fold higher for BChE as compared with AChE, in spite of the slower rate of ACh hydrolysis by BChE (Schwarz et al., 1995). This emphasizes the pivotal importance of BChE for determining the cholinergic status in the human circulation. Parallel findings were obtained in an Israeli cohort (Bryk et al., 2005), suggesting accumulation of cholinesterases in the circulation of aging individuals from various ethnic origins.

The possibility of causal involvement of BChE in the progression of AD has long been discussed in the literature (Ballard et al., 2005), but its possible mode of action remained obscure. Our findings demonstrate that BChE is capable of delaying and suppressing fibril formation,

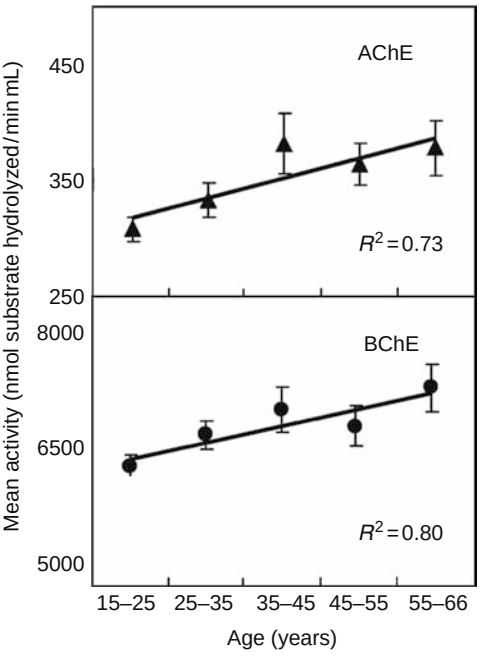

FIGURE 9.1 Progressive elevation of serum AChE and BChE activities with age. Shown are the average values for a U.S. cohort of 472 volunteers. (From Sklan, E.H., Lowenthal, A., Korner, M., Ritov, Y., Landers, D.M., Rankinen, T., Bouchard, C., Leon, A.S., Rice, T., Rao, D.C., Wilmore, J.H., Skinner, J.S. and Soreq, H., *Proc. Natl. Acad. Sci. U.S.A.*, 101, 5512, 2004.) R^2 values for the age-dependant increases in AChE and BChE activities were calculated by using the two-tailed Student's *t* test.

inverse to the facilitation of this process by the highly homologous AChE-S protein. Intriguingly, the core of BChE's action on amyloid fibrils lays in its C terminus, whereas the corresponding peptide of AChE-S failed to affect this process.

B. Butyrylcholinesterase Attenuates Fibril Formation in a Dose Dependent Manner

Fibril formation from 33 μM Aβ peptide was first quantified by measuring online changes in ThT fluorescence in reaction mixtures including increasing concentrations of purified human BChE, recombinant AChE-S, or both. The presence of AChE-S or BChE conferred distinct kinetics of amyloid-β sheets formation in vitro. Our preassumption was that in the immediate vicinity of nerve cells, Aβ concentrations would exceed those of BChE by far. Therefore, we used 1:100 ratios of cholinesterases to Aβ. Purified BChE added to Aβ prolonged the lag and reduced the apparent rate of amyloid fibril formation in a dose-dependent manner. Thus, addition of 2.4 μg BChE to 14.3 μg Aβ in a total volume of 100 μL prevented fibril formation for over 600 min. In contrast, addition of similar doses of AChE-S to Aβ caused an inverse effect by shortening by half the lag time before fibril formation (from 240 to 150 min) and increasing the rate of fibril formation in a dose-dependent manner. Compatible with the findings of others, 1:100 molar amounts of AChE-S increased the apparent maximal rate of amyloid fibril formation from 0.36 fluorescence units/h (McLean et al., 1999) for 14.3 μg Aβ alone to 0.696 for Aβ in the presence of 2.3 μg AChE-S, in a total volume of 100 μL (Figure 9.2). Thus, the effects of AChE and BChE on this process were inverse to each other. Next, we challenged the capacity of BChE to compete with the AChE effect. BChE doses added to a mix of 33 μM Aβ in the presence of 0.4 μM AChE-S caused dose-dependent interference with the fibril formation process (Diamant et al., 2006). Importantly, the rate of fibril formation in the presence of both enzymes receded to the slow rate observed for Aβ

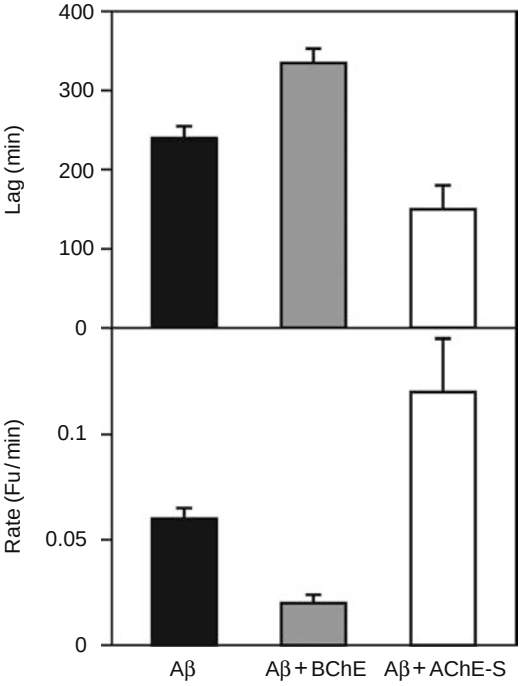

FIGURE 9.2 BChE prolongs the lag and reduces the rate of fibril formation. Shown are the average values for the lag period preceding fibril formation and the rate of changes in ThT fluorescence for Aβ alone, BChE + Aβ, and AChE-S + Aβ.

alone. This demonstrated a capacity of BChE to attenuate the fibril formation-accelerating effect of AChE-S when both proteins are present at equal doses.

C. Butyrylcholinesterase–Aβ Interactions Affect Its Catalytic Properties

The BChE effect could be relevant to each or all of the different intermediates of the Aβ fibril formation process. To address this issue, we incubated 40 μM $A\beta_{40}$ peptide with 1:100 (0.4 μM) BChE or AChE-S and separated the soluble and insoluble fractions by centrifugation. We then assessed the presence of catalytically active cholinesterases in each of these fractions by measuring their enzymatic activities. AChE-S predictably disappeared within 2–4 days from the soluble fraction and appeared in the pellet (data not shown), supporting the notion that it facilitates fibril formation by interacting with the forming fibrils and compatible with the findings of others (Alvarez et al., 1998). In contrast, BChE was found to be present almost exclusively (>95%) in the soluble fraction of Aβ over the entire time range of fibril formation (up to 7 days) (Diamant et al., 2006). A similar distribution of BChE activity was obtained when the rate of fibril formation was enhanced by shaking and followed the time scale of hours (data not shown). These findings indicate that the attenuating effect of BChE involved interaction with soluble Aβ. To challenge this prediction, the enzymatic properties of the soluble BChE–Aβ mixture were tested for assessing possible effects of BChE interaction with Aβ on its hydrolytic capacity. BChE's affinity to its substrate was therefore determined following 22 h preincubation in the presence or absence of Aβ. To avoid dissociation of BChE–Aβ complexes, the preincubation mixture was rapidly cooled to 4°C. Supernatant and pellet were separated and butyrylthiocholine hydrolytic activities were determined at 4°C. The substrate affinity of soluble BChE incubated without Aβ was reflected by a K_m of 0.7 mM. In contrast, BChE preincubated with Aβ showed substrate binding with a K_m value of 2.9 mM, reflecting an over 4 times lower affinity than that for the enzyme incubated alone (Figure 9.3). This decrease in substrate affinity likely reflects binding of BChE to one or several soluble intermediates of the Aβ fibrillation process, present in the labile, soluble BChE–Aβ complexes in dynamic equilibrium.

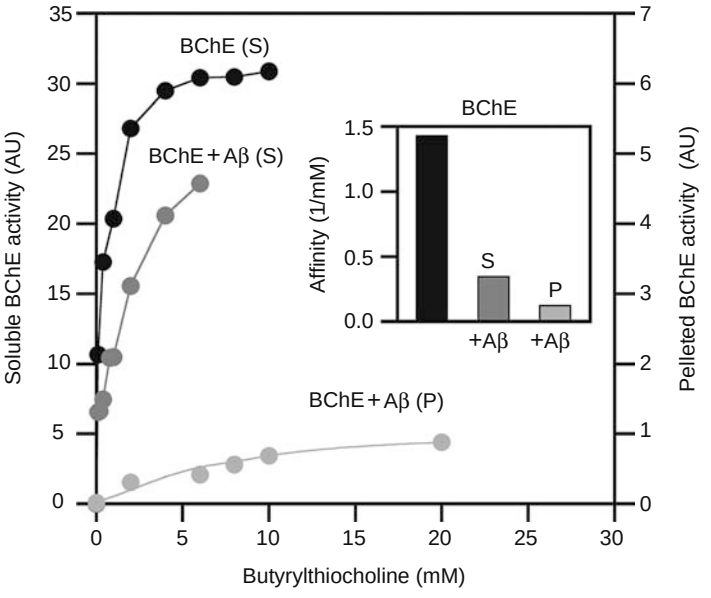

FIGURE 9.3 BChE forms soluble complexes with Aβ. Substrate affinity decreases in the bound form. BChE without or with Aβ was incubated for 22 h with shaking. The mixture was separated into soluble (S—left axis) and pelleted (P—right axis) fractions. The K_m values were calculated using Kaleidagraph program (Synergy Software, Reading, PA). Affinity is expressed as K_m^{-1}.

Importantly, enzyme activity in the soluble fraction represents the activity of both free and Aβ-bound enzyme species, suggesting that in this fraction the measured change in the affinity of BChE to its substrate is likely an underestimation. Additionally, the small fraction of BChE (3%), which was associated with the Aβ pellet (resuspended in the original volume of PBS buffer and exhibiting high binding of ThT), showed drastically different interaction with its substrate, demonstrating over 10-fold lower affinity and a K_m of 8.1 mM, as compared with the soluble unbound enzyme (Figure 9.3). Parallel decreases in substrate affinity were reported for AChE present in AChE–Aβ complexes of the insoluble fibrillar fraction (Alvarez et al., 1998), and were similarly interpreted to reflect interaction of the enzyme with these peptide chains. In an in vivo context, this may imply underestimation of BChE's increases, evaluated by its activity in the AD brain (especially when present within the plaques) (Ballard et al., 2005). However, the inverse consequences of such AChE and BChE interactions attribute to them distinct effects on fibril formation.

D. Synaptic Acetylcholinesterase and Butyrylcholinesterase Have High Homology but Distinct Peripheral Anionic Sites and C Termini

Our next attempts were directed at delineating the specific domains in BChE, which attenuate the nucleation and progression of the fibril formation process. Others reported that within the AChE-S molecule, the peripheral anionic site (PAS) domain is casually involved with the enzyme's capacity to accelerate amyloid fibril formation (Alvarez et al., 1998). Since AChE-S and BChE show high levels of homology, this raised the question of why BChE's PAS does not exert similar effects. Superposition of the corresponding region in the two enzymes (Figure 9.4) highlights three of the four aromatic residues that exist in AChE-S's PAS as missing in BChE's PAS. Importantly, BChE's PAS region displays inverse biochemical properties to that of AChE-S (namely, it presents substrate activation in BChE rather than the substrate inhibition in AChE [Glick et al., 2003]). This suggested that BChE possesses an inert PAS domain, with regards to the fibril formation process, yet raised another question, namely, which region in BChE exerts the fibrils attenuating effect.

A promising region, which is distinct in the two enzymes, was their C terminus. The C-terminal domains of both AChE-S and BChE are encoded by individual exons, suggesting specific biological

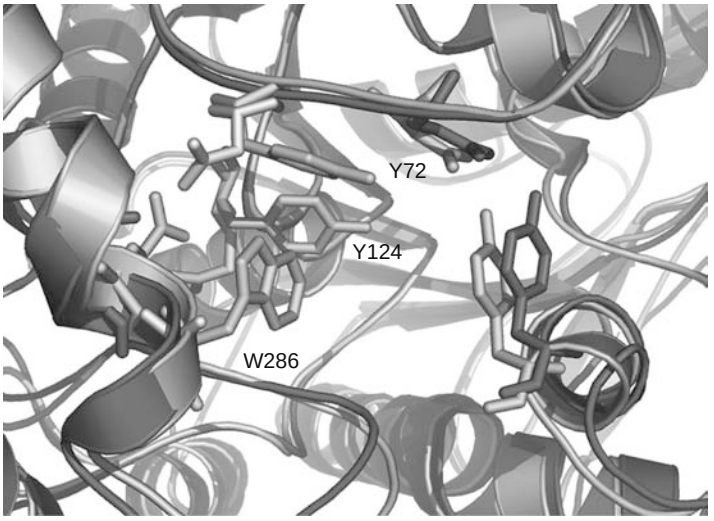

FIGURE 9.4 Distinct PAS domains in AChE and BChE. BChE's PAS region includes polar residues (asparagine, glutamine, and alanine), and lacks three out of the four aromatic residues in the corresponding positions of the AChE (tyrosine72, tyrosine124, and tryptophan286).

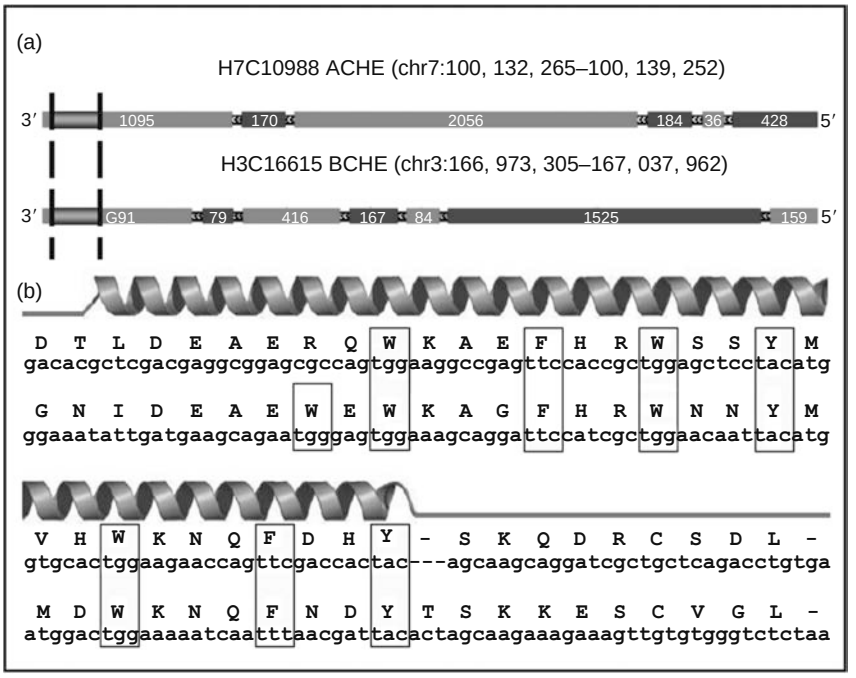

FIGURE 9.5 Symmetric aromaticity in the C-terminal domains of AChE and BChE. (a) The chromosomal location of the *ACHE* and *BCHE* genes is shown along with the composition of exons and introns as well as the highlighted C-terminal sequences. (b) The coil and line indicate the helical and random-coil content of the C-terminal sequences. The DNA and protein sequences (lowercase and uppercase, respectively) are aligned. Protein, but not DNA, sequences show a significant similarity, which is even higher within the helical part of the sequences. Pairs of aromatic residues (framed), spaced perfectly to form amphipathicity in both sequences, are framed; the unpaired BSP W8, spaced ''incorrectly'' for amphipathicity, is framed.

functions (Figure 9.5a). Both regions (namely from the splice site between exon 4 and exon 6 in AChE-S and downstream, and the equivalent region in BChE) share 80% similarity and display abundant aromatic residues (Figure 9.5b), implicated by others in the fibril formation process (Gazit, 2002). However, certain sequence differences between the domains appeared to entail a structural context. Synthetic peptides having the C-terminal sequence of both proteins were hence prepared and examined for their effects on amyloid fibril formation. The 41 amino acid long BChE-derived peptide, BSP41, suppressed the fibril formation process in a dose-dependent manner and in similar molar ratios as those of BChE (Figure 9.6). Like BChE, BSP41 increased the lag and decreased the rate of fibril formation. In absolute quantity terms, complete attenuation of Aβ fibril formation for over 200 min was achieved with 2 μg/mL BSP41 as compared with 30 μg/mL BChE. In contrast, a synthetic ASP40 peptide, derived from the corresponding domain in AChE-S, showed no capacity to activate or inhibit fibril formation (data not shown).

E. Structural Modeling Implications

Structural modeling revealed that both ASP40 and BSP41 peptides form symmetric amphipathic helices, compatible with findings of others (Dvir et al., 2004) and consistent with CD analyses, which showed a clear positive band at 192 nm and two negative bands at 209 and 220 nm, characteristic of α helical structures (Diamant et al., 2006). However, BSP41 displayed imperfect amphipathicity, locally disturbed by the protruding aromatic W8 residue in the polar side of this

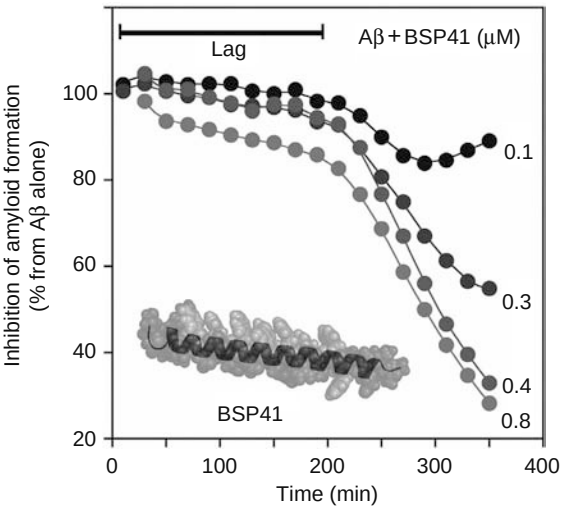

FIGURE 9.6 BSP41 suppresses fibril formation in a dose-dependent manner. Decreasing the rate of fibril formation was found for increasing peptide concentrations. Inset: BSP41 helical peptide (shown as both cartoon and space-filling model).

helix. This indicated that the aromatic W8 residue in the polar side of BSP41 could potentially be responsible for the functional difference between ASP40 and BSP41. To challenge this hypothesis we replaced W8 with arginine, by preparing a W8R mutant of BSP41, and reciprocally replaced R8 in ASP40 with tryptophan, by preparing a R8W mutant of ASP40.

Intriguingly, W8R BSP41 lost its ability to attenuate fibril formation, but R8W ASP40 did not gain such activity (Figure 9.7 and data not shown). This indicated that W8 was not the only determinant of such activities and called for further search.

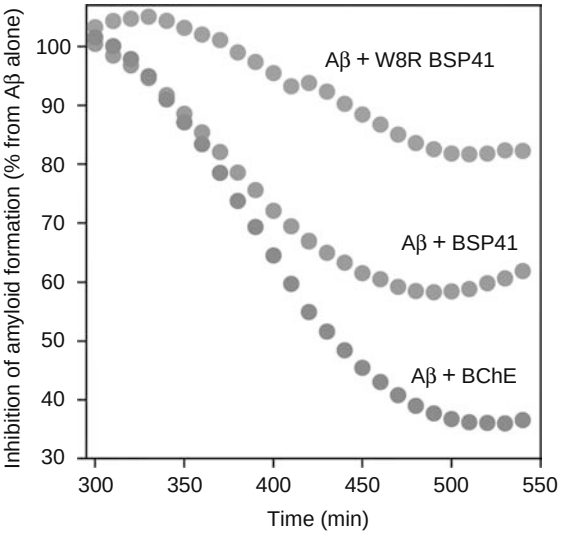

FIGURE 9.7 Site-directed mutagenesis. Fibril formation was inhibited by incubation of 1:100 molar ratios of BChE or BSP41 with Aβ. The BSP41 inactive mutant W8R was ineffective. Data is presented as percentage of the basal rate obtained with Aβ alone over time.

F. Aromaticity Considerations

Aromatic residues are frequent in the cross-β core of fibrils but are not necessarily required for fibril formation. Recent studies demonstrate that aromatic residues can establish a network of hydrophobic interactions, which are casually involved in the formation of the cross-β structure (Bemporad et al., 2006). The finest example comes from the $A\beta_{40}$ model itself, based on solid-state nuclear magnetic resonance experiments (Petkova et al., 2002). In this study, two β-strands formed by residues 12–24 and 30–40 gave rise to two parallel β-sheets in register, which interact through two phenylalanine residues that form interstrand π-stacking. Specifically, the contacts between phenylalanine residues in fibrils are thought to be important for stabilizing interactions (Alvarez et al., 1998; Gazit, 2002; Porat et al., 2003, 2004). However, the forces that maintain the aromatic residues in close contact and stabilize fibrils do not arise from specific interactions involving the π-electrons or the aromatic nature of these residues. Rather, these powers are due to their high hydrophobicity and high tendency to form β-sheets. The stabilizing effect of aromatic residues on the resulting fibrils is also compatible with their high frequency in amyloidogenic sequences (Bemporad et al., 2006). Supporting this notion, in vitro experiments have demonstrated the importance of amphipathic molecules for inhibiting the fibril formation process: Addition of an amphiphilic surfactant, *n*-dodecylhexaoxyethylene glycol monoether (Lomakin et al., 1996), or the amphiphilic di-C6-PC (Gazit, 2002; Wang et al., 2005) to $A\beta_{40}$ was shown to decrease the rate of Aβ aggregation in a concentration-dependent fashion and cause a concentration-dependent reduction in the length, but not the diameter, of Aβ fibrils.

G. Inverse Roles for Butyrylcholinesterase and Synaptic Acetylcholinesterase in Fibril Formation

In the AChE-S molecule, the hydrophobic PAS domain located close to the lip of the active site gorge would be free to form complexes with growing fibrils, thus supporting the Aβ assembly process (Alvarez et al., 1998). In variance with AChE-S, BChE (and its synthetic peptide BSP41) emerges from our study as being capable of attenuating the fibril formation process through its aromatic W8 residue, positioned outward in the polar side of the BSP helix, and in a competitive manner with AChE-S.

Last, but not least, one should consider the multimeric composition of the BChE and AChE-S molecules. In the majority of such molecules, the hydrophobic (mostly aromatic) part of the amphipathic helix of the C terminus is likely engaged in G4 homo-oligomers (Fernandez et al., 1996; Altamirano and Lockridge, 1999). Because of the amphipathic nature of the C-terminal helices, their hydrophobic sides would tend to turn inward, exposing the hydrophilic surface to the outside environment. In AChE-S, the hydrophilic nature of this surface would be intact, because the outward-turned arginines do not impair it. In contrast, BChE peptides would yield outward-protruding aromatic tryptophans, enabling Aβ interactions (Figure 9.8). These protruding residues can form hetero-aromatic complexes with soluble, monomeric or low oligomeric, Aβ conformers. In the naturally formed tetramers, the protruding tryptophan would likely interfere with oligomerization and the side chain stabilization of the β-sheet structure and inhibit propagation of the fibril formation process to form toxic protofibrils and insoluble fibers.

H. Therapeutic Considerations

In the human brain, AChE mRNA is 20-fold more abundant than BChE mRNA (Soreq and Zakut, 1990), whereas in human blood, BChE is threefold more abundant than AChE (Schwarz et al., 1995). In the AD brain, the declining AChE (Talesa, 2001) and increasing BChE (Ballard et al., 2005) would change this ratio considerably. In blood, the physiological importance of this ratio stems from BChE's capacity to interact with Aβ in the soluble phase and due to the continuous communication between the brain and circulation (Shaked and Soreq, 2006).

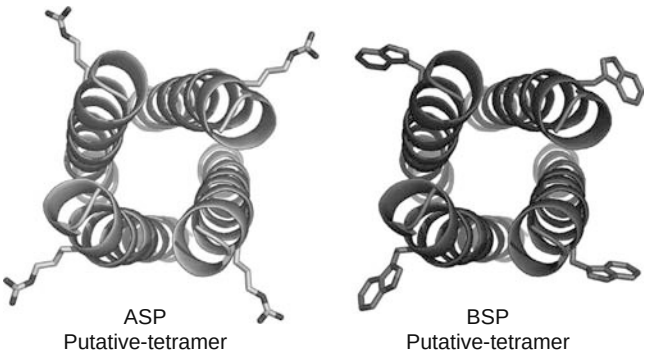

FIGURE 9.8 Illustrative four helical bundles of ASP40 and BSP41. Illustrative figures of the four helical bundles of ASP40 and BSP41 (represented here as cartoon) formed by their corresponding C-terminal peptides to yield tetramers. The aromatic side is turned inward in both, resulting in a protruding residue in position 8. In the BSP tetramer, the tryptophan side chains (W8; represented here as sticks) of each helix would be turned outward, readily available for attenuating fibril formation, unlike the nonaromatic ASP's arginine residue (R8; represented here as sticks).

Putative therapeutic uses of BChE and the relatively short BSP peptide may involve injection, similar to erythropoietin or granulocyte/macrophage colony stimulating factor (GM-CSF) (Arndt et al., 2005; Zhang et al., 2005). Transfecting bone marrow cells for autologous transplantation, with a BSP expression vector similar to the gene therapy protocols used for adenosine deaminase replacement, would be an alternative (Aiuti et al., 2003). Intranasal administration, like that of nerve growth factor (NGF) (De Rosa et al., 2005), is also plausible. In either way, the reportedly disrupted blood–brain barrier in AD (Ballabh et al., 2004) and that cholinergic imbalances induce blood–brain barrier disruption (Meshorer et al., 2005) predicts effective BSP41 penetration into the brain.

V. SUMMARY AND CONCLUSIONS

BChE, in a 1:100 ratio with synthetic amyloid peptide ($A\beta_{40}$), delayed the formation of Aβ fibrils in a dose- and time-dependent manner. Moreover, BChE competed with AChE and slowed down the AChE-S-facilitated rate of fibril formation.

A synthetic peptide derived from the BChE C terminus (BSP), but not from the AChE-S C terminus (ASP), delayed the onset and decreased the rate of Aβ fibril formation in vitro. The helical conformation of the tested peptides (confirmed by CD measurements and molecular modeling), which is presumably present in the native protein, is amphipathic. Nevertheless, BSP's amphipaticity is interrupted due to an aromatic tryptophan residue in the polar side of the C terminus, whereas ASP shows symmetric amphipathicity. Supporting the notion of the importance of this structural distinction, single amino acid substitutions in that position impaired the amyloidogenic potential of the BSP peptide. Most importantly, our findings are in favor of the therapeutic use of BChE, but not AChE-S for treating OP-poisoned patients and avoiding long-term amyloidogenic damages.

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10 Novel Medical Countermeasure for Organophosphorus Intoxication: Connection to Alzheimer's Disease and Dementia

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CONTENTS

I. INTRODUCTION

Since their development just before World War II, the organophosphorus (OP) nerve agents have been potential and actual threats to soldiers on the battlefield (Wiener and Hoffman, 2004; Geoghegan and Tong, 2006). Their more recent use in 1994–1995 terrorism incidents in Japan (Tokuda et al., 2006) has added a threat to the general civilian population as well, which was already present for farm workers or exterminators using OP insecticides (Karalliedde and Senanayake, 1989). These agents' acute toxic effects are primarily due to irreversible inhibition of acetylcholinesterase (AChE), the enzyme that breaks down the neurotransmitter acetylcholine at the neuromuscular junction and elsewhere (Maxwell et al., 2006). Other mechanisms, particularly in the brain, have also received

some support (Duysen et al., 2001). Symptoms vary according to the OP and route and the extent of exposure, but usually include a nicotinic cholinergic syndrome characterized by skeletal muscle fasciculations followed by desensitizing neuromuscular blockade, and a variety of other effects resulting from overstimulation of muscarinic receptors. A more complete clinical list of symptoms includes miosis, excess sweating, headache, tightness in the chest, labored breathing, dizziness, excess salivation, muscle cramps, difficulty in walking, convulsions, diarrhea, wheezing, abdominal cramps, muscle fasciculations, unconsciousness, coma, respiratory failure, and death. As suggested by this list, death is usually due to respiratory failure, resulting from both diaphragm paralysis and failure of central respiratory drive (Rickett et al., 1986). Currently used treatments for OP poisoning are far from satisfactory. They consist of atropine to block muscarinic effects, oximes to reactivate the inhibited AChE before ''aging'' of the phosphorylated enzyme occurs, and benzodiazepines to control seizures (Marrs, 2004). More recently, the Food and Drug Administration approved the use of pyridostigmine, a reversible inhibitor of AChE and butyrylcholinesterase (BuChE), as a pretreatment for military personal under threat of exposure to nerve agents (Layish et al., 2005). The intent was to protect a portion of the AChE molecules against irreversible blockade by the nerve agent by occupying them with the reversible inhibitor. This is particularly important for exposure to soman, because the soman-inhibited AChE ages rather rapidly.

Symptoms observed in people intoxicated with nerve agents and other OPs are also evident in neurological disorders in which the cholinergic system is compromised. Thus, the muscle weakness and fatigue seen in OP poisoning resemble that observed in patients with myasthenia gravis, a neuromuscular disorder characterized by varying degrees of skeletal muscle weakness in the body due to the autoimmune destruction of nicotinic receptors (nAChRs) at the neuromuscular junction (Schwendimann et al., 2005). Likewise, there are reports that people who are chronically exposed to OP insecticides show measurable deficits in cognitive functions (Kamel et al., 2003) and that an association may exist between past occupational exposure to OP pesticides and increased risk to develop neurodegenerative disorders, particularly Alzheimer's and Parkinson's diseases (Baldi et al., 2003). Based on evidence that impairments of brain cholinergic systems contribute to the cognitive dysfunctions in Alzheimer's disease, reversible cholinesterase inhibitors, including galantamine, rivastigmine, and donepezil, are currently approved for treatment of the disease (Lleo et al., 2006). Some members of the same class of drugs, specifically pyridostigmine, are also used to treat patients with myasthenia gravis. The primary difference between reversible cholinesterase inhibitors used for Alzheimer's disease and myasthenia gravis lies in their lipid solubility. While the cholinesterase inhibitors used for Alzheimer's disease promptly cross the blood–brain barrier, those used for myasthenia gravis, pyridostigmine included, do not. This consideration suggests the possible utility of Alzheimer's disease drugs in protecting against central as well as peripheral effects of nerve agents. One such drug, galantamine (also called galanthamine), has recently been found by our group to be a superior antidote against intoxication by soman and other nerve agents, effective when administered both before and soon after exposure (Albuquerque et al., 2006).

II. GALANTAMINE

A. History

Amazingly enough, the history of galantamine may reach back to Homer's Odyssey, more than a 1000 years ago. One theory of Circe's bewitchment of Odysseus's men is that it involved use of jimsonweed (*Datura stramonium*), which contains atropine, and that Odysseus's potion to counteract it was made from the common snowdrop (*Galanthus nivalis*), a source of galantamine (Plaitakis and Duvoisin, 1983). Further mention of galantamine had to wait until modern times, about 1950 in Bulgaria, when a pharmacologist may have noted people rubbing their heads with snowdrops to relieve pain (reviewed in Heinrich and Lee Teoh, 2004). This or other observations evidently led to a 1951 Russian paper describing galantamine as an AChE inhibitor (Mashkovsky and

Kruglikova-Lvova, 1951) and its isolation and structural determination the next year. Further research led to its commercialization (trade name Nivalin) in Bulgaria in 1958, and its use since then in Eastern Europe to help treat the loss of motor neurons (and acetylcholine) produced by polio, as well as to restore neuromuscular function after anesthesia involving use of neuromuscular blocking agents (Pearson, 2001). It was not until 1996 that clinical trials tested the effectiveness of galantamine (as Nivalin) in patients with Alzheimer's disease. Its chemical synthesis was followed by its approval (as Reminyl) for treating Alzheimer's disease in the United States in 2001. In April 2005, its manufacturer, Ortho-McNeil Neurologics, Inc., changed the trade name from Reminyl to Razadyne. Galantamine is also available as a nutritional supplement extracted from plants, now primarily species of *Narcissus* (daffodil) and *Leucojum* (snowflake).

B. Use in Alzheimer's Disease

Alzheimer's disease is a form of senile dementia described by Alois Alzheimer more than 100 years ago (Goedert and Spillantini, 2006). It is a progressive neurodegenerative disorder characterized by an incremental decline in mental function that affects memory, thinking, language, and behavior in general. As the disease progresses, cognitive impairments become severe enough to interfere with the daily activities of patients and eventually lead to death (Chung and Cummings, 2000).

Even though the causes of Alzheimer's disease remain obscure, numerous studies have reported that incremental loss of cholinergic neurons in the brain and progressive reduction of nAChR function/expression correlate well with the severity of the symptoms (Coyle et al., 1983; Perry et al., 2000; Nordberg, 2001). In particular, loss of α7 nAChRs is pronounced in the hippocampus and not as significant in the cerebral cortex of patients with Alzheimer's disease as revealed by binding studies that have used [^{125}I]α-bungarotoxin to label α7-containing nAChRs in post-mortem brain tissue samples from patients and age-matched control subjects (Hellstrom-Lindahl et al., 1999; Wevers et al., 1999). In patients with more advanced stages of Alzheimer's disease, such a correlation between nAChR loss and cognitive decline does not appear to exist (Sabbagh et al., 2001).

Epidemiological findings that brain region specific losses of cholinergic neurons and nAChRs correlate well with the cognitive decline in the mild-to-moderate stages of Alzheimer's disease and the evidence that nAChR activation is neuroprotective and improves memory, both support the concept that increasing nAChR activity in the brain should slow down the progression of the disease (Maelicke and Albuquerque, 1996; Coyle and Kershaw, 2001).

At the neuromuscular junction, nicotinic function is enhanced by inhibition of AChE. However, unlike muscle nAChRs, some neuronal nAChRs, particularly those bearing the α7 subunit, recognize both acetylcholine and its metabolite choline as full agonists (Pereira et al., 2002). Therefore, cholinesterase inhibition may not necessarily enhance functions mediated by these nAChRs. In fact, cholinesterase inhibitors do not affect α7 nAChR-mediated synaptic transmission evoked by low-frequency stimulation of cholinergic fibers in chick ciliary ganglia (Zhang et al., 1996).

An alternative means to increase nicotinic functions in the brain is to sensitize the nAChRs to activation by the endogenous agonists using the so-called nicotinic allosteric potentiating ligands (APLs), of which galantamine is the prototype (Maelicke and Albuquerque, 1996). As discussed below, in addition to acting as a weak inhibitor of AChE, galantamine binds to and potentiates the activation of nAChRs by classical agonists (Pereira et al., 2002).

Recent studies have reported that galantamine also improves the cognitive performance of patients with autism (Nicolson et al., 2006) and, unlike other cholinesterase inhibitors, decreases the negative symptoms in patients with schizophrenia (Schubert et al., 2006). For more severe Alzheimer's disease, memantine, an antagonist of *N*-methyl-D-aspartate (NMDA) receptors, has also been approved. A number of newer drugs undergoing clinical trials for Alzheimer's disease work by a variety of other mechanisms, although a common theme appears to be neuroprotection (Robertson and Mucke, 2006).

C. Mechanisms of Action

As mentioned above, galantamine has two basic mechanisms of action. It reversibly inhibits cholinesterases, being approximately 50-fold more potent as an AChE inhibitor than a BuChE inhibitor. It also interacts directly with nAChRs, potentiating their activation by subsaturating concentrations of classical agonists (Pereira et al., 2002).

Physostigmine was the first cholinesterase inhibitor shown to activate muscle and neuronal nAChRs by directly interacting with them (Shaw et al., 1985; Sherby et al., 1985; Okonjo et al., 1991; Pereira et al., 1993, 1994). Later on, galantamine was found to act on nAChRs similar to physostigmine. Direct activation of nAChRs by galantamine or physostigmine was shown to be very weak compared to that induced by classical agonists. More importantly, activation of nAChRs by physostigmine or galantamine was detected even when the receptors were desensitized by high agonist concentrations and was found to be insensitive to blockade by competitive nicotinic antagonists (Shaw et al., 1985; Sherby et al., 1985; Okonjo et al., 1991; Pereira et al., 1993, 1994; Schrattenholz et al., 1993). Instead, it was found to be sensitive to blockade by the monoclonal antibody FK1, which recognizes an extracellular epitope that is very well conserved on the nAChR α subunits cloned to date.

Evidence has been provided that the agonistic activity of physostigmine and galantamine, initially referred to as noncompetitive agonists (Storch et al., 1995), results from their binding to a site close to, but distinct from, the acetylcholine-binding site on nAChR α subunits (Pereira et al., 1993; Schrattenholz et al., 1993). The region flanking the amino acid Lys-125 on the nAChR α subunits contains essential elements of the physostigmine site and is highly conserved across species (Pereira et al., 1993; Schrattenholz et al., 1993; Schroder et al., 1994). Even though physostigmine and galantamine induce opening of nAChR single channels, the probability of channel openings by these compounds is so low that the single-channel currents they activate do not give rise to macroscopic responses (Pereira et al., 1994; Storch et al., 1995; Schrattenholz et al., 1996). In different systems, however, these compounds potentiate the nAChR activity induced by subsaturating concentrations of classical nAChR agonists, and are, therefore, referred to as nicotinic allosteric potentiating ligands (Schrattenholz et al., 1996). The nicotinic allosteric potentiating action is not common to all cholinesterase inhibitors; for instance, donepezil and rivastigmine are devoid of this action (Samochocki et al., 2000).

The allosteric potentiating action of galantamine has been suggested to contribute importantly to its effectiveness in Alzheimer's disease (Maelicke et al., 2001). Acting primarily as a nicotinic allosteric potentiating ligand, galantamine improves synaptic transmission in different areas of the brain (Santos et al., 2002) and protects neurons from different neurodegeneration-inducing insults (Geerts, 2005).

III. GALANTAMINE IN ORGANOPHOSPHORUS POISONING

A. Existing Treatments

Existing treatments for OP poisoning (Bajgar, 2004; Newmark, 2004a, 2004b, 2005; Cannard, 2006) start with pyridostigmine as prophylaxis. Pyridostigmine is most appropriate for anticipated exposure to soman and was approved in 2003 for pretreatment use by military personnel under the threat of exposure to nerve agents, specifically soman (Couzin, 2003). It had already been used by military personnel in the Gulf War (1991). Its use is always to be accompanied by immediate treatment after OP exposure with atropine and an oxime such as pralidoxime (2-PAM), the previous standard treatments after exposure. Pyridostigmine was chosen not to cross the blood–brain barrier to avoid possible central nervous system impairment or incapacitation, an effect particularly undesirable (and possibly fatal) in soldiers, at least those not being exposed to OPs. Several other anticholinesterases that do cross the blood–brain barrier, including physostigmine, tacrine, and

huperzine A, have been found to offer better protection than pyridostigmine, but at the cost of undesirable central effects (Deshpande et al., 1986; Fricke et al., 1994; Grunwald et al., 1994; Lallement et al., 2002, 2002b). None of the current treatments offer any real protection against central effects of the OPs, which can contribute to acute lethality (e.g., through respiratory depression or convulsions) and presumably also lead to the delayed neurological and cognitive deficits observed after OP exposure in both animals and humans, for example, following the Tokyo sarin subway attack in 1995 (Abou-Donia, 2003; Yanagisawa et al., 2006). These deficits are accompanied by visible structural changes. They cannot be prevented merely by controlling seizures, for example, with diazepam (Bhagat et al., 2005), although seizure control does help (Shih et al., 2003). Another problem with pyridostigmine is that it blocks BuChE as well as AChE, thus reducing OP binding to this scavenger site not associated with OP toxicity.

B. Promise of Galantamine

Galantamine, at doses used for Alzheimer's disease, is relatively devoid of side effects (Loy and Schneider, 2006). When they do appear, they are generally gastrointestinal; the most common are nausea, vomiting, and diarrhea. These side effects might be expected to be reduced for a nonoral route of administration, such as intramuscular. Such doses clearly must be blocking a significant portion of brain AChE to provide benefit. Biochemical evidence indicates that there is actually a greater blockade of peripheral AChE (Albuquerque et al., 2006), and this may account for part of the superiority of galantamine over other centrally acting anticholinesterases in terms of side effects. Unlike pyridostigmine, galantamine inhibits BuChE poorly, leaving intact this scavenger site for OPs in blood. Additionally, galantamine has been reported to have anticonvulsant (Kilimov, 1965) and neuroprotective (Arias et al., 2004, 2005) properties, both of which are promising in the context of OP poisoning.

C. Pretreatment with Galantamine

These and later experiments were performed in guinea pigs, the best nonprimate model of OP poisoning (Maxwell et al., 1988; Maxwell and Brecht, 1991). Mice and rats have high levels of carboxylesterases, rendering them considerably less sensitive to OPs than guinea pigs and primates and giving reduced benefit of antidotes. Pretreatment is most relevant for first responders, police and firemen reporting to a scene of chemical attack, or for military personnel anticipating such an attack. Given at 30 min before OP exposure, galantamine (5–16 mg/kg, i.m. as hydrobromide) protected completely (in terms of 24 h lethality) against 1.5 LD_{50}s of soman (42 μg/kg, s.c.) or sarin (56 μg/kg, s.c.), when given in conjunction with atropine at 1 min postexposure (10 mg/kg, i.m.) (see Figure 10.1). With the combination of pretreatment with 8 mg/kg galantamine and posttreatment with 10 mg/kg atropine, all of the animals thus protected for 24 h after soman exposure survived with no signs of toxicity for 7 days after exposure. Galantamine (8 mg/kg, i.m.) administered at 30 min prior to exposure of the guinea pigs to $1 \times LD_{50}$ soman afforded full protection against lethality in the absence of any posttreatment.

In addition to effectively preventing the toxicity and lethality of nerve agents, galantamine protected guinea pigs from lethality and toxicity of paraoxon, the active metabolite of parathion, an extremely toxic OP insecticide. Although the use of paraoxon has been recently banned in the United States, Europe, and Australia, numerous countries still use it in agriculture. It was estimated that approximately 50% of atropine-treated guinea pigs (10 mg/kg, i.m.) would die within 30–40 min after they received 1.5 mg/kg paraoxon (s.c.). Galantamine (8 mg/kg) administered at 30 min prior to the exposure of the guinea pigs to 2 mg/kg paraoxon afforded full protection against lethality and toxicity of this OP (Figure 10.2).

The protection afforded by a single dose of galantamine can extend up to several hours. At times up to 3 h before OP exposure, galantamine (10 mg/kg, i.m. as hydrobromide) afforded full

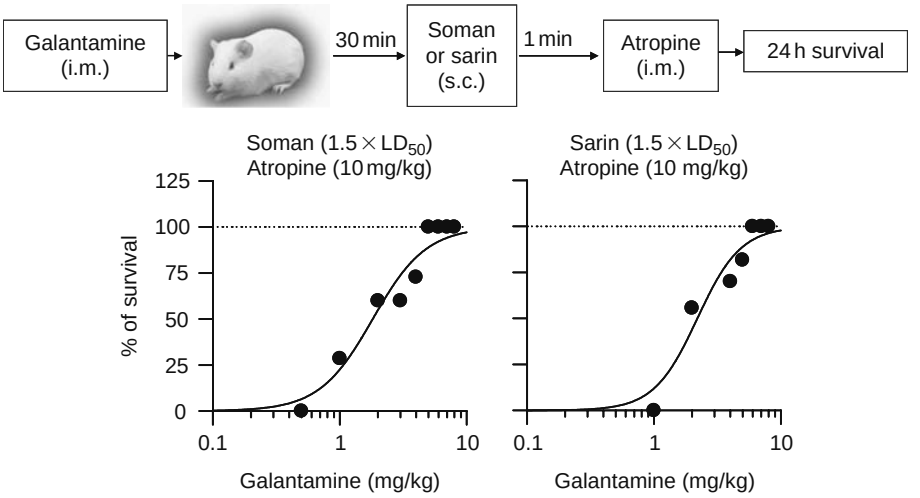

FIGURE 10.1 Effectiveness of galantamine/atropine as an antidote against the acute toxicity and lethality of $1.5 \times LD_{50}$ soman or sarin in guinea pigs. Note that there is no need for additional supportive therapy. (From Albuquerque, E.X. et al., *Proc. Natl. Acad. Sci. USA*, 103, 13220, 2006. With permission.)

protection against the lethality and toxicity of 1.5 LD_{50}s of soman, when used in conjunction with atropine postexposure treatment (10 mg/kg, i.m.) (Figure 10.3). A slightly lower dose (8 mg/kg), half of the galantamine dose at which mild side effects began to appear, protected completely for up to 1 h (Figure 10.3). Presumably oral doses could have protected for even longer than these intramuscular doses because of slower galantamine absorption. The time to peak drug concentration after oral dosing in humans is 52 min and the plasma half-life is 5.7 h (Sramek et al., 2000). Protection by galantamine was superior to that provided by pyrodostigmine, which exhibited a peak protection (about 65% survival) at 52 μg/kg (Figure 10.4). At higher doses, there was less protection, presumably because of pyridostigmine's removal of scavenging of OPs by BuChE. The protection by galantamine was equivalent to that afforded by huperzine A (Figure 10.4),

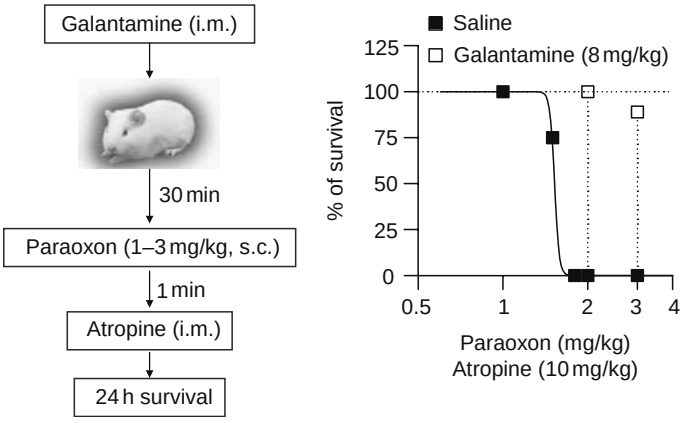

FIGURE 10.2 Galantamine/atropine is an effective antidote against the acute toxicity and lethality of paraoxon, the active metabolite of the OP insecticide parathion. Thus, the antidotal therapy counteracts the toxicity of OP insecticides as well as it counteracts the toxicity of nerve agents. (From Albuquerque, E.X. et al., *Proc. Natl. Acad. Sci. USA*, 103, 13220, 2006. With permission.)

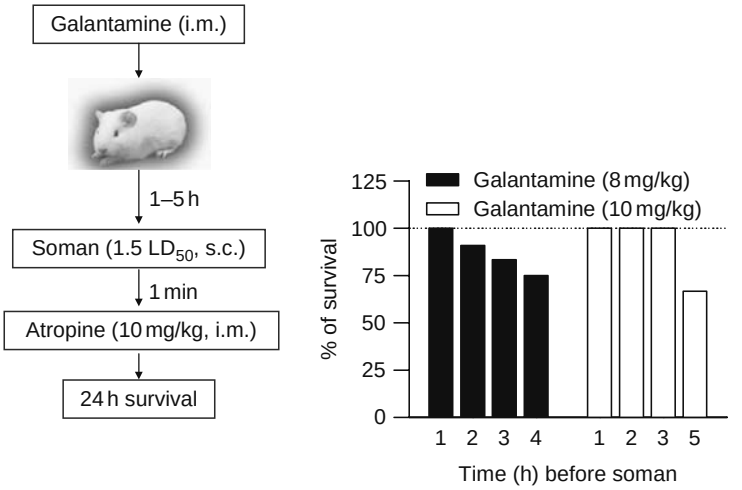

FIGURE 10.3 The safety window of time for pretreatment with galantamine to prevent soman-induced lethality in guinea pigs can be up to several hours. (From Albuquerque, E.X. et al., *Proc. Natl. Acad. Sci. USA*, 103, 13220, 2006. With permission.)

previously tried for protection against OP exposure (reviewed in Lallement et al., 2002a). However, galantamine produced no detectable behavioral effects (Figure 10.5) whereas even the minimal doses of huperzine A needed to afford full protection against lethality of $1.5 \times LD_{50}$ soman increased locomotor activity and stereotypic behavior (Figure 10.5) as well as obvious and potentially incapacitating side effects (profuse secretions, muscle fasciculations, respiratory distress, and tremors). Furthermore, this dose of huperzine A, given only once, reduced the rate of weight gain of peripubertal guinea pigs over the subsequent 7 days, again in contrast to galantamine (and atropine). More complete results are described in Albuquerque et al. (2006).

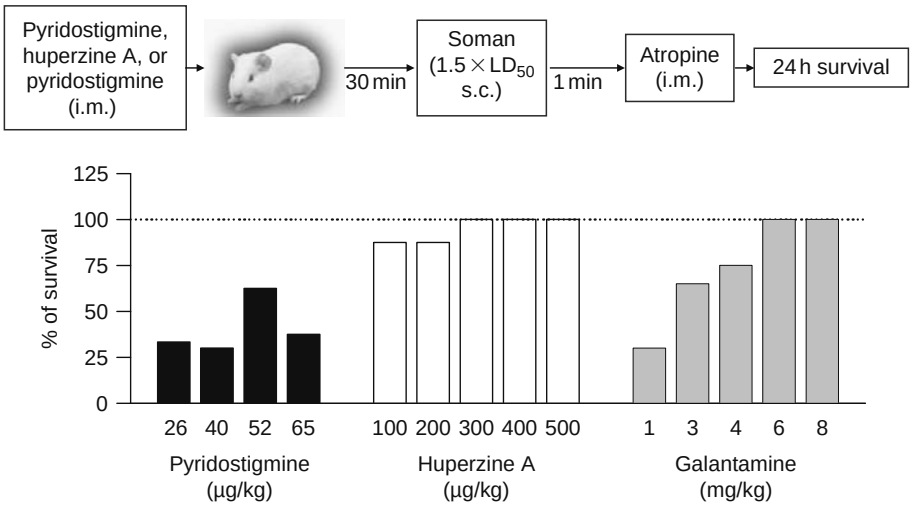

FIGURE 10.4 Galantamine/atropine is more effective than pyridostigmine/atropine and as effective as huperzine A against the acute toxicity and lethality of soman. (From Albuquerque, E.X. et al., *Proc. Natl. Acad. Sci. USA*, 103, 13220, 2006. With permission.)

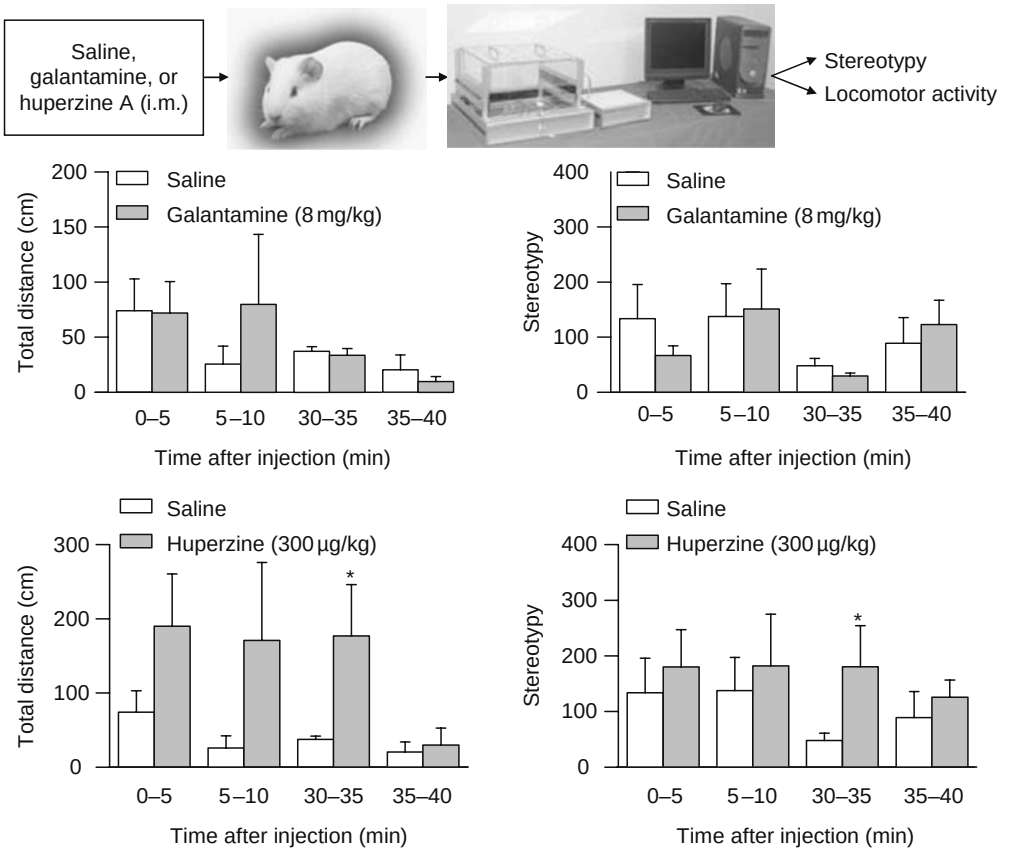

FIGURE 10.5 At doses sufficient to afford full protection of guinea pigs against the lethality and acute toxicity of $1.5 \times LD_{50}$ soman, galantamine has no significant effect on the animals' gross behavior whereas huperzine A significantly increases locomotor activity and stereotypic behavior. (From Albuquerque, E.X. et al., *Proc. Natl. Acad. Sci. USA*, 103, 13220, 2006. With permission.)

D. Posttreatment with Galantamine

Because chemical attacks, or accidents with insecticides, usually cannot be anticipated, postexposure antidotes against OP intoxication are even more desirable than preventative pretreatments. If given sufficiently soon after OP exposure, before major symptoms appear, galantamine appears to be such an antidote. In guinea pigs, galantamine (8 or 10 mg/kg, i.m.), given in conjunction with atropine (10 mg/kg, i.m.), protects completely against soman lethality ($1.5 \times LD_{50}$, s.c., measured at 24 h) at times up to 5 min after exposure (Figure 10.6), with similar protection against paraoxon (Figure 10.6) and sarin (not shown). For lower doses of OP, the time after exposure for which galantamine provides effective treatment is prolonged. Thus, for guinea pigs treated with only $1 \times LD_{50}$ of soman subcutaneously, complete survival was afforded by galantamine (8 mg/kg, i.m., + atropine 10 mg/kg, i.m.) even at 20 min after the soman injection (Albuquerque et al., unpublished results). Treatment could presumably be delayed further still when OP exposure was by routes, for example, transdermal, giving slower OP absorption. These findings of the postexposure effectiveness of galantamine are the most exciting reported in this chapter.

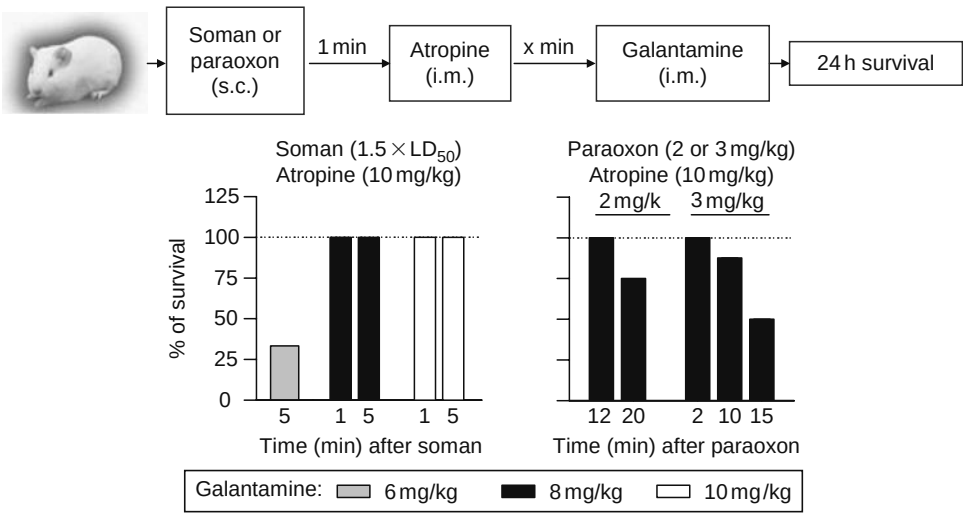

FIGURE 10.6 Posttreatment with galantamine/atropine effectively counteracts nerve agent and insecticide toxicity in guinea pigs. Lower the level of OP exposure, longer the time within which the antidotal therapy effectively maintains 100% survival of the animals with no signs of toxicity. (From Albuquerque, E.X. et al., *Proc. Natl. Acad. Sci. USA*, 103, 13220, 2006. With permission.)

E. Protection Against Brain Damage

The above results in terms of survival are extremely promising. However, survival alone is not enough if there is brain damage that can potentially lead to delayed cognitive or neurological effects. Neurodegeneration in the pyriform cortex, the amygdala, and the hippocampus is characteristic of (nonfatal) OP exposure in animals and presumably contributes to delayed cognitive effects reported in victims of the Japanese sarin attacks and others (DiGiovanni, 1999) and certain neurological effects in animal models (Jamal, 1995, 1997). Galantamine (8 mg/kg, i.m., with atropine at 1 min after OP exposure), given 30 min before or 5 min after soman ($1.5 \times LD_{50}$, s.c.), protected guinea pigs virtually completely against the extensive neurodegeneration seen at 24 h in these three areas (visualized with Fluro-Jade B) in surviving animals treated with atropine (Figure 10.7). The neuroprotection afforded by galantamine is likely to result from its direct actions in the brain, as evidenced by the findings that galantamine can prevent neurodegeneration induced by treatment with staurosporine (STS) of primary cultures of the rat hippocampus. In these experiments, primary hippocampal cultures were exposed to medium-containing vehicle, galantamine, STS, or galantamine followed by STS plus galantamine. Number of apoptotic cells visualized by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) in cultures treated with STS alone was compared to the number of apoptotic cells in cultures subjected to each of the other treatments. As shown in Figure 10.8, STS-induced cell death was significantly decreased by the treatment with galantamine. It has been suggested that the neuroprotective effect of galantamine is a result of activation of a number of kinases, including the extracellular signal regulated kinase, secondary to the nicotinic allosteric potentiating action of the alkaloid (Capsoni et al., 2002; Kihara et al., 2004; Arias et al., 2005).

It is tempting to speculate that the neuroprotection afforded by galantamine against the OP insult would contribute to protection against delayed cognitive or neurological effects, but this remains to be established. Such findings should also be correlated with effects on gene expression (Damodaran et al., 2006). It is interesting that the chronic benefit of galantamine in terms of ameliorating

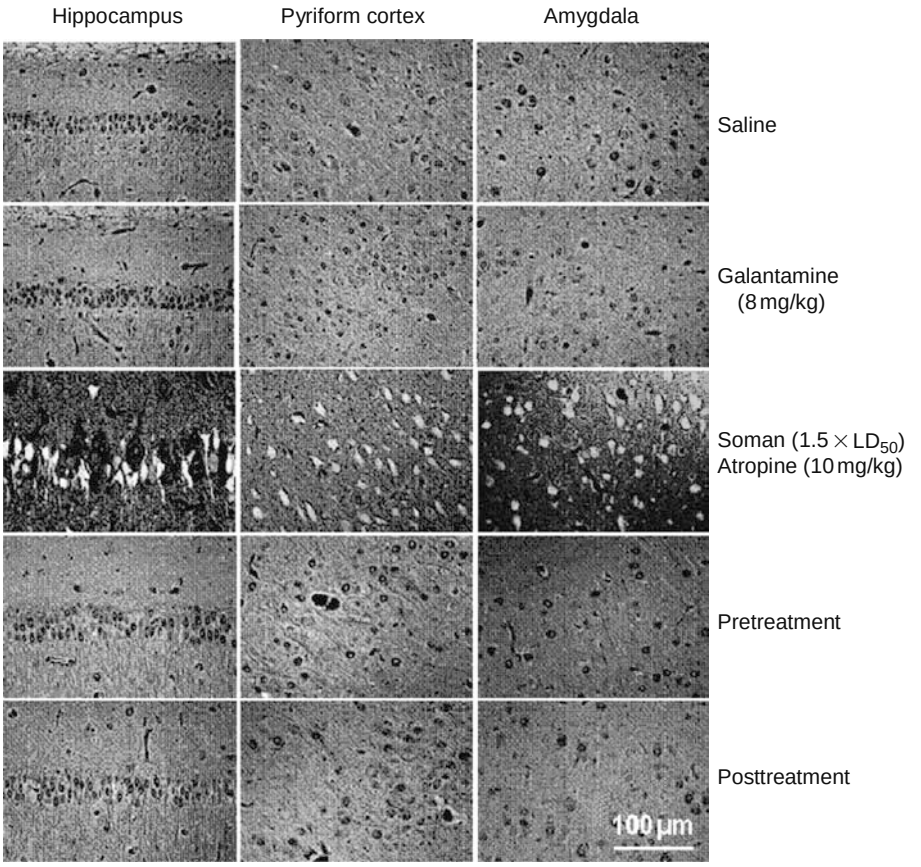

FIGURE 10.7 Pre- or posttreatment with galantamine counteracts the neurotoxic effects of lethal doses of nerve agents. Note the relative absence of staining with Fluro-Jade B in brain regions of all animals except those exposed to soman and treated only with atropine. In the pretreatment paradigm, guinea pigs received galantamine (8 mg/kg, i.m.) at 30 min before $1.5 \times LD_{50}$ soman (s.c.). In the posttreatment paradigm, guinea pigs received galantamine (8 mg/kg, i.m.) at 5 min after soman. At 1 min after the soman challenge, all animals were treated with atropine (10 mg/kg, i.m.). (From Albuquerque, E.X. et al., *Proc. Natl. Acad. Sci. USA*, 103, 13220, 2006. With permission.)

cognitive effects of Alzheimer's disease seems likely to be paralleled by an acute benefit in terms of preventing delayed cognitive effects of OP exposure, with a common primary mechanism (inhibition of AChE) but different secondary mechanisms (increasing acetylcholine at remaining synapses versus occupying the enzyme reversibly to protect it against irreversible inactivation). For treatment of both Alzheimer's disease and OP intoxication, it seems likely that other mechanisms may contribute as well.

IV. CONCLUSIONS

Galantamine, used for many years for polio and for accelerated recovery from anesthesia involving neuromuscular blockers, and more recently for Alzheimer's disease, appears to possess a fortuitous combination of properties which render it a superior preventative and therapy for OP exposure as well. It is not yet fully apparent what all these properties may be. However, it is likely that selective block of AChE versus BuChE, ability to penetrate the blood–brain barrier, and allosteric

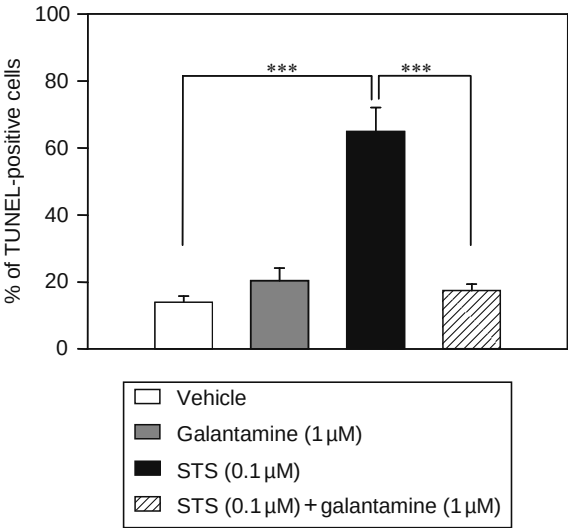

FIGURE 10.8 Staurosporine (STS)-induced apoptosis in primary hippocampal cultures is reduced by pretreatment with galantamine. Primary hippocampal cultures were treated with medium containing (1) vehicle (water, 1:10,000 and DMSO 1:10,000) for 48 h, (2) galantamine for 48 h, (3) STS for 24 h, or (4) galantamine for 24 h followed by STS plus galantamine for an additional 24 h. In each group, the number of cells counted in each of three microscopic fields in a coverslip or culture dish was taken as 100% and used to normalize the number of TUNEL-positive cells. Experiments were carried out in triplicate. *** indicates that results differ from one another with a $p < 0.001$ according to the ANOVA. (From Albuquerque, E.X. et al., *Proc. Natl. Acad. Sci. USA*, 103, 13220, 2006. With permission.)

potentiation of nAChRs all contribute to the effectiveness of galantamine as a medical countermeasure against OP poisoning. The data summarized here indicate that the introduction of galantamine into clinical practice for OP poisoning will provide a major advance.

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11 Inhalation Toxicology of Nerve Agents

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CONTENTS

I. INTRODUCTION

The chemical warfare nerve agents are a class of extremely toxic chemicals developed during the period surrounding World War II. The nerve agents are cholinesterase (ChE)-inhibiting organophosphorus compounds that are related through structure and mechanism of action to pesticides, but are much more potent. Five of the most common nerve agents are tabun, sarin, soman, cyclosarin, and VX.

Exposure to nerve agent vapor or aerosol can result in a variety of toxicological effects. As with other chemical compounds, the severity of these effects depends on the concentration of the agent and the duration of the exposure. The initial signs and symptoms of nerve agent vapor involving whole-body or head-only exposure include miosis, rhinorrhea, and tightness in the chest. These responses are generally attributed to direct, local effects of nerve agent in ocular and respiratory tissue (Grob, 1956). In contrast, exposures to nerve agents resulting from routes other than inhalation (e.g., intramuscular, intravenous, intraperitoneal, or subcutaneous injection; percutaneous absorption) typically do not produce these local effects on the ocular and respiratory systems as their first noticeable effects (Frederiksson et al., 1960). Instead, local effects, such as muscle fasciculation, may be seen at the site of application or injection. Regardless of the route of exposure, systemic toxic signs will begin to appear as the agent absorbs into the systemic circulation and is distributed throughout the body.

This chapter presents an overview of the toxic effects associated with inhalation of a nerve agent vapor or aerosol. Many of the studies cited were conducted at the U.S. Army Chemical Biological Center from the 1950s up to the present day. One of the objectives of these studies was to

characterize the health hazards of chemical nerve agent atmospheric exposure with regard to their potential impact on the performance of military operations. The results of such studies enhance human risk assessment modeling tools, support the operational risk management decision process, and help define nerve agent detection thresholds which are physiologically relevant.

II. EXPOSURE TO NERVE AGENT IN THE ATMOSPHERE: THE IMPACT OF PHYSICAL PROPERTIES

The potential for exposure to nerve agents in a real-world situation is likely to occur as a result of military operations, a terrorist incident, or accidental exposure, including demilitarization of weaponized material. In these cases, the exposure would be limited to two likely routes: percutaneous and inhalation. Percutaneous exposure is possible through contact with contaminated surfaces. Inhalation of nerve agent would be a major route of exposure in unprotected individuals following explosive dissemination of nerve agent material. In this case, an aerosol cloud containing respirable particles, typically less than 10 μm in diameter, would be generated (Witschi and Last, 2001). It is also possible that explosive dissemination could produce toxicity through the percutaneous route due to settling of aerosol particles onto the skin. However, if a more volatile agent such as sarin was used, the percutaneous component of the exposure would be minimal. This is because sarin has a vapor pressure of 2.9 mmHg at 25°C and would likely evaporate before having a chance to be absorbed through the skin. Indeed it has been demonstrated in open-air testing that sarin has minimal effectiveness in humans via the percutaneous route of exposure (Marzulli and Williams, 1953). Therefore, the primary hazard of sarin is through the inhalation route. For a less volatile agent such as VX, with a vapor pressure 3000 times less than that of sarin (Table 11.1), very little evaporation of material deposited onto the skin would occur. Any aerosol that did deposit onto the skin would remain until it is either removed or absorbed, and the chances of a percutaneous component to the toxic response are increased (Bramwell et al., 1963). Therefore, exposure to aerosolized VX poses potentially both an inhalation and a percutaneous exposure hazard.

In addition to aerosols, nerve agent vapor also poses an inhalation hazard. Overall, greater the vapor pressure of a nerve agent, greater the inhalation hazard it poses. Relatively recent examples of real-world nerve agent vapor exposure incidents include two attacks reported in Japan. In 1994, sarin was disseminated in a residential neighborhood of Matsumoto, Japan by the group Aum Shinrikyo using a heater and an automobile fan to vaporize the agent and disperse it (Yanagisawa et al., 2006). The attack left seven people dead and over 600 casualties. In 1995 in Tokyo, the same group disseminated liquid sarin from plastic bags by poking holes in the bags. The agent leaked out from the bags and evaporated, resulting in the exposure of thousands of subway commuters to sarin vapor. This attack left 12 people dead and resulted in over 600 casualties being treated in the immediate aftermath. In both cases, inhalation of sarin vapor was the primary route of exposure.

TABLE 11.1
Physical Properties of the Nerve Agents Relevant to Vapor Generation

	Tabun	Sarin	Soman	VX
Vapor pressure	0.037 mmHg at 20°C	2.1 mmHg at 20°C	0.40 mmHg at 25°C	0.0007 mmHg at 20°C
Vapor density (relative to air)	5.6	4.86	6.3	9.2
Volatility at 25°C	610 mg/m^3	22000 mg/m^3	3900 mg/m^3	10.5 mg/m^3

Note: The "G" agents, namely sarin, soman, and tabun, are all relatively volatile compounds when compared with VX, making them better candidates for vapor generation (Sidell, 1997).

A. Laboratory Scale Generation of Nerve Agent Atmospheres

In the laboratory, several methods have been employed to produce nerve agent vapors and aerosols. These methods are able to generate stable vapor or aerosol atmospheres, and the exposure durations can be easily controlled, lasting from a few minutes to several hours. Although these methods attempt to reproduce a real-world exposure, it is important to realize that significant differences exist between the two exposure scenarios. Unlike many atmospheres generated in a laboratory, a real-world exposure atmosphere is likely to be much more dynamic and can have unpredictable and wide variations in the nerve agent concentration over time due to variations in wind speed, temperature, humidity, degree of ventilation, presence of objects that could alter airflow, as well as many other factors. This variation in concentration can complicate the extrapolation of toxicological data collected in a laboratory setting to a field setting.

Several methods will be briefly described in this chapter; however, more complete descriptions of the methods for the generation and analysis of nerve agent atmospheres exist (Carpin et al., 2003; Anthony et al., 2004; Allon et al., 2005; Benton et al., 2006; Muse et al., 2006). Several commonly used systems are (1) the spray atomizer generation system, (2) the dual-needle air-blast generation system, and (3) the saturator-cell generation system. A spray-atomization generation system employs a syringe drive to supply a constant amount of agent to a spray atomizer (Muse et al., 2006). As liquid nerve agent enters the atomizer, compressed air is used to atomize the liquid nerve agent into a fine aerosol. With more volatile agents, such as sarin, these droplets quickly evaporate, and the resulting vapor is drawn into an inhalation chamber. This method is useful for generating vapors of relatively volatile agents, such as the ''G'' series nerve agents. Additionally, this methodology could also be used to produce aerosols of less volatile agents.

A similar method has been used previously to generate nerve agent aerosols (Carpin et al., 2003). VX aerosol has been generated using a syringe drive to pump liquid nerve agent into a dual-needle air-blast generator. In the dual-needle design, liquid agent is fed down a tube, which is sheathed by a larger needle. Compressed air is delivered through the larger needle and as the liquid agent exits the smaller needle, it is blasted into a fine aerosol.

A saturator-cell generation system can be used to generate a nerve agent vapor. The saturator cell generates agent vapor by passing nitrogen through a multiple-pass glass vessel containing a porous ceramic cylinder saturated with liquid nerve agent. As the nitrogen carrier gas flows over the liquid agent, nerve agent vapor is carried from the glass saturator cell to the inhalation chamber. The flow of nitrogen through the saturator cell can be manipulated to generate various concentrations of nerve agent vapor. Additionally, the concentration of nerve agent vapor can be manipulated by changing the temperature of the saturator cell, thereby changing the rate of evaporation of the nerve agent (Muse et al., 2006). Due to the ability to manipulate the temperature of the saturator cell, this system can be used to generate vapors of either the volatile ''G'' series nerve agents or less volatile agents such as VX (Benton et al., 2005, 2006).

For systems that generate nerve agent vapor, the stability of the generated atmosphere can be continuously monitored by using Hydrogen Flame Emission Detection, a selective monitor for phosphorus containing species. Additionally, a quantitative measurement of the agent concentration in the chamber can be determined using solid phase sorbent tubes. Small samples of the nerve agent vapor containing environment are pulled through glass tubes containing a sorbent material, such as TENAX TA. Once the sample is collected, the amount of agent is quantified using gas chromatography. By knowing the amount of air drawn through the sorbent tube, the concentration of nerve agent (mg/m^3) can be calculated (Muse et al., 2006).

For systems that generate a nerve agent aerosol, there are several ways to characterize the chamber atmosphere. Real-time optical aerosol monitors can be used to monitor the concentration and stability of the chamber atmosphere. Alternatively, glass fiber filters can be used to collect aerosol from the chamber. However, this method only provides information about the average concentration of the chamber atmosphere during the period when the sample was collected and does

not provide information about the stability of the chamber atmosphere. Particle size distribution can be monitored in real time using aerodynamic particle sizer spectrometers connected to the chamber. Alternatively, particle size can be assessed using multistage impactors. As with the glass fiber filters, multistage impactors only provide an average particle size of the chamber aerosol during the period when the sample was collected and do not provide real-time information about changes in particle size. Aerosol chamber concentrations are usually expressed in mg/m^3, although other units are acceptable. Particle size is usually expressed as the mass median aerodynamic diameter (MMAD), a calculation of particle size based on the weight of the collected sample and normalized to particle density. By weight, 50% of the particles will be larger than the MMAD and 50% of the particles will be smaller than the MMAD.

III. OCULAR EFFECTS OF NERVE AGENT VAPOR/AEROSOL EXPOSURE

One of the first noticeable effects of a whole-body exposure to nerve agent vapor or aerosol is constriction of the pupils, or miosis (Figure 11.1). In the Tokyo and Matsumoto terrorist incidents, miosis with subsequent dim vision and narrowing of the visual field was one of the most common signs seen in exposed victims (Ohbu et al., 1997; Okudera, 2002; Yanagisawa, 2006). Additionally, a report of accidental exposures to sarin at Rocky Mountain Arsenal occurring in 1953 and 1954 found that miosis was the most common clinical effect of exposure seen (Gaon and Werne, 1955). A study conducted by the Chemical Defence Establishment at Porton Down, UK found miosis to be the only consistent sign of exposure in volunteers exposed to a low level of sarin vapor for 30 min (Baker and Sedgwick, 1996). Because it is a common finding in exposed individuals, and because it can occur at concentrations much lower than those necessary to cause other signs of toxicity, miosis is often used as a marker of exposure to sarin or cyclosarin vapor or aerosol.

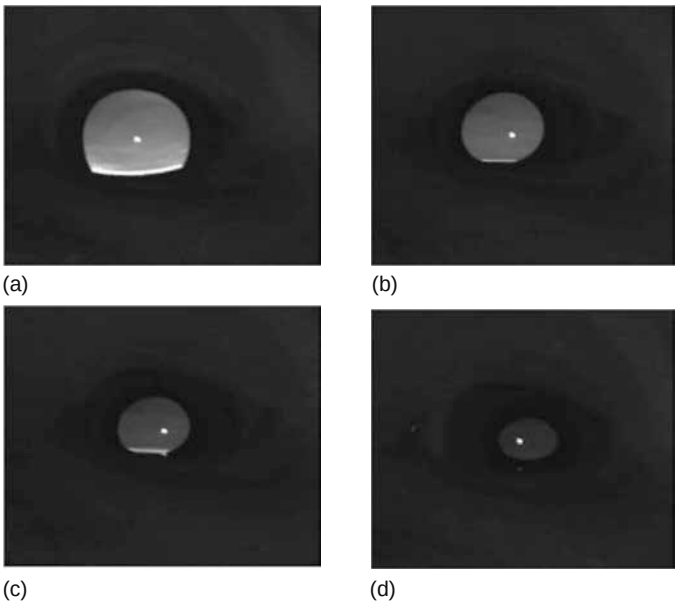

FIGURE 11.1 Representative infrared images showing the progression of miosis in the Gottingen minipig during exposure to sarin vapor. Gottingen minipigs exposed to a lethal concentration of sarin vapor (LCt_{99}, or 4.0 mg/m^3) develop miosis rapidly. Once the pupil begins constricting, the progression from normal pupil size (image A) to a miotic pupil (images B through D) takes less than 3 min. Infrared images were acquired as described previously. (Hulet S.W. et al., *Inhal Toxicol*, 18, 143, 2006a.)

The ocular effects of nerve agents are readily explainable given the physiology of the eye. Normally, both the sympathetic and parasympathetic branches of the autonomic nervous system play a role in the control pupillary size. In both humans and rats, stimulation of the sympathetic branch releases norepinephrine onto α-adrenergic receptors located on the radial muscles of the iris, resulting in dilation of the pupil (Moroi and Lichter, 1996; Yu and Koss, 2002, 2003). Muscarinic acetylcholine receptors are present on the pupillary sphincter, and administration of muscarinic antagonists results in pupillary dilation, demonstrating the role for muscarinic receptors and the parasympathetic nervous system in the control of pupil diameter (Moroi and Lichter, 1996; Smith et al., 1996; Sokolski and Demet, 1996; Furuta et al., 1998). Nerve agent induced miosis is due to inhibition of ocular acetylcholinesterase, which results in excessive stimulation of muscarinic acetylcholine receptors and contraction of the pupillary sphincter. In guinea pigs, it has been shown that nerve agent induced contraction of the pupillary sphincter muscle is proportional to the degree of inhibition of acetylcholinesterase in the iris (Soli et al., 1980). As predicted from the physiology, administration of the muscarinic receptor antagonist atropine prior to nerve agent vapor exposure is able to prevent the miotic response (Dabisch et al., 2005a). The miotic effect of nerve agent vapors and aerosols, unlike other effects of exposure to nerve agent vapor or aerosol, is mainly due to direct contact of nerve agent vapor with the eye and not inhalation and subsequent distribution of the agent to the eye. This direct effect of nerve agents on the eye has been demonstrated previously in several studies. In the 1950s, Sim (1956) demonstrated that covering soldiers' eyes with a bandage decreases the magnitude of the miotic response to sarin vapor exposure, suggesting that penetration of the vapor through the surface of the eye plays an important role in the development of miosis. Additionally, direct application of soman drops to the eye produced miosis in guinea pigs, again suggesting that miosis can result from agent penetration through the ocular surface (Soli et al., 1980).

Different nerve agents have different miotic potencies. Several studies performed in rats have demonstrated that VX is approximately an order of magnitude more potent than sarin and cyclosarin (Mioduszewski et al., 2002a; Whalley et al., 2004; Benton et al., 2005). VX exposure data from humans (Bramwell et al., 1963) is in agreement with the animal potency data, and human estimates reflect this (Reutter et al., 2003). In addition to overall potency differences between agents, gender differences in the miotic potency of nerve agent have also been reported. Female rats appear to be more sensitive to the miotic effects of sarin, cyclosarin, and VX than male rats (Mioduszewski et al., 2002a; Whalley et al., 2004; Benton et al., 2005). Additionally, Hulet et al. (2006a) reported that in the Gottingen minipig, females were more sensitive to sarin for longer duration exposures. However, the same study reported that males were more sensitive to cyclosarin-induced miosis. In mice, males are consistently more sensitive to the effects of sarin vapor than are female mice (Long et al., 1951; McPhail, 1953). Thus, gender differences in miotic sensitivity appear to depend on both the agent being studied and the species being used to study it. The reason for the differing sensitivities between males and females remains unclear. However, several studies have reported gender differences in ocular physiology. In the rat, levels of several drug-metabolizing ocular enzymes were found to be significantly higher in females than in males (Nakamura et al., 2005). Additionally, gender differences in activities of other enzymes, including sorbitol dehydrogenase and glucose-6-phosphate dehydrogenase (Bours et al., 1988), and the structure of the tear-producing lacrimal gland (Sullivan et al., 1990) have been identified in the rat eye. In humans, lacrimal fluid peroxidase activity was found to be different between males and females (Marcozzi et al., 2003). Gender-related differences in humans have also been identified in lacrimal gland structure (Ueno et al., 1996), intraocular pressure (Qureshi, 1997), and Goblet-cell density (Connor et al., 1999). Thus, although the exact reason is unknown, it is plausible that gender differences in the sensitivity to the miotic effect of nerve agent vapor exist. However, data to date suggest that gender differences are not constant across species and type of nerve agent vapor. This makes accounting for gender in human estimates problematic.

Several studies have described the effects of repeated exposure to nerve agents on the eye (Soli et al., 1980; Dabisch et al., 2005a, 2005b). These studies reported that following multiple exposures

to nerve agent, the miotic response to the nerve agent is reduced for several days. The mechanism of this response likely involves desensitization of the pupillary muscarinic receptors, resulting in a loss of parasympathetic function in the eye, and, subsequently, the response to the nerve agent. Although repeated exposures to a nerve agent are unlikely in a real-world scenario, the threshold dose for the toxicological response seen in the eye following such an exposure sequence has not been determined. Thus, it is unknown whether a single exposure in which a sufficient dose of nerve agent is received could result in decreased parasympathetic function and a diminished pupillary light reflex.

IV. RESPIRATORY EFFECTS OF NERVE AGENT VAPOR/AEROSOL EXPOSURE

In addition to the ocular effects of a whole-body nerve agent exposure, respiratory effects due to direct contact of nerve agent with the upper airways are also among the first noticeable signs of exposure. These respiratory effects include tightness in the chest, salivation, and rhinorrhea. A report of accidental exposures to sarin at Rocky Mountain Arsenal occurring in 1953 and 1954 found tightness in the chest present in more than half of the exposed personnel (Gaon and Werne, 1955). Similarly, Baker and Sedgwick (1996) reported dyspnea in sarin exposures that also produced 40%–50% inhibition of red cell acetylcholinesterase. In the Matsumoto and Tokyo terrorist incidents, salivation, rhinorrhea, and dyspnea were commonly reported among exposed individuals (Yanagisawa et al., 2006).

As in the eye, the toxicological effects of inhalation of nerve agent vapor into the respiratory tract can be explained by examining the physiology of the respiratory system. The airways are surrounded by smooth muscle, with the muscular thickness decreasing from the bronchioles to the alveoli (Levitzky, 1999). As in the eye, innervation by the autonomic nervous system plays a major role in controlling the tone of this smooth muscle. Beta-adrenergic receptors present in the large airways and bronchioles mediate the bronchodilator response to beta agonists (Barnes 2004; Proskocil and Fryer, 2005). Additionally, stimulation of beta receptors results in an increase in mucus clearance (Proskocil and Fryer, 2005). Muscarinic receptors located on airway smooth muscle mediate the bronchoconstriction induced by cholinergic agonists by several mechanisms. Stimulation of muscarinic receptors produces a direct increase in airway tone through a phospholipase C mediated mechanism. Additionally, stimulation of muscarinic receptors on airway smooth muscle results in inhibition of beta-receptor mediated relaxation of airway smooth muscle (Fryer and Jacoby, 1998). Normally, acetylcholine released from parasympathetic neurons terminating near these receptors is responsible for the control of airway tone. Stimulation of muscarinic receptors also increases mucus secretion from nasal and submucosal glands (Proskocil and Fryer, 2005). The initial effects of a nerve agent vapor or aerosol in the respiratory system are due to direct action of the agent on the airways as it is inhaled. Inhibition of acetylcholinesterase associated with parasympathetic neurons results in the buildup of acetylcholine, producing bronchoconstriction and increased secretions from cholinergic glands. This results in the tightness in the chest, salivation, and rhinorrhea that are commonly noted following exposure to nerve agent vapor (Gaon and Werne, 1954; Baker and Sedgwick, 1996; Yanagisawa et al., 2006). In severely poisoned individuals, airway obstruction due to increased secretions and bronchoconstriction can be life threatening (Grob, 1956).

The degree to which the toxic effects of inhalation of a nerve agent vapor or aerosol are manifested depends on the amount of nerve agent that is deposited in the airways or absorbed systemically. Nerve agents can be inhaled as a vapor, an aerosol, or a combination of both. The distribution of nerve agent vapor in the respiratory tract will follow normal respiratory gas flow patterns (Urbanetti, 1997). The inspiration of nerve agent contaminated air leads to the distribution of the agent vapor over the nasal and oral cavities, the pharynx, and the trachea, through the primary, secondary, and tertiary bronchi, into the bronchioles and eventually the alveoli (Figure 11.2). Nerve agent vapor deposition in the upper airways of the respiratory tract is responsible for the early respiratory signs of nerve agent vapor exposure. Nerve agent penetration to the distal

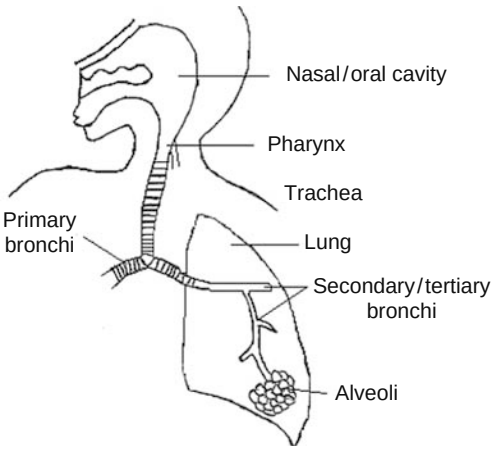

FIGURE 11.2 Anatomy of the human respiratory tract. Deposition of nerve agent vapor or aerosols in the different regions of the lung can lead to different symptomology. Upper airway deposition can lead to immediate respiratory distress. Alveolar deposition leads to systemic distribution of the nerve agent.

regions of the respiratory system increases at higher flow rates, such as during exercise (Ainsworth and Sheppard, 1961). In the alveoli, the inhaled nerve agent vapor is absorbed through the alveolar epithelium into the systemic circulation, resulting in distribution throughout the body.

The deposition of aerosols in the lung is more complicated than the distribution of nerve agent vapor. The geometry of the respiratory tract and the diameter of the inhaled liquid aerosol determine the deposition of the inhaled material. The geometry of the respiratory tract varies from species to species. However, it is consistent within a species. Therefore, within a given species, the deposition of aerosols is primarily dependent on the aerodynamic size of the inhaled aerosol droplet. Classically, aerosols are classified into four groups with each having significantly different deposition behavior (Anderson et al., 1988; Casillas et al., 1999). In humans, aerosols greater than 10 μm in diameter are classified as nonrespirable because they do not penetrate the respiratory tract deeper than the larynx due to impaction. Very small particles, with aerodynamic diameters less than 0.01 μm, are also trapped in the upper airways and are considered nonrespirable (Witschi and Last, 2001). The largest respirable aerosols in humans have an aerodynamic diameter between 2.5 and 10 μm. Aerosols in this size range deposit in the nose, mouth, and airways greater than 2 mm in diameter. As the aerodynamic size of the aerosols becomes less than 2.5 μm, deposition begins to occur in more distal region of the respiratory tract, mainly in airways less than 2 mm in diameter. Aerosols with very small diameters (<0.1 μm, but >0.01 μm) tend to deposit primarily in the alveoli.

V. GASTROINTESTINAL EFFECTS OF NERVE AGENT AEROSOL EXPOSURE

For aerosol delivery systems used for drugs such as albuterol, a high percentage of the inhaled aerosol is swallowed (Serafin, 1996). Thus, it is possible that an individual exposed to a nerve agent aerosol may swallow a significant amount of nerve agent. As with the other systems discussed, the gastrointestinal system has a significant degree of cholinergic innervation. Thus, a nerve agent would be expected to produce local signs of cholinergic overstimulation in the gastrointestinal tract. Indeed, oral administration of sarin by humans resulted in abdominal cramps and loose stool in some exposed individuals (Grob and Harvey, 1958). Additionally, it is possible that absorption through the gastrointestinal tract may represent an additional route of absorption for an aerosol exposure. Data from several studies support this. It has been shown previously that ingestion of VX-contaminated drinking water can result in a decrease in red blood cell cholinesterase activity

(Sim et al., 1964), suggesting that VX is absorbed through the human gastrointestinal tract into the systemic circulation. Similarly, orally administered sarin produced significant systemic signs of intoxication in exposed individuals (Grob and Harvey, 1958).

VI. OTHER EFFECTS FOLLOWING DISTRIBUTION THROUGHOUT THE BODY

Irrespective of the exposure route, once the nerve agent enters the systemic circulation it will be distributed throughout the tissues of the body, possibly resulting in a cascade of additional toxic signs (Frederiksson et al., 1960). These effects are discussed in detail in other chapters of this volume.

VII. MODELING CHEMICAL NERVE AGENT DOSAGE

A. Haber's Rule versus Toxic Load

It is widely known that the toxicity caused by inhalation of vapor of a harmful agent is dependent on dosage which, in turn, is a function of both the concentration of the substance in the air (C) and the duration of the exposure (T). As early as 1924, Haber developed a formula for assessing the toxic affects of harmful vapors. Haber's rule is described by the equation

$$C \times T = \mathrm{k}$$

where k is a constant for some biological effect or response (Derelanko, 2002). Toxicologists use the term LCt_{50} to describe the product of the atmospheric concentration of a vapor and the duration of exposure that results in lethality to 50% of the exposed population. Similarly, other endpoints are defined using the term ECT_{50}, or an effective concentration $\times$ time that results in a designated biological endpoint. Effective concentrations as a result of inhalation of nerve agent vapors have been described for miosis (Mioduszewski et al., 2002a; Whalley et al., 2004; Benton et al., 2005; Hulet et al., 2006a), incapacitation (Cresthull et al., 1957; Oberst and Woodson, 1957), and cholinesterase depression (Marzulli and Callahan, 1954). However, the most common effects investigated are miosis and lethality. Miosis is often referred to as the ''first noticeable effect'' of a G-agent vapor exposure. As discussed previously, the pupil constriction is generally attributed to a local inhibition of cholinesterase in the eye rather than a systemic effect (Grob, 1956). In contrast, after intravenous, subcutaneous, or percutaneous exposures miosis occurs after other signs of toxicity or is an irregular finding (Frederiksson et al., 1960). Pupil constriction is often used as a biological endpoint for investigating G-agent vapor exposures because it occurs at concentrations much lower than those necessary to produce severe or lethal signs of toxicity; for V-agent vapor, this issue is still under study.

Previously, military and other organizations dealing with inhalation toxicology have accepted the principle of dosage (concentration $\times$ time) as constant over time (Haber's rule) when assessing the impact of exposures to nerve agent vapor. Vapor studies conducted during the 1950s and 1960s on experimental animals and human volunteers used durations of exposures ranging from several seconds to several minutes. Haber's rule was then used to extrapolate dose–response data based upon relatively short exposure times to predict response probabilities involving longer exposure times. It was assumed that a calculated LCt_{50} was valid regardless of the duration of exposure. However, this concept has been found to be inadequate for assessing biological effects resulting from exposure to many acutely toxic gases and aerosols (ten Berge et al., 1986; Sommerville et al., 2006). ten Berge varied both exposure concentration and duration of exposure to several toxic vapors and determined that the concentration and the exposure duration were not equally important in determining the toxic effect. ten Berge modeled the relationship between the concentration, exposure duration, and resulting biological effect using a toxic load model. The toxic load model can be described using the following equation:

$$C^n T = \mathrm{k}$$

where

C is the concentration

T is exposure duration

k is a constant for some biological effect or response

n is the toxic load exponent, which is unique to a particular vapor and exposure scenario

In the toxic load model, dosage is not constant over time. Rather, the effective dosage increases with exposure time in a nonlinear relationship. If the toxic load exponent is 1, then $CT = k$ and the data are best modeled with Haber's rule. If the toxic load exponent is greater than 1, then the concentration of the agent plays a larger role in determining the biological response than time. If the toxic load exponent is less than 1, then the time of exposure plays a greater role in determining the biological response than concentration. Sommerville et al. (2006) discuss the mathematics of the toxic load model in detail. A comparison between the toxic load model and Haber's rule is shown in Figure 11.3.

ten Berge's findings suggesting that the toxicity of gases was not constant over time necessitated the reexamination of nerve agent toxicity estimates. Subsequently a focused research effort by investigators (Mioduszewski et al., 2002a, 2002b; Anthony et al., 2003; Whalley et al., 2004; Benton et al., 2005, 2006: Hulet et al., 2006a, 2006b, 2006c) at the Edgewood Chemical Biological Center was undertaken to assess the effects of exposure to low concentrations of nerve agent vapors for extended exposure durations. These reports address the vapor toxicity of the nerve agents sarin, cyclosarin, and VX in rats and swine with separate endpoints for miosis and lethality. The conclusion of all the reports is that toxicity caused by exposure to nerve agent vapors is not constant over time as would be predicted by Haber's rule. Rather the data were best modeled with toxic load equations. Table 11.2 gives the toxic load exponents for vapor exposures to sarin, cyclosarin, and VX in rats and swine. Subsequently, even for a clear toxicological endpoint such as lethality, historical assumptions previously used to extend the prediction of exposures out in time have been shown to be overly conservative for sarin, the best-studied agent.

As stated previously, higher the toxic load exponent, greater the influence that the vapor concentration has on toxicity. The only instance in which the toxic load exponent was not greater

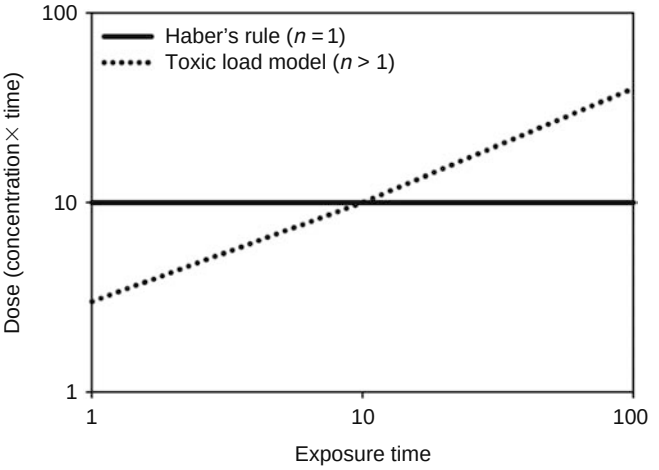

FIGURE 11.3 Comparison of Haber's rule and toxic load model. If Haber's rule applies ($n = 1$), then the dose required to produce a given response is constant relative to exposure time. However, for a toxic load model with an $n > 1$, the dose required to produce a given response increases as the exposure time increases. For a toxic load model with an $n < 1$ (not shown), the dose required to produce a given response decreases as the exposure time increases.

TABLE 11.2
Experimentally Determined Toxic Load Exponents for Nerve Agent Vapor in Rat and Swine

Endpoint	Species	Agent	Toxic Load Exponent	References
Miosis	Rat	sarin	1.96	Mioduszewski et al. (2002a)
		cyclosarin	1.98	Whalley et al. (2004)
		VX	1.65	Benton et al. (2005)
	Swine	sarin	1.32	Hulet et al. (2006a)
		cyclosarin	1.60	Hulet et al. (2006a)
Lethality	Rat	sarin	1.66	Mioduszewski et al. (2002b)
		cyclosarin	1.27	Anthony et al. (2003)
		VX	0.92	Benton et al. (2006)
	Swine	sarin	1.38	Hulet et al. (2006b)
		cyclosarin	1.28	Hulet et al. (2006c)

than 1 was in the study of Benton (2006) investigating lethality of vapor VX in the rat where a toxic load exponent of 0.92 was calculated. A toxic load exponent less than 1 suggests that the duration of the exposure has more influence on the toxicity than does the concentration. This finding is most likely due to the physical properties of VX. As seen in Table 11.1, VX is several orders of magnitude less volatile than any of the G agents making evaporation of VX deposited on the rats' fur less likely. Thus, for longer exposure durations it is possible that there was sufficient time for VX to enter the systemic circulation by percutaneous absorption and oral ingestion (due to grooming habits of rodents). This absorption of VX would be in addition to the vapor inhalation component. Because of their relatively higher volatilities, G-agent vapor exposures are not associated with significant additional body burden from percutaneous (Marzulli and Williams, 1953) and oral routes. Therefore, the primary hazard of sarin vapor is inhalation whereas VX may have both inhaled and percutaneous components when generated as a vapor. However, because of the extremely low vapor pressure of VX it requires temperatures in excess of 40°C to generate significant amounts of VX vapor (Benton et al., 2005, 2006). Although this requirement makes generation of VX vapor less likely, it is still reasonable to assume VX vapor could be present given the right environmental conditions.

B. Use of Animal Model Toxicity Data to Estimate Risks of Human Exposure to Nerve Agent Atmospheres

In the approximately 70 years since the discovery of the toxic G agents and 50 years since the subsequent development of the V agents, humans have only occassionally served as test subjects in laboratory studies designed to determine threshold toxic effects associated with low-level (nonlethal) sarin and VX vapor exposures (2–10 min) (Johns, 1952; Sim, 1956; Bramwell et al., 1963). In addition, although the toxic effects of accidental exposures and nonexperimental exposures from terrorist or military attacks are documented, critical information related to the exposure conditions can only be estimated at best. Thus, estimates of human dose–responses to nerve agent vapor exposures from such sources are often associated with significant uncertainty and are of limited utility in predicting health hazard risks.

The current human estimates for nerve agent vapor and aerosol toxicity are based upon data from animal models. Rodents are popular animal models in that they are readily available, cheap, and convenient to handle. Additionally, there is a great amount of background data on rodents available in the literature for comparison to many biological endpoints. However, nonrodent models are preferred for studies of prophylactic and therapeutic efficacy associated with nerve agent exposure due to the protective buffering impact of relatively high blood carboxylesterase levels in

TABLE 11.3
LCt_{50} Values for 10 min Exposures to Sarin Vapor

Species	LCt_{50} (mg min/m^3)	References
Mouse	380	McGrath and Fuhr (1948)
Rats	231	Mioduszewski et al. (2002b)
Rabbits	115	McGrath and Fuhr (1948)
Cats	79	McGrath and Oberst (1952)
Monkeys	74	Cresthull et al. (1957)
Swine		
Gottingen	73	Hulet et al. (2006b)
Yorkshire	34	Crook et al. (1952)
Human (estimate)	60	Reutter et al. (2003)

rodents (Maxwell et al., 1987). Nevertheless, rodents are routinely used as an initial component in a multispecies database for extrapolation to human estimates. An example of the variation in species sensitivity to the lethal effects of sarin vapor is listed in Table 11.3 where experimentally obtained LCt_{50} values for 10 min sarin vapor exposures are compared in several species. Table 11.3 also includes current human lethal estimates for comparison.

In general, it is obvious that the larger animals (pigs, dogs, cats, and monkeys) have lower threshold values than do smaller animals (mice, rats, and rabbits). The differences are most likely due to the variations in body mass, respiratory parameters, and the presence of relatively high carboxylesterase levels in rodents. Reutter et al. (2003) estimated the human LCt_{50} for a 10 min sarin vapor exposure to be 60 mg min/m^3. The estimates from this report were incorporated into U.S. Army Field Manual 3–11.9 (2005). This estimate was extrapolated based on data taken from studies involving eight species (mice, rat, guinea pig, rabbit, cat, dog, swine, and monkey). The estimate took into account the nonanesthetized minute volume (MV) to body weight (BW) ratio for most of the species (Bide et al., 2000). The small rodents (guinea pigs, rats, and mice) all had MV/BW ratios above 0.594. The MV/BW ratio in dogs was 0.328. The MV/BW ratio in monkeys was 0.384. The MV/BW ratio of pigs was 0.225 (Denac et al., 1977). The calculated MV/BW ratio for humans was 0.223. These findings highlight the importance of the choice of animal model and exposure route in investigating nerve agent toxicity and the difficulties associated with extrapolating data obtained from one species and exposure route to data obtained by another species and exposure route.

VIII. SUMMARY

Exposure to chemical nerve agent atmospheres leads to a myriad of toxic signs that are primarily mediated though acetylcholinesterase inhibition at target tissues. The initial signs of chemical nerve agent vapor and aerosol exposure are attributed to direct effects on target tissues which are most accessible to vapor/aerosols including the eyes and respiratory tract. Thus, some of the first noticeable effects of nerve agent vapor or aerosol inhalation exposure are miosis, rhinorrhea, and tightness in the chest. Because it is a common finding in exposed individuals, and because it can occur at concentrations much lower than those necessary to cause other signs of toxicity, miosis is often used as a marker of exposure to a nerve agent vapor or aerosol. In a real-world situation, the most common scenarios of exposure would involve the percutaneous or inhalation routes. In the Inhalation Toxicology Laboratory at the U.S. Army Edgewood Chemical Biological Center, several methods have been employed to produce nerve agent vapors and aerosols that act via these routes and, in that regard, mimic likely exposure scenarios. The challenge for laboratory studies is to safely generate stable vapor or aerosol atmospheres and verify their chamber atmospheric concentration, chemical characterization, and stability throughout the exposure period. The combination of these

methods with toxicity studies in multiple animal models has resulted in significantly improved confidence intervals on estimates of human exposure risks to nerve agent atmospheres. This in turn, provides the basis for defendable criteria for chemical nerve agent detection, protection, decontamination, and countermeasure technologies.

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12 Vesicants and Oxidative Stress

Milton G. Smith, William Stone, Ren-Feng Guo, Peter A. Ward, Zacharias Suntres, Shyamali Mukherjee, and Salil K. Das

CONTENTS

I. BACKGROUND

The primary treatment of vesicant exposure is decontamination and supportive therapies; there is only one vesicant that has an antidote. The paucity of the treatment options continues to confer their tactical advantage. The development of an ameliorative or antidote would accomplish, at a minimum, two goals: (1) effective treatment, if needed, and (2) decreased tactical advantage.

Vesicants are considered to be of low technology and are relatively simple to manufacture. Barrier protection gear, otherwise known as Mission Oriented Protective Posture (MOPP), provides significant protection if donned before exposure. During battlefield conditions, errors would be expected in the use of the MOPP gear because of stressful situations, tears in the suit, false positive or negative alarms, etc.

Vesicants share some common properties that are noteworthy in attempts to achieve a better understanding of their pathogenesis. One characteristic is that they all induce an acute inflammatory reaction (Sidell et al., 1997; Sciuto, 1998; Ricketts et al., 2000; Naghii, 2002; Segal and Lang, 2005), of which a subcomponent is oxidative stress (OS) (Bartsch and Nair, 2006). A consequence of OS is the oxidation of thiol groups, which is seen in all of the vesicant exposures (Vissers and Winterbourn, 1995; Pant et al., 2000; Carr et al., 2001).

The oxidation of thiol groups disrupts redox balance (defined later in this chapter), which can set into motion a cascade of events, such as apoptosis, oxidant production, and increased activity of redox-regulated transcription factors (e.g., nuclear factor kappa beta [NF-κB]). The occurrence of OS is not isolated to the vesicant class of weapons of mass destruction (WMD). Radiation (Kang et al., 2006), bacterial infections (e.g., anthrax) (Hanna et al., 1994; Kuhn et al., 2006), viral infections (e.g., influenza) (Ghezzi and Ungheri, 2004), and ricin (Kumar et al., 2003; Suntres et al., 2005) exposures also induce OS as part of the host pathogen response, which is acute inflammation.

Sublethal OS induces varying degrees of damage in cellular organelles. Lethal OS occurs when antioxidant defenses completely fail, resulting in necrosis (Virag, 2005) or apoptosis (Haddad, 2004). In this chapter, OS will be discussed as a concept, along with its occurrence in the organ systems that are most notably affected by the vesicants. Antioxidant defenses that are critical to the maintenance of redox balance are also discussed.

Achieving a deeper understanding of OS is important for the elucidation of all acute inflammatory disorders. The OS component of vesicant exposure has not been a focal point of research, by

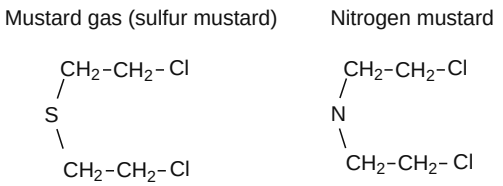

FIGURE 12.1 Chemical structure of sulfur and nitrogen mustards.

and large for vesicants or other WMD. It is hoped that this chapter will help to spur future research in the area.

II. INTRODUCTION

There are four primary vesicating agents–mustard, phosgene, chlorine, and Lewisite. Strategically, they are poor weapons because they are subject to being redirected by the wind. Sulfur mustards (SM) are considered to be one of the vesicants that cause the most concern. There are two types of mustards, sulfur- and nitrogen-based compounds (see Figure 12.1). The nitrogen mustards have been found to be unsuitable for warfare; therefore, there will be no further mention of them. Any further references to mustards will be to SM. Strategically, Lewisite is the least important since there is a proven antidote. British anti-Lewisite, the antidote for Lewisite, has been known for decades (Goldman and Dacre, 1989).

A significant component of acute inflammation is OS (Nonas et al., 2006). One aspect of OS is the production of oxidants and proinflammatory cytokines. It is defined as an imbalance of antioxidant/oxidant ratio, which has a consequent effect on gene expression. The maintenance of the antioxidant/oxidant ratio in the nonstressed cell would be termed redox homeostasis. Thiol groups that are part of intracellular proteins and glutathione (GSH) are critical for the maintenance of redox homeostasis. The vesicants as a class are either oxidants themselves, or they indirectly produce oxidants. Oxidants can be water soluble and fat soluble; therefore, they can arise in any compartment of the cell. They are able to attack any cellular structure, rendering them partially or completely dysfunctional.

The chemical WMD classified as vesicants cause blistering of the skin, which is why they are referred to as blistering agents. They incapacitate more so than kill the exposed person because burns occur on any tissue that it contacts. They all have prolonged systemic effects. The reader is referred to the individual sections of this book and other excellent reviews on the agents for a more detailed classic description of their effects, usage, and history (Goldman and Dacre, 1989; Naghii, 2002; Sciuto and Hurt, 2004; Segal and Lang, 2005). The agents themselves will be reviewed with the features of OS highlighted where information was available.

III. MUSTARD

Vesicants were first deployed against troops during World War I (WWI). They were used as recently as 1988 by Saddam Hussein against the Kurds in Halabja. SM (2-bis-chloroethyl ethyl chloride) is a straw-colored liquid that disseminates with a garlic-like odor on evaporation (Duke-Elder and MacFaul, 1972; Dahl et al., 1985). SM is composed of small, oily droplets with volatility significantly higher in warmer climates (e.g., the Middle East). At a temperature of 38°C, it can be present in the environment for 7 h, whereas at 10°C, it persists for 100 h. The density of mustards is 5.4-fold greater than air and tends to be found 6–12 inches above the ground. This ground-hugging characteristic causes it to sink into trenches and gullies. In WWI, soldiers frequently removed their masks in the morning, assuming that the mustard threat had subsided, and were unaware that the

mustard persisted in the environment. As the ambient temperature increased at sunrise, there was an increase in evaporation of the mustard at the ground-level atmosphere, and the soldiers would unknowingly inhale the newly evaporated gas. Mustard has a high freezing temperature (57°F). This freezing temperature can be reduced by mixing it with other agents, such as chlorobenzene or carbon tetrachloride (Borak and Sidell, 1992; Sidell et al., 1997). Similar reductions in freezing point can be accomplished by mixing it with Lewisite.

SM is commonly referred to as an alkylating agent, but recent evidence has shown it to be a potent inducer of oxidants (Levitt et al., 2003; McClintock et al., 2006). Mustard gas has a strong, irritating effect on living tissue and induces long-lasting, toxic effects (Safarinejad et al., 2001). Additionally, its destructive effects are not localized to the site of application; remote cells and tissues are also affected. SM damages DNA mainly by alkylating and cross-linking the purine bases (Fox and Scott, 1980). Cysteine groups in proteins make them sensitive to SM, resulting in covalently cross-linked dimers on exposure (Byrne et al., 1996). This cross-linking causes conformational change or dysfunction of enzymes. The potent alkylating activity of SM is due to the formation of highly electrophilic ethylene episulphonium derivative (Lundlum et al., 1984; Papirmeister et al., 1991). A portion of the inhalation pathogenesis of SM is due its cholinergic properties (mediating the action of acetylcholine) that stimulates both muscarinic and nicotinic receptors (Anslow, 1946).

On contact with human skin, 80% of the liquid evaporates and 20% penetrates (half remains on the skin and the other half is absorbed systemically). Systemic absorption results in partitioning to organs, such as the spleen, liver, and bone marrow (Langenberg et al., 1998). It is mutagenic and carcinogenic (Papirmeister et al., 1985; Somani and Babu, 1989; Wormser, 1991; Langenberg et al., 1998). The lethal dose for humans is 200 mg if ingested and 3 g with cutaneous exposure (Javadi et al., 2005). A property that has received little attention, but should be kept in mind, is that SM is also a radiomimetic, a property that is shared with other radiation-emitting agents. Additional properties that both radiation and mustards share are the inducement of apoptosis, burns, and OS.

A. Poly ADP-Ribose Polymerase

Alkylation of DNA by SM leads to the activation of poly ADP-ribose polymerase (PARP), which reduces the availability of oxidized nicotinamide adenine dinucleotide (NAD^+) and ATP in the cell (Fox and Scott, 1980). The consequent change in cellular bioenergetics leads to the inhibition of glycolysis, activation of hexosemonophosphate shunt, induction of plasminogen activator, and ultimately, production of skin lesions (Mol et al., 1989). Other alterations in cellular metabolism due to the loss of NAD^+ are microfilament architecture and function in keratinocytes (Papirmeister et al., 1985). PARP uses NAD^+ for a two-electron donor in an oxidation step to catalyze the polymerization of ADP-ribose units on target proteins to attach PARP. PARP has a multiplicity of functions, such as DNA damage sensor and repair chromatin modification, transcription, cell death pathways, insulator function, and mitotic apparatus function. These processes are important to several physiological and pathophysiological processes that involve genome maintenance, carcinogenesis, aging, inflammation, and neuronal function. Poly ADP-ribose polymerase-1 (PARP-1) is the most abundantly expressed member of the family of PARP proteins; it binds to DNA structures that have single and double strand breaks, crossovers, cruciforms, and supercoils (Kim et al., 2005). During normal cellular metabolism, the level of PARP-1 is very low. The enzymatic activity is greatly increased, as much as 500-fold, with allosteric activators, such as damaged and undamaged DNA occurrence (D'Amours et al., 1998, 1999; Kun and Bauer, 2001; Oei and Shi, 2001).

The attachment of PARP or PARrylation, in response to oxidation, alkylation, or ionizing radiation, is dramatic and immediate. At minimal levels of DNA damage, PARP-1 acts as a survival factor involved in DNA damage detections and repair. At high levels of DNA damage, PARP-1 promotes cell death (Burkle, 2001). Elevated levels of PARP in response to DNA damage canpromote necrosis secondary to exhaustion of cellular NAD^+ and ATP (cellular energy failure) (Decker and Muller, 2002; Bouchard et al., 2003). PARP-1 even facilitates apoptosis by a caspase-independent

apoptotic cell death via apoptosis inducing factor (AIF). AIF, a potent trigger of apoptosis, is a flavoprotein, which resides in the mitochondrial intermembrane (similar to cytochrome *c*). However, it is unclear what the exact trigger of AIF release is, but it could be the result of the depletion of NAD^+ due to excessive PARP synthesis and activity. PARP preferentially depletes cytosolic NAD^+ (Zong et al., 2004), which would be expected to affect the overall cellular bioenergetics.

B. Metabolites of Sulfur Mustard

The metabolites of SM are noteworthy because of their utilization of thiol compounds. These metabolites, in addition to OS, diminish the thiol cellular pool. Some metabolites occur by direct hydrolysis, but the majority are conjugates of GSH (Black et al., 1992; Black and Read, 1995); other metabolites are *N*-acetyl-L-cysteine (NAC) conjugates or methylthio/methylsulfhinyl derivatives. Enzymatic conjugation of alkylating agents utilizing GSH can occur through glutathione-S-transferase (GST); but alkylating agents can also combine directly with thiols (Moore and Ray 1983; Colvin et al., 1993). Spontaneous or enzymatic conjugation of alkylating agents occurs through an aziridium intermediate (Colvin et al., 1993).

C. Signaling

There are a multitude of activators of NF-κB, such as viral infections, bacterial infections, radiation, interleukin (IL)-1, and tumor necrosis factor (TNF). Most of the activators of NF-κB can be blocked with the use of antioxidants (Schulze et al., 1997). Other transcription factors, such as AP-1 (Xanthoudakis et al., 1994), MAF, and NRL (Kerppola and Curran, 1994), and NF-IL6 (Hsu et al., 1994) are regulated by oxygen-dependent mechanisms that cause redox cycling of cysteinyl residues.

NF-κB regulates many genes involved in inflammation, such as inducible nitric oxide synthase (iNOS), proinflammatory cytokines, IL-1, tumor necrosis factor-alpha (TNF-α), IL-6, chemokine, IL-8, E-selectin, vascular cell adhesion molecule 1 (ICAM-1), and granulocyte-macrophage colony stimulating factor (GM-CSF) (Brennan et al., 1995; Akira and Kishimoto, 1997; Barnes and Adcock, 1998; Rahman and MacNee, 1998; McClintock et al., 2002). Arroyo et al. (2000) found a dose-dependent increase in TNF-α, IL-6, and interleukin-1-beta (IL-1-beta) in SM-treated human keratinocyte cells.

Although inflammatory cytokine profiles differ depending on the skin models used (Ricketts et al., 2000; Sabourin et al., 2002), they were shown to be elevated in response to SM. In contrast, in guinea pig lungs exposed to 2-chloroethyl ethyl sulfide (CEES) (a mustards analog), the inflammatory cytokine TNF-α was found to be markedly elevated (Das et al., 2003). The inflammatory cytokines exacerbate the effects of CEES (Stone et al., 2003), which would imply that there is an amplification of the initial injury by the mustards. Lipopolysaccharide (LPS), a ubiquitous entity in our environment, also upwardly modulates the damage that CEES inherently causes to cells (Stone et al., 2003).

SM activates phospholipase A2 and liberates arachidonic acid by acting on linolenic acid found in cellular membranes. Activated neutrophils show an amplification of their respiratory burst in the presence of arachidonic acid (Bostan et al., 2003). Metabolites of arachidonic acid produce oxidants, which would be additional contributors to OS. Arachidonic acid itself and several of its metabolites, such as 12 HETE and 15 HETE, induce FOS and JUN expression (Haliday et al., 1991; Sellmayer et al., 1991; Rao et al., 1996).

One class of signaling pathways that has received considerable attention involves the action of cytokine-stimulated sphingomyelinase (SHM-ase) (Hannun and Obeid, 1997). Ceramide generated from SHM-ase activation plays a critical role in cytokine-mediated apoptosis, cellular differentiation, and senescence, each of which may be important in the inflammatory response.

Ceramide can be generated through several different pathways, such as synthesis within the cell, hydrolysis of SHM by SHM-ase, and breakdown of glycosphingolipids. The degradation of ceramide by acid ceramidase liberates sphingosine, a free fatty acid in the lysosomal compartment

(Alphonse et al., 2002). Ceramide can also be increased by the inhibition of ceramide breakdown by ceramidase and the inhibition of sphingomyelin synthase.

SHM-ase has been proposed as a key enzyme involved in stress-induced ceramide formation (Hannun and Obeid, 1997). Multiple pathways may be regulated, which in turn ultimately determine the levels of ceramide.

D. Tumor Necrosis Factor-Alpha Increases with CEES Exposure

A single, intratracheal injection (0.5 mg/kg body weight) of CEES in guinea pigs was done at different time points. The guinea pigs were dissected and the lung was removed after perfusion. The lung was lavaged and TNF-α concentrations were measured in lung lavage fluid, lung lavage macrophages, and in lung tissue. The level of TNF-α in lavage fluid was very low, whereas high levels accumulated in lung as well as in lung macrophages within 1 h of CEES exposure. The level of TNF-α decreased rapidly after 1 h and returned to normal levels within 24 h of CEES exposure (Figure 12.2a). Further studies revealed that the induction of TNF-α by CEES is dose dependent, and optimal TNF-α accumulation was observed at 2 mg/kg dose of CEES exposure (Figure 12.2b).

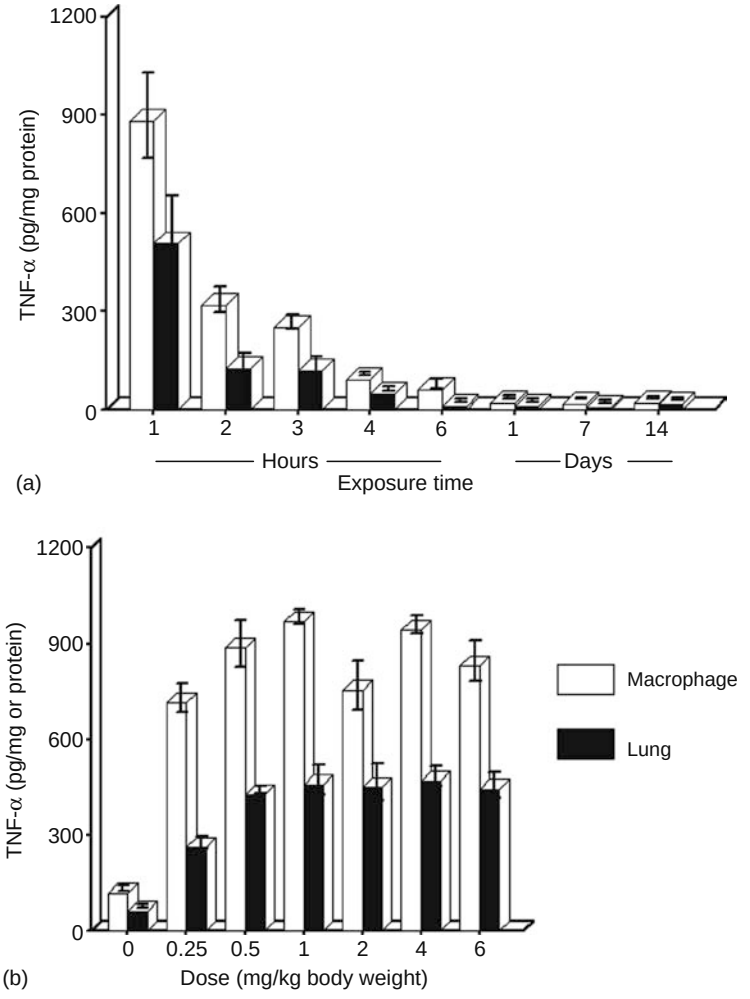

FIGURE 12.2 Accumulation of TNF-α in guinea pig lung and macrophages after exposure to CEES. (a) Time-dependent induction of TNF-α after intratracheal injection of CEES (0.5 mg/kg body weight). (b) Accumulation of TNF-α 1 h after exposure to CEES at different doses (ranging from 0.5 to 6.0 mg/kg body weight).

E. Activation of Sphingomyelinase Activities after CEES Exposure

Both the neutral and acid SHM-ase activities showed fourfold to fivefold increases after CEES treatment. The basal level of acidic SHM-ase activity (Figure 12.3b and d) was much higher than the basal level of neutral SHM-ase (Figure 12.3a and c).

Both neutral and acid SHM-ase activities started to increase, along with the increase of TNF-α; these activities reached a maximum peak between 4 and 6 h in the lung and between 3 and 4 h in macrophages. It is not known at this time whether this difference between lung and macrophages is functionally significant or not. The SHM-ase activities (both neutral and acidic) were found to be higher in lavage macrophages than those in the lung tissue. It is possible that macrophages are more sensitive to CEES than other cell types in the lung. The level of SHM-ase decreased rapidly and returned to near normal level within 24 h. It was also found that a 2 mg/kg dose of CEES is sufficient to reach the maximum level of both neutral and acid SHM-ase activity (Figure 12.3c and d).

F. Accumulation of Ceramide in Lungs after CEES Exposure

The ceramide accumulation after CEES exposure demonstrated a biphasic pattern. Within 1 h of CEES exposure, ceramide levels became very high and reached a peak accumulation within 2 h (Figure 12.4a). After 2 h, there was some decrease in the ceramide level, but then the level increased very high and remained almost to a steady state, even up to 14 days later (Figure 12.4a). CEES-induced ceramide accumulation was found to be saturated at 4 mg/kg dose of CEES. At 2 mg/kg dose of CEES, about 90% induction of ceramide was achieved (Figure 12.4b).

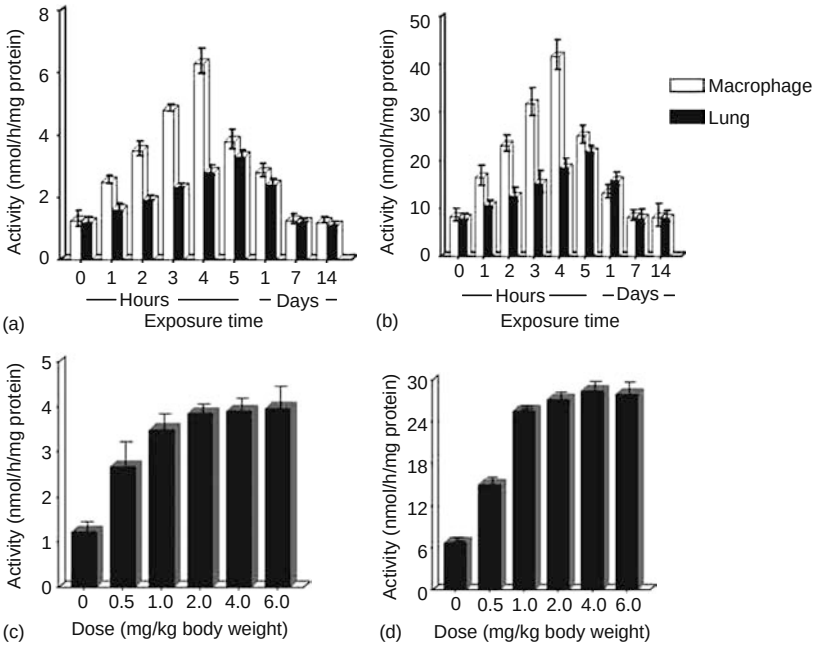

FIGURE 12.3 Activation of acid and neutral sphingomyelinase in guinea pig lung and macrophages following CEES exposure. Time course of induction of neutral (a) and acid (b) sphingomyelinase after intratracheal injection of CEES (0.5 mg/kg body weight). Accumulation of neutral (c) and acid (d) sphingomyelinase at 4 h after exposure to CEES at different doses (ranging from 0.5 to 6.0 mg/kg body weight).

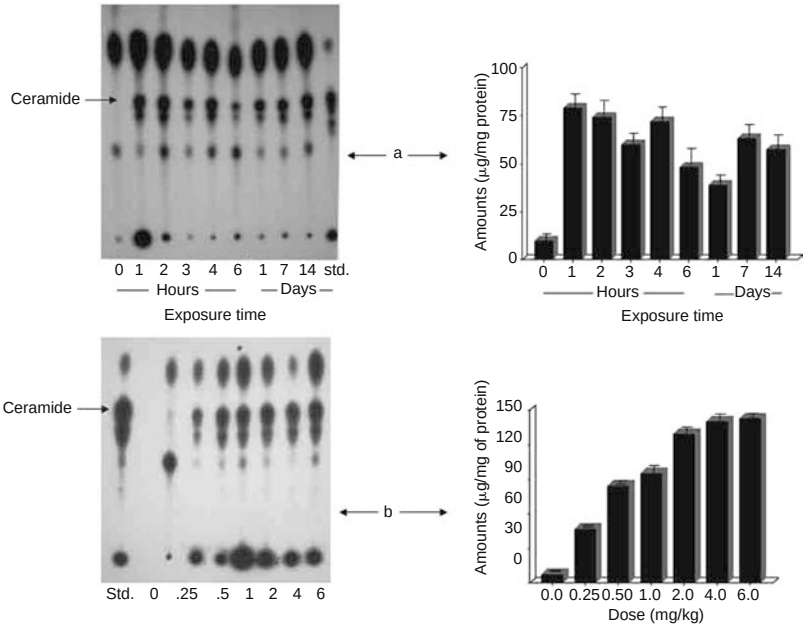

FIGURE 12.4 Accumulation of ceramide in guinea pig lung after CEES exposure. (a) Time course of induction of ceramide after intratracheal injection of CEES (0.5 mg/kg body weight). (b) Accumulation of ceramide at 1 h after exposure to CEES at different doses (ranging from 0.5 to 6.0 mg/kg body weight). In both cases (a and b), the left panel is the autoradiograph showing the accumulated ceramides, and the right panel represents the quantitative analysis of accumulated ceramide as determined by ^{32}P incorporation.

G. Activation of Nuclear Factor Kappa in Lungs after CEES Exposure

The activation of NF-κB was measured in the nuclear extracts of lungs after exposure to CEES. NF-κB, which is well known to inhibit TNF-α-mediated apoptosis, showed activation only up to 1–2 h after CEES exposure (Figure 12.5a). This may explain the biphasic effect of CEES on the lung.

After initial lung damage by TNF-α, within 2 h there was some recovery due to activation of NF-κB. After 2 h, the NF-κB level went down, ceramide level increased, and secondary lung damages were observed. Dose-dependent studies revealed that 4 mg/kg CEES was needed for the optimum activation of NF-κB (Figure 12.5b). Super shift assay using specific antibodies to p50 and p65 revealed that both the p50 and p65 subunits were activated in lung because of CEES exposure (Figure 12.5c).

H. Activation of Caspases after CEES Exposure

Activation of different caspases in guinea pig lung after the CEES exposure is shown in Figure 12.6. Within 1 h of exposure, some activation was observed for all four caspases, but the activity came back to the basal level within 2 h of exposure. The activity of caspase 2, caspase 3, caspase 8, as well as caspase 9 increased significantly between 4 and 6 h of exposure and then declined again. No activity for any of the caspases was observed at 24 h and thereafter. Here also, the activation of caspases was found to be optimum at 2 mg/kg body weight of CEES.

Our study clearly demonstrated the involvement of an SHM-ase/ceramide signal transduction pathway in the mustard gas-mediated lung injury. After intratracheal injection of CEES to guinea pigs, the TNF-α level increased sharply within 1 h of exposure. The TNF-α level started, declined after 1 h, and returned to basal level within 24 h.

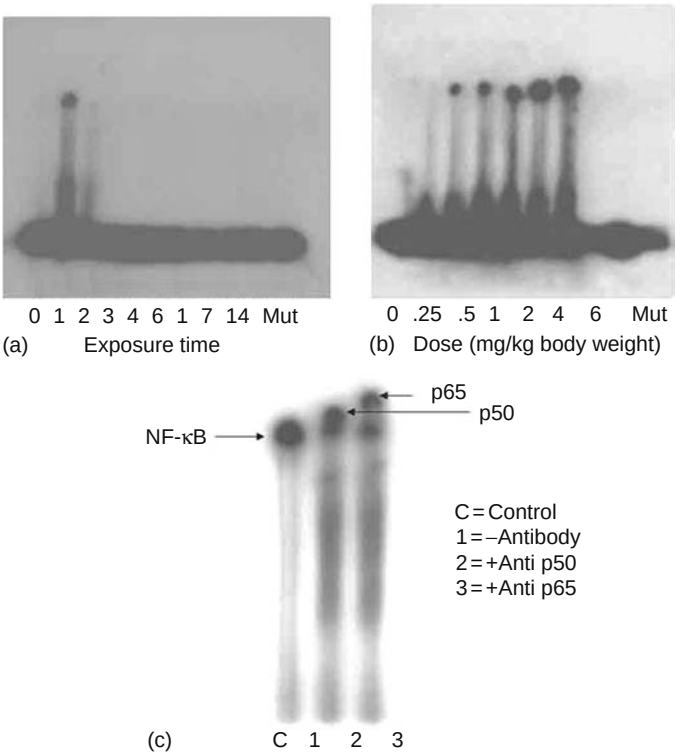

FIGURE 12.5 Activation of NF-κB in guinea pig lung following CEES exposure. (a) Time-dependent activation of NF-κB after intratracheal injection of CEES (0.5 mg/kg body weight), as observed by mobility shift assay. (b) Mobility shift assay showing the accumulation of NF-κB after 1 h of CEES exposure at different doses (ranging from 0.5 to 6.0 mg/kg body weight). (c) Supershift assay, using subunit specific antibodies, to identify the subunits (p50 and p65) of NF-κB activated due to CEES exposure in guinea pig lung.

Followed by the accumulation of TNF-α, both the acid and neutral SHM-ase activities were stimulated, peaking within 4–6 h after CEES exposure. Though both the acid and neutral SHM-ase activities were stimulated, the level of acid SHM-ase was found to be much higher after CEES exposure.

The higher levels of TNF-α, as well as both the acid and neutral SHM-ase activities in lung macrophages compared with those in lung tissue were expected because lung tissue consists of several types of cells, all of which were not responsive to CEES. We observed a biphasic effect of CEES on lung. After initial damage by TNF-α, there was some recovery due to activation of NF-κB within 2 h. This biphasic pattern was also observed in caspases activation.

Significant but small activation of caspase 2, caspase 3, caspase 8, and caspase 9 was observed within 1 h of CEES exposure. This activation of caspases declined thereafter and reappeared within 4–6 h, initiating the cell apoptosis in lung as observed by light as well as electron microscopy (unpublished observation). One explanation for this biphasic action of mustard gas is that NF-κB is activated by TNF-α through a phosphatidylcholine-specific phospholipase C/diacylglycerol (DAG)/protein kinase C (PKC) or phosphatidylcholine-specific phospholipase C/DAG/acid SHM-ase/ceramide model (Chatterjee et al., 2004).

These studies indicate that CEES exposure causes accumulation of TNF-α-activated SHM-ases, resulting in the production of ceramides and simultaneous activation of caspases and finally apoptosis. Ceramides are known to cause apoptosis via the activation of caspases (Alphonse et al., 2002; Hearps et al., 2002; Hetz et al., 2002).

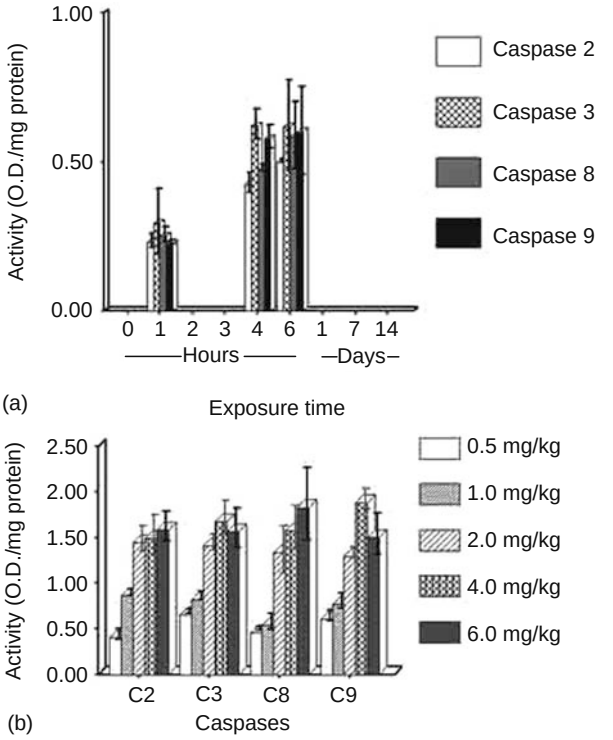

FIGURE 12.6 Activation of different caspases in guinea pig lung after CEES exposure. (a) Time course of activation of different caspases after intratracheal injection of CEES (0.5 mg/kg body weight). (b) Accumulation of different caspases at 4 h after exposure to CEES at different doses (ranging from 0.5 to 6.0 mg/kg body weight).

Our study also revealed that there was some initial damage of the lung tissue when exposed to CEES, but self-defense mechanisms of the lung played a significant role in the recovery from the damage and the prevention of any further damage. Furthermore, the present investigation enhances our understanding of mustard gas-mediated proapoptotic signaling pathways and characterizes the events of mustard gas-induced lung dysfunction.

Mustard gas exposure also causes inflammatory lung diseases, including acute respiratory distress syndrome (ARDS) (Calvet et al., 1994; Sohrabpour, 1984). A defective secretion of surfactant by alveolar type II cells has been implicated as one of the causative factors for the development of ARDS (Ansceschi, 1989). A major component of lung surfactant is DPPC (Stith and Das, 1982). The precursor of DPPC is normally 1-palmitoyl-2-oleolyl PC. DPPC is produced by deacylation and subsequent reacylation with palmitic acid at 2-position of glycerol moiety of the unsaturated phospholipid.

The CDP-choline pathway is the major pathway for the synthesis of PC in the lung, and cholinephosphotransferase (CPT) is a terminal enzyme in this pathway. Regulation of PC metabolism is one of the vital aspects of the cell cycle with implications in the control of cell proliferation as well as in apoptosis (Cui et al., 1996; Baburina and Jackowski, 1998).

PC is the most abundant phospholipid in mammalian cells, and it is synthesized via the CDP-choline pathway (Kent, 1995). CPT is the terminal enzyme of this pathway and plays a direct role in the final production of PC in the lung. This pathway is important for both cell proliferation and cell death (Ghosh et al., 2002; Ryan et al., 2003), and selective inhibition of this pathway has been shown to induce cellular apoptosis (Miquel et al., 1998). Any modulation in the expression and activity of this enzyme is expected to result in abnormal functioning of the cells.

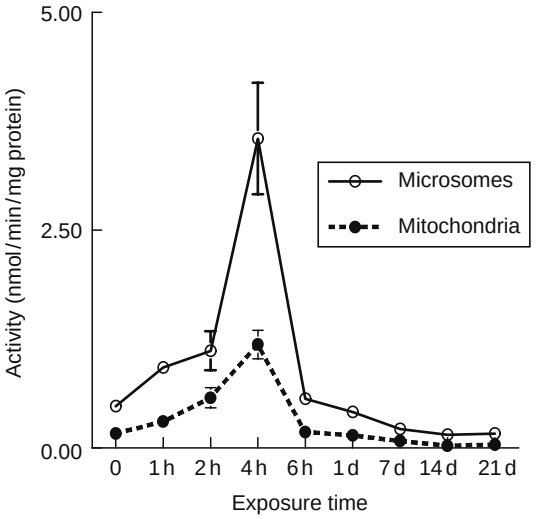

FIGURE 12.7 Time-dependent effects of 0.5 mg/kg body weight. CEES treatment on the mitochondrial and microsomal CPT activity. $N = 3$.

The time-dependent effects of CEES treatment showed a biphasic effect on CPT activity in both mitochondria and microsomes. The time-dependent studies indicated that a single infusion of CEES (0.5 mg/kg body weight) caused an increase in the activity for a short time after CEES exposure (up to 4 h), followed by a decrease (6 h onward) (Figure 12.7). The dose-dependent studies indicated that CEES treatment caused an initial increase in the CPT activity at low doses (0–2 mg/kg body weight), followed by a decrease at higher doses (4 and 6 mg/kg body weight) at incubation times of 1 and 4 h. This decrease was more acute in microsomes than in the mitochondria (Figure 12.8).

We have previously demonstrated that in addition to its predominant localization in the microsomes, CPT also exists in the mitochondria (Stith and Das, 1982; Sikpi and Das, 1987). Thus, it is possible that during the early stage of lung injury as observed in this study, cells try to repair the membrane damage by stimulating PC synthesis. Therefore, with increased CPT activity

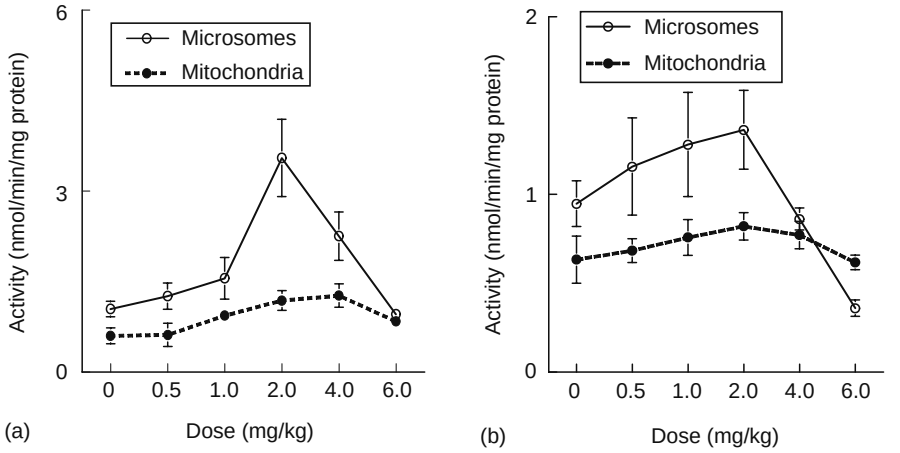

FIGURE 12.8 Dose-dependent effects of CEES on cholinephosphotransferase activity in mitochondria and microsomes. (a) After 1 h CEES treatment. (b) After 4 h CEES treatment. $N = 3$.

over time, lung cells lose their ability to repair membrane damage, as evident from decreased CPT activity in both mitochondria and microsomes isolated from lungs of CEES-treated animals (Figures 12.7 and 12.8). Hence, CEES has both short-term (stimulation) and long-term (inhibition) effects on lung CPT activity. Since CPT activity is crucial to the synthesis of surfactant, these effects may cause ARDS with long-term CEES exposure because of lack of surfactant synthesis.

I. Effects of Ceramide Treatment on Lung Microsomal CPT Activity

Ceramides are intracellular signaling molecules implicated in the induction of cellular apoptosis (Kolesnick and Krönke, 1998; Hannun and Luberto, 2000), and are known to induce several protein kinases and phosphatases (Mathias et al., 1991; Dobrowsky et al., 1993; Vietor et al., 1993). Ceramide analogs have been shown to inhibit PC synthesis (Bladergroen et al., 1999; Allan, 2000; Ramos et al., 2000; Vivekananda et al., 2001). Ceramides may directly affect the biosynthesis of PC and phosphatidylethanolamine (PE) by inhibiting the enzymes of the CDP-choline and CDP-ethanolamine pathways (Bladergroen et al., 1999; Awasthi et al., 2001; Ramos et al., 2002).

It has been reported that cells treated with ceramides may undergo programmed cell death, become growth arrested, or in rare cases, become stimulated to proliferate. The diversity of biological responses of cells to ceramides reflects the complexity of the role of these sphingolipids as second signal molecules (van Blitterswijk et al., 2003). Furthermore, ceramide treatment of lung cancer-derived A-549 cells promotes apoptosis in a caspase-dependent process (Kurinna et al., 2004).

Other laboratories have also shown that enzymes of the CDP-choline pathway for the production of PC in the cells, including CPT, show reduced activity when cells are incubated with cell-permeable C_2/C_6 ceramides (Bladergroen et al., 1999; Ramos et al., 2002), and it has been predicted that this inhibition may be due to the competitive inhibition by ceramides owing to the similarity in the structure to one of the substrates for CPT, DAG.

The downstream signal transduction events in lung following CEES exposure involve the induction of TNF-α, which in turn activates both acid and neutral SHM-ases, resulting in the subsequent accumulation of ceramides in the lung (Chatterjee et al., 2003, 2004). The level of ceramide was found to be ~60 μg/mg protein after 7 days (with 0.5 mg/kg body weight of CEES) and ~130 μg/mg protein for 2 mg/kg body weight of CEES (after 1 h; Figure 12.4).

When the lung microsomal fraction from control animals was incubated with C_2 ceramide at different concentrations (50, 100, and 200 μM) and time periods (0, 0.5, 1, and 6 h) before the assay for CPT activity, CPT activity decreased significantly in a time- and dose-dependent manner (Figure 12.9).

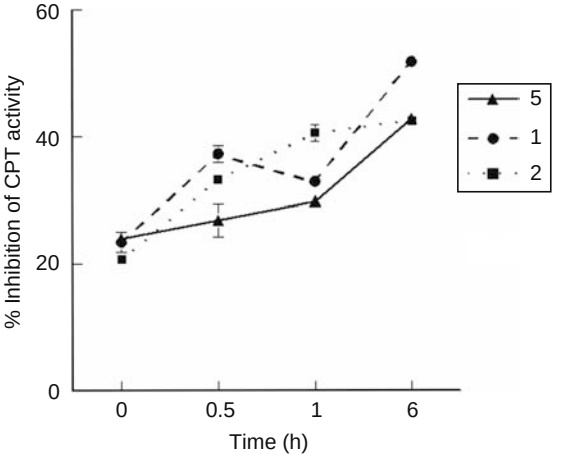

FIGURE 12.9 Effect of C_2 ceramide treatment on lung microsomal CPT activity. $N = 3$.

However, the effect was more pronounced when the microsomal fraction was preincubated with the ceramide before assay of the CPT activity. The degree of inhibition was increased with the increase in incubation time (0.5, 1, and 6 h). The highest inhibition (50%) was achieved after 6 h of incubation. However, only 20% inhibition was observed when ceramide was directly added into the assay mixture.

It has been shown that a 30% inhibition could be obtained in CPT activity when ceramides were directly added to the assay mixture at 50 μM concentrations (Bladergroen et al., 1999). Since this inhibition of 30% was less than the 64% obtained when cells were incubated directly, it would indicate competitive inhibition was not the only mechanism. In the present work, we found similar results with lung microsomal fraction, that is, with an increase in the incubation time with ceramides, the inhibition of the enzyme activity increases. Therefore, we support the observations by Bladergroen et al. (1999) that ceramide inhibition of CPT activity may be only partially through direct competitive inhibition with DAG; ceramide may act through interaction with other CPT enzyme inhibitors present in the microsomal fraction.

It is known that the short-chain ceramides often do not mimic the endogenous long-chain ceramides produced as a result of SHM-ase activity. However, the lipophilic nature of the both short- and long-chained ceramides makes these molecules likely candidates for altering biological processes as components of the lipid bilayer (Gidwani et al., 2003). We can therefore predict that ceramides accumulated in the lung because of exposure of CEES can alter the activity of the membrane-bound enzymes like CPT and can also act as a membrane perturbant.

This ceramide-induced membrane perturbation can result in mitochondrial release of cytochrome *c* and subsequent release of different caspases (Figure 12.6) (Gidwani et al., 2003; Chatterjee et al., 2004) as a part of the apoptotic pathway. Furthermore, CEES exposure (2.0 mg/kg body weight for 7 days) caused a significant decrease of both CPT activity (~1.5-fold; Figure 12.10) and gene expression (~1.7-fold; Figure 12.11) in the lung (Gidwani et al., 2003; Sinha Roy et al., 2005). This decrease in CPT activity was not associated with any mutation of the CPT gene. Thus, the inhibition of CPT activity as a chronic effect of CEES exposure may be directly responsible for the

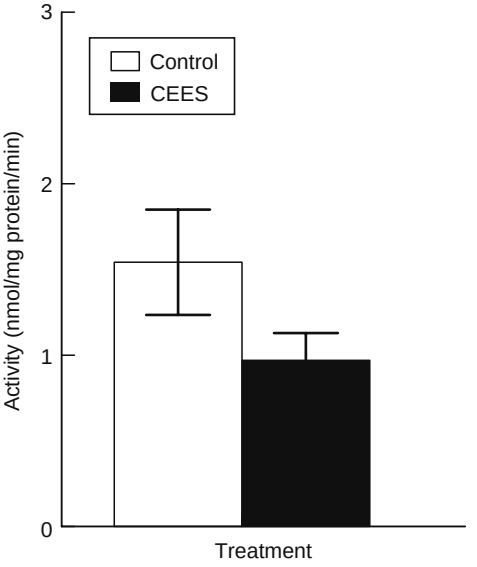

FIGURE 12.10 CPT enzyme activity in the lung microsomal fraction of 2 mg/kg body weight CEES (for 7 days) treated lung as compared with the control (only vehicle) showing significant decrease in activity. $p \leq 0.05$, $N = 3$.

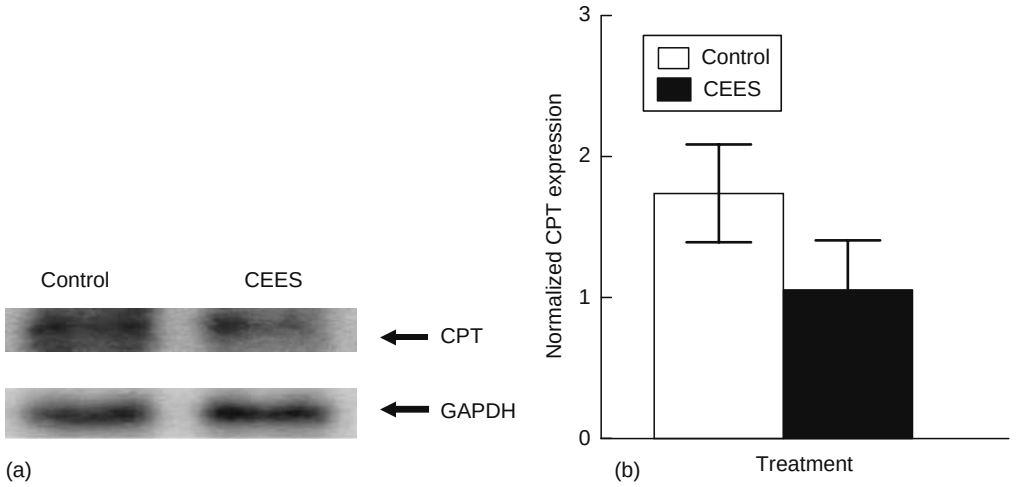

FIGURE 12.11 Northern blot analysis for the expression of the CPT gene. (a) One representative blot for CPT mRNA from control and CEES-treated guinea pig lung and same blot reprobed for GAPDH expression. (b) Graph showing downregulation of CPT expression as the result of mustards gas treatment normalized with GAPDH. $N = 3$.

reduction in the lung surfactant production, resulting in subsequent development of ARDS and pulmonary fibrosis, and is probably mediated by accumulation of ceramides.

J. CEES Induces Oxidative Stress

Stone et al. (2003) have found that OS and inflammatory agents play a key role in the toxicity of CEES. Both HD and CEES are known to provoke acute inflammatory responses in the skin (Blaha et al., 2000a, 2000b; Sabourin et al., 2002). It is interesting that RAW 264.7 macrophages stimulated with LPS or inflammatory cytokines are more susceptible to the cytotoxic effect of CEES than unstimulated macrophages. LPS (bacterial endotoxin) is a well-characterized inflammatory factor found in the cell wall of Gram-negative bacteria and is a ubiquitous natural agent found in the environment. LPS is present in serum, tap water, and dust. Military and civilian personnel would always have some degree of exposure to environmental LPS. Very low levels of LPS (20 ng/mL) were found to dramatically enhance the toxicity of CEES at concentrations greater than 400 μM (Stone et al., 2003). CEES alone is not toxic to RAW 264.7 macrophages at levels lower than about 500 μM (Stone et al., 2003).

LPS is known to trigger a variety of inflammatory reactions in macrophages and other cells having CD14 receptors (Wright et al., 1990; Downey and Han, 1998). In particular, LPS is known to stimulate the macrophage secretion of inflammatory cytokines such as TNF-α and IL-1-beta (Shapira et al., 1994). It is interesting, therefore, that both TNF-α and IL-1-beta were also found to enhance the cytotoxic effects of CEES but to a lesser extent than LPS (Stone et al., 2003). LPS stimulation of macrophages is known to involve the activation of protein phosphorylation by kinases as well as the activation of nuclear transcription factors such as NF-κB (Fujihara et al., 1994; Shapira et al., 1994, 1997; Chen et al., 1998). The activation of PKC by DAG is also a key event in LPS macrophage activation (Downey and Han, 1998). In vitro experiments have shown that the secretion of TNF-α and IL-1-beta by LPS-stimulated monocytes is dependent on PKC activation (Shapira et al., 1997; Coffey et al., 2000). Stone et al. (2003) also determined that phorbol myristate acetate (PMA) activation of PKC also enhanced CEES toxicity. These data suggest that the activation of PKC may play a key role in the molecular mechanism whereby LPS and inflammatory cytokines enhance the cytotoxicity of CEES (and potentially other vesicant weapons

as well). Evidence suggests that LPS (deRojas et al., 1995; Fu et al., 2001) as well as TNF-α (Ye et al., 1999) also stimulate the production of free radicals by macrophages. Collectively, this information supports the view that inflammatory factors and OS are key factors in understanding vesicant toxicity, and are important factors in designing effective countermeasures (Veness-Meehan et al., 1991; Yourick et al., 1991; Elsayed et al., 1992; Pant et al., 2000; Schlager and Hart, 2000; Kadar et al., 2001; Naghii, 2002; Levitt et al., 2003).

IV. CHLORINE

Chlorine is a pulmonary irritant that affects both the upper and the lower respiratory tracts. It is similar to SM in that its density is greater than that of air. Therefore, it characteristically hugs the ground, as does SM when deployed. It is only slightly water soluble; on contact with water or moisture, it forms hypochlorous acid (HClO) and hydrochloric acid (HCl). HClO is unstable and readily decomposes to oxygen-centered radicals. In animal models, 2000 ppm exposure will induce respiratory arrest. Subacute exposures of 9 ppm and acute exposures of 50 ppm can cause chemical pneumonitis (an inflammatory process) and bronchiolitis olbiterans (Segal and Lang, 2005).

The LD_{50} is in the range of 800–1000 ppm. HOCl, whether exogenously obtained from Cl_2 gas, or from an oxidative burst from neutrophils, the product reacts with a number of functional thiol groups present in enzymes (Winterbourn, 1985; Pullar et al., 2002). HOCl reactivity with thiol groups could inactivate such enzymes as GSH peroxidase and catalase (Aruoma and Halliwell, 1987). Inactivation of enzymes involved in antioxidant defense system renders the cell vulnerable to OS (a disruption of redox homeostasis).

Glutamylcysteine synthetase, cysteine, or methionine was 100 times more reactive to hypochlorous acid in comparison with amino acids that did not contain thiol groups (Folkes et al., 1995). Sublethal exposures to HOCl decreased GSH levels in several cell types (Vissers and Winterbourn, 1995; Pullar et al., 1999). In a study by Pullar et al. (1999) using human umbilical vein endothelial cells, doses of 25 nmol of HOCl and less were sublethal; when the exposure was done over 10 min, there was a concentration-dependent loss of intracellular GSH. Tissue exposure to HOCl resulted in a reduction of GSH. The metabolite of the HOCl interaction with GSH was an unexpected cyclic sulfonamide that was exported from the cell. The expected metabolites of glutathione disulfide (GSSH) and GSH sulfonic acid were actually minimal (Pullar et al., 2001). Inactivation of acetylcholinesterase by HOCl could be a contributory cause of airway hyperreactivity (den Hartog et al., 2002).

V. PHOSGENE

Phosgene ($COCl_2$), also referred to as carbonic dichloride, is extensively used as an industrial chemical. The gas dissolves slowly in water and is hydrolyzed to CO_2 and HCl. It produces little damage to the upper airway, although the lower airways sustain the bulk of the necrosis and inflammation. There is typically a delayed onset of symptoms in the pulmonary system occurring 1–24 h after the initial exposure. The respiratory symptoms of hypoxia (shortness of breath), and in extreme cases, respiratory arrest (discontinuation of spontaneous breathing), are apparently due to leaky alveolar capillaries and the resulting pulmonary edema (Noltkamper and Burgher, 2004); also contributory to the clinical symptoms are arachidonic acid mediators and lipid peroxides (Sciuto and Hurt, 2004).

In rodent models, phosgene elicits decreases in total GSH in lung tissue 48% within 45–60 min after exposure (Sciuto, 1998). Jaskot et al. (1991) confirmed similar results 2 years after the Sciuto publication. The concentration of phosgene exposure inversely affected the GSH levels (Jaskot et al., 1991). In gene expression studies in the inhalation mouse model, GSH regulation and redox regulation, in particular, were affected (Sciuto et al., 2005). A gene expression response can be seen as early as 30 min, wherein glutamate cysteine ligase increases and continues to increase at 8 h. Glutamate cysteine ligase continued to be elevated, approaching control levels in the 24–72 h time period. There was an upregulation of GST α-2, GSH peroxidase-2, glutamate, and γ-glutamyl

cysteine synthetase. At 4–12 h, GSH peroxidase-2, GSH reductase was elevated. GSH synthetase increased in the 4–24 h time frame. Further evidence of the crippling of the antioxidant defense system was the decreased SOD3 gene expression and enzyme activity. Similar results were found in independent experiments by Qin et al. (2004), as were reported by Sciuto in his examination of the antioxidant enzyme defense system. In the exposed rats, the antioxidant defense enzymes GST, superoxide dismutase (SOD), catalase, GSH peroxidase, and nitric oxide synthase in serum, blood, or liver were all increased. In contrast, the nitric oxide content was decreased. Similar changes in enzymes were also noted for other gaseous toxins, such as O_3 and NO_2 (Jaskot et al., 1991).

VI. LEWISITE

The Germans developed several arsenical-based warfare chemical agents circa 1917 (Goldman and Dacre, 1989). The allies, on the other hand, developed Lewisite (2-chlorovinydichloroarsine), adamsite (diphenylaminechloroarsine), methyldichloroarsine, and arsine. Lewisite is soluble in organic solvents; it is readily absorbed by rubber, paint, varnish, and porous materials. There are labile chlorine atoms, trivalent arsenic, carbons, and multiple bonds that make it quite reactive. Some of its reactions are due to nucleophilic substitution by water, hydrogen sulfide, thiols, and acid salts.

The reactions with thiol groups (e.g., those that are found in proteins) form an alkylarsine sulfide. Mustard gas, at the same LCt_{50}, induces vesication, whereas Lewisite does not. Apparently, Lewisite is more irritating initially to the pulmonary systems than mustard. The hydrolysis products are more persistent in soil in comparison with mustard. In ambient air, Lewisite is about 10 times more volatile than mustard.

The clinical sequelae after Lewisite absorption referred to as ''Lewisite Shock'' (L shock) are similar to that of severe burns. In dog models at high dosages, exposure resulted in retching, vomiting, extreme salivation, labored breathing, inflammation of the entire respiratory system, and pulmonary edema; respiratory distress was also common, and 80% died. Inhalation of 0.05 mg/L for 15 min results in intoxication and incapacitation for several weeks, whereas 0.5 mg/L for 5 min is lethal. The exact pathophysiology of the pulmonary symptoms seen is unknown, but is likely due to the dilatation of the capillaries. Apparently, the innate immune system is also compromised because of the occurrence of bronchopneumonia. In L shock, a hemolytic anemia can occur; however, it is unclear if this is due to an autoimmune reaction or OS (McMillan et al., 2004; Sato et al., 2006). Other complications of exposure are edema and bleeding that occur in the liver and kidneys (Wardell, 1941).

It is the arsenic in Lewisite that reacts with thiol groups to form alkylarsine sulfides, which is the basic reaction with thiol groups in tissues (Goldman and Dacre, 1989). The degree of enzyme dysfunction is dependent on the affinity of the enzyme to arsine. For example, it binds to the alpha and gamma thiol groups of lipoic acid, a component of pyruvate oxidase, which forms a stable six-membered ring (Stocken, 1949; Johnstone, 1963). Several other known enzymes are also inhibited–alcohol dehydrogenase, succinic oxidase, hexokinase, and succinate dehydrogenase (van Heyningen, 1941; Barron, 1947; Peters, 1946). Lewisite contact with skin results in the immediate elicitation of pain, in contrast to mustard, which would take 4–5 h (Wardell, 1941); it penetrates skin more rapidly than does mustard.

VII. ANTIDOTES OR AMELIORATIVE AGENTS

A. Lewisite

Partial protection was afforded by monothiols, such as cysteine or GSH, when tested against pyruvate oxidase (Goldman and Dacre, 1989), which was thought to be one of the prime targets of Lewisite. British anti-lewisite or 2,3-dimercaptopropanol preferentially binds with arsenicals over the thiol rings in proteins to form a nontoxic five-membered stable ring, which reverses the lesion induced by

Lewisite. British anti-lewisite is also effective against other heavy metals, such as gold, excessive copper deposition seen in Wilson's disease (El-Youssef, 2003), and mercury (Goldman and Dacre, 1989). Newer analogs of BAL have been synthesized to improve efficacy, such as DMSA, 2,3-dimercapto-1-propane sulfonic acid, and *N*-(2,3 dimercaptopropyl-phthalamidic acid) (DMPA).

B. Mustard

Elsayed et al. (1992) performed subcutaneous injections of CEES that resulted in increased GST activity, increased lipid peroxides, and depleted, reduced GSH in lung tissue (Elsayed et al., 1992). Similar findings occurred in the in vitro mouse model, a neuroblastoma–rat glioma hybrid cell line, in that there was a reduction in GSH as a function of time after exposure (Moore and Ray, 1983). Within the last 5 years, there has been a greater emphasis on the inhalation effects of SM.

Peter Ward and his group developed a rat instillation CEES model (McClintock et al., 2002). Leakage of radiolabeled albumin ([^{125}I] BSA) was used as a measure of lung damage, and the rats were sacrificed after 4 h after the installation of CEES into the rat lung with CEES. Neutrophil and complement depletion was found to significantly reduce the injury. Both enzymatic and nonenzymatic protocols were used to examine their ameliorative effect on the model. Dimethyl sulfoxide (DMSO) and dimethyl thiourea (DMTU) resulted in a 51% and 35% reduction in injury, respectively. The enzymatic antioxidants catalase and SOD exhibited 47% and 23% protection, respectively. In contrast, the antioxidant found in red wine, resveratrol (a phytoalexin), showed protection at 61%. Interestingly, the iron chelators 2,3-dihydroxybenzoic acid (DHBA) and deferoxamine mesylate (desferal) did not have any protective effect. NAC was administered 10 min before instillation of CEES. A dose–response curve was generated for 5, 10, 20, 30, and 40 mg/kg^{-1} body weight. All of the dosages of NAC were found to be protective. The dosage of NAC at 20 mg/kg^{-1} conferred the best protection out of all antioxidants at 70% protection. In vitro, the lethal effects of SM on L-cells were reduced by thiol reagents, namely, dithiothreitol and NAC (Walker, 1967; Walker and Smith, 1969). Several observations support the concept of a pathogenic role for toxic oxygen species in CEES-induced acute lung injury.

Other groups investigated the beneficial effects of agents that scavenge free radicals and other oxidant species (Wormser et al., 2000). They monitored the beneficial effects of those agents mainly by showing the recovery from damaged skin or by showing inhibition of the induction of proteolytic enzymes (Cowan et al., 1992; Cowan and Broomfield, 1993). Systemic and topical steroids administered revealed ameliorative properties in the rabbit SM skin model (Vogt et al., 1984).

The Das Group investigated the effects of orally administered antioxidants on signal transduction in the guinea pig model. A single dose of NAC could not block the CEES-induced lung injury at all. On the other hand, about 76% of lung injury could be blocked by long-term pretreatment (30 days) with NAC. No significant protection of lung injury was observed by pretreatment with either Ondrox (an over-the-counter antioxidant supplement as single-dose or 3 days pretreatment) or GSH (single; Table 12.1). Similarly, a single-dose treatment of NAC just before CEES exposure was found to be ineffective, whereas 76% of lung injury could be blocked by long-term pretreatment (30 days) with NAC. There was no significant protection of lung injury by pretreatment with either Ondrox (single-dose or 3 days pretreatment) or GSH (single dose).

A single-dose treatment of NAC just before CEES exposure was found to be ineffective, whereas 62% of TNF-α induction was inhibited by 3 days pretreatment with NAC. Long-term treatment with NAC gave more protection (inhibition of 73% of TNF-α induction). There was no protection for TNF-α accumulation by single dose of either Ondrox or GSH. Three days pretreatment with Ondrox and DMT could only block 13% and 8% of the TNF-α induction (Table 12.2). Additional support for an involvement of oxidants in the pathogenesis of CEES-induced acute lung injury was provided by the protective effects seen after treatment of CEES-exposed rat with NAC (McClintock et al., 2002). The protective effect of NAC was confirmed in the guinea pig lung model, as well as by others (Wormser et al., 1997).

TABLE 12.1
Effects of *N*-acetyl-L-Cysteine Pretreatment on CEES-Induced Lung Injury in Guinea Pigs

Treatment	Permeability Index
Control	0.20 ± 0.04
Control + NAC (3 days)	0.08 ± 0.01
CEES	2.01 ± 0.16
CEES + NAC (single dose)	2.07 ± 0.11
CEES + NAC (3 days)	0.62 ± 0.09[a]
CEES + NAC (30 days	0.48 ± 0.06[a]
CEES + Ondrox (single dose)	2.04 ± 0.03
CEES + Ondrox (30 days)	2.00 ± 0.08
CEES + GSH (single dose)	1.98 ± 0.10

Note: CEES was infused (6 mg/kg body weight) intratracheally into guinea pigs with or without pretreatment with NAC. NAC was given either in single dose (5 mg) by gavage directly into stomach 10 min before infusion of CEES or with drinking water (0.5 g/day/animal, for either 3 days or 30 days before CEES infusion). Information on treatment with ondrox and GSH is given in text. The lung injury was measured after 1 h of CEES exposure and expressed by permeability index, which is a measure of ^{125}I-BSA leakage from damaged blood vessels into lung tissue. Each group had six animals. Values are mean ± SE ($n = 6$).

[a] NAC supplementation in drinking water blocked the CEES-induced lung injury significantly ($p < 0.05$).

TABLE 12.2
Inhibition of CEES-Induced TNF-α Accumulation in Guinea Pig Lung by NAC Treatment

Treatment	Level of TNF-α (pg/mg Protein)
Control	20 ± 7
CEES	708 ± 38
CEES + NAC (single dose)	705 ± 24
CEES + NAC (3 days)	270 ± 40[a]
CEES + NAC (30 days)	190 ± 64[a]
CEES + Ondrox (single dose)	716 ± 22
CEES + Ondrox (3 days)	610 ± 54
CEES + DMT (3 days)	648 ± 62
CEES + GSH (single dose)	720 ± 41

Note: CEES was injected (4 mg/kg body weight) intratracheally into guinea pigs with or without pretreatment with NAC. NAC was given either in single dose (5 mg) by gavage directly into stomach 10 min before injection of CEES or with drinking water (0.5 g/day/animal, for either 3 days or 30 days before CEES injection). Information on treatment with ondrox, DMT and GSH is given in the text. TNF-α was measured after 1 h exposure of CEES. Values are mean TNF-α ± SE ($n = 6$).

[a] NAC supplementation in drinking water blocked the CEES-induced accumulation significantly (p of TNF-α < 0.05).

1. Effect of NAC on Signal Transduction

Short-term (3 days) and long-term (30 days) pretreatment with NAC blocked significantly the activation of acid (4% and 49%, respectively, Figure 12.12) and neutral (46% and 61%, respectively, Figure 12.12) SHM-ases and decreased the levels of ceramide by 71% and 77% (Table 12.3). However, pretreatment with a single dose of NAC did not inhibit the activity of either neutral or acid SHM-ase and accumulation of ceramide.

Exposure to CEES significantly inhibited ($p < 0.05$) the activity of SOD (31%), GSH peroxidase (67%), and catalase (25%) (Figure 12.13). Pretreatment of guinea pigs for 3 days with NAC before CEES infusion significantly ($p < 0.05$) decreased CEES-induced inhibition of SOD (from

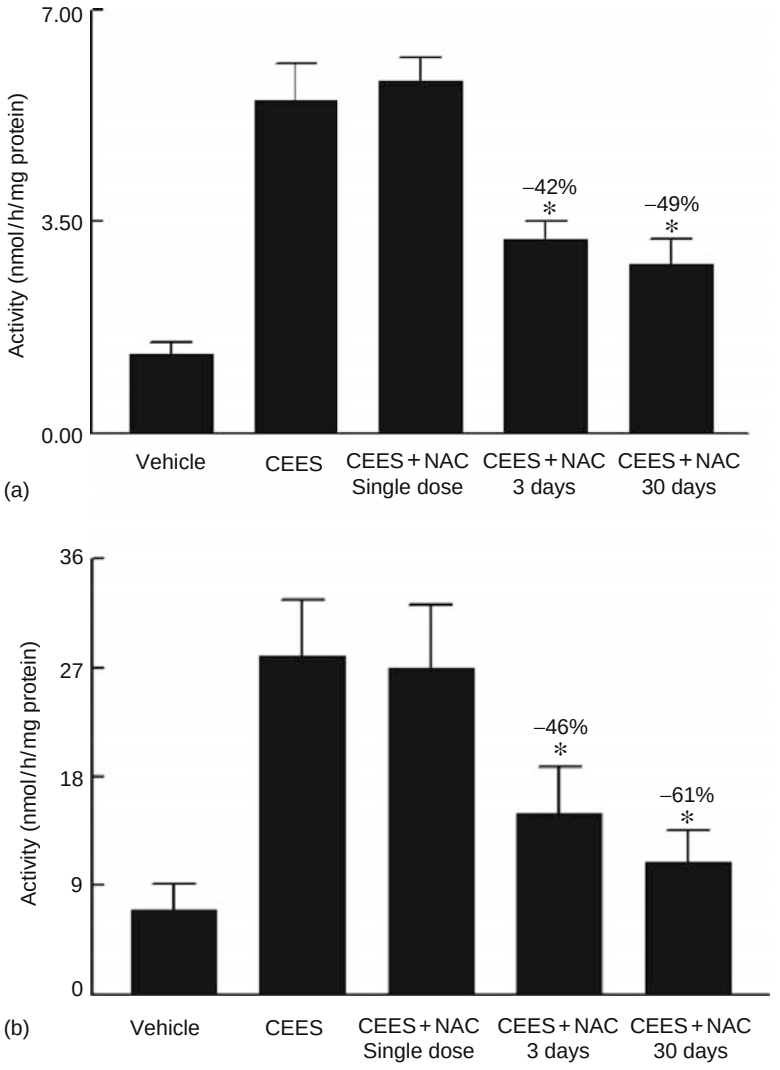

FIGURE 12.12 Inhibition of CEES-induced activation of neutral and acid sphingomyelinases in guinea pig lung by NAC treatment: Accumulation of neutral (a) and acid (b) sphingomyelinases at 4 h after exposure to CEES (4 mg/kg body weight) and their inhibition by 3 and 30 days pretreatment with NAC. Values are mean ± SE ($n = 6$). Pretreatment with single dose of NAC did not inhibit the activity of either neutral or acid sphingomyelinase. *Pretreatment with NAC for 3 and 30 days before CEES exposure caused a significant inhibition of both neutral and acid sphingomyelinases ($p < 0.05\%$).

TABLE 12.3
Inhibition of CEES-Induced Ceramide Accumulation in Guinea Pig Lung after NAC Treatment

Treatment	Levels of Ceramide (μg/mg Protein)
Control	9 ± 2
CEES	148 ± 14
CEES + NAC (single dose)	146 ± 10
CEES + NAC (3 days	39 ± 8[a]
CEES + NAC (30 days)	28 ± 12[a]

Note: CEES was infused (4 mg/kg body weight) intratracheally into guinea pigs with or without pretreatment with NAC. NAC was given either in single dose (5 mg) by gavage directly into stomach 10 min before infusion of CEES or with drinking water (0.5 g/day/animal, for either 3 days or 30 days before CEES infusion. Ceramide accumulations were assayed after 1 h of infusion of CEES. Values are mean ± SE ($n = 6$).

[a] NAC supplementation in drinking water blocked the CEES-induced accumulation of ceramide significantly ($p < 0.05$).

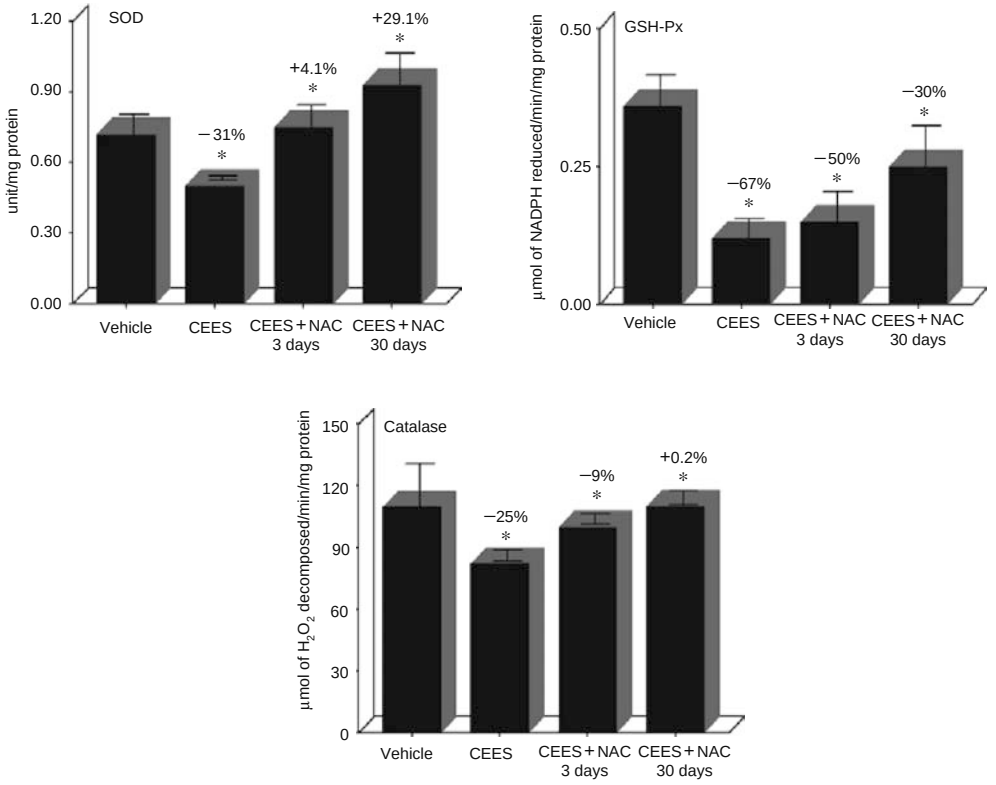

FIGURE 12.13 Effects of NAC pretreatment on the CEES-induced alterations in the free radical-metabolizing enzymes: Guinea pigs were infused with CEES (4 mg/kg body weight) intratracheally and the free radical-metabolizing enzymes (SOD, GSH pretreatment, catalase) were assayed in perfused lung after 4 h of CEES exposure. The samples were taken from guinea pigs with or without pretreatment with NAC for 3 days. *CEES exposure caused a significant decrease in the activity of SOD, GSH pretreatment, and catalase ($p < 0.05$). NAC treatment blocked the CEES-induced changes significantly ($p < 0.05$) for all enzymes.

31% decrease to 4.1% increase), GSH pretreatment (from 67% decrease to 50% decrease), and catalase (from 25% decrease to 9% decrease). Pretreatment of guinea pigs for 30 days with NAC provided additional resistance. For example, the activity of SOD was increased by 29% over the basal value, the activity of GSH pretreatment was decreased by only 30%, and the activity of catalase was brought back to the basal level.

Both short-term (3 days) and long-term (30 days) treatments significantly ($p < 0.05$) blocked the CEES-induced activation of NF-κB that was observed 1 h after CEES infusion (Figure 12.14a). Furthermore, pretreatment with NAC for 3 days also blocked the activation of caspase 2, caspase 3, caspase 8, and caspase 9 by 41%, 44%, 55%, and 51% (Figure 12.14b).

Protection by NAC from half-mustard, gas-induced, acute lung injury has also been demonstrated recently in rats by McClintock et al. (2006). However, in these studies, NAC was administered by liposome encapsulation directly into the lung as a method of treatment for acute exposure to mustard gas. The co-instillation with CEES of liposomes containing the pegylated (PEG)-catalase, PEG-superoxide, NAC, GSH, resvertrol, or combination greatly attenuated development of rat lung injury (McClintock et al., 2006). Thus, we suggest the following model for the action of NAC on CEES-induced guinea pig lung injury (Figure 12.15).

Morphological analysis indicates that animals exposed to CEES showed symptoms of a chemical burn within 1 h; however, the severity of damage progressively increased with time. At 21 days postexposure, severe bronchial constriction with occasional apoptotic nucleus and accumulation of viscid secretion of mucins were observed in CEES-treated animals. Furthermore, both polymorphonucleus (PMNs) and eosinophilic leukocytes migration were observed in both alveoli and bronchi. However, pretreatment with NAC protected the lung from all these changes remarkably, except mucin secretion (Figure 12.16). How CEES functions as a powerful oxidant and what lung cells are targets of CEES is unclear.

C. Antioxidant Liposomes

Another method of increasing antioxidant tissue levels is facilitated by using liposomes. Liposomal drug delivery is advantageous to using the oral route in that there is a multiplicity of possible administration routes. They can be used topically, orally (using the gastrointestinal tract), or delivered by aerosol to the lung.

Intact skin allows the passage of small lipophilic substances, but in most cases, efficiently retards the diffusion of water-soluble molecules. Lipid-insoluble drugs generally penetrate the skin slowly in comparison with their rates of absorption through other body membranes. Absorption of drugs through the skin may be enhanced by iontophoresis if the compound is ionized. Certain solvents (e.g., DMSO) may facilitate the penetration of drugs through the skin, but their use for therapeutic applications is controversial. In the formulation of topical dosage forms, attempts are being made to utilize drug carriers to ensure adequate localization or penetration of the drug within or through the skin in order to enhance the local and minimize the systemic effect. For dermatopharmacotherapy, there is a need for a drug delivery system that enhances the penetration of the active ingredient into the skin, localizes the drug at the site of action, and reduces percutaneous absorption. Antioxidant liposomes may prove very useful in this regard.

Liposomes are phospholipid vesicles composed of lipid bilayers enclosing an aqueous compartment. Hydrophilic molecules can be encapsulated in the aqueous spaces, and lipophilic molecules can be incorporated into the lipid bilayers. Liposomes, in addition to their use as artificial membrane systems, are used for the selective delivery of antioxidants and other therapeutic drugs to different tissues in sufficient concentrations to be effective in ameliorating tissue injuries (Stone et al., 2002). Antioxidant liposomes containing combinations of water- and lipid-soluble antioxidants may provide a unique therapeutic strategy for mustard gas exposure because (1) the antioxidants are nontoxic and could, therefore, be used at the earliest stages of exposure; (2) the liposomes themselves are composed of nontoxic, biodegradable, and reusable phospholipids;

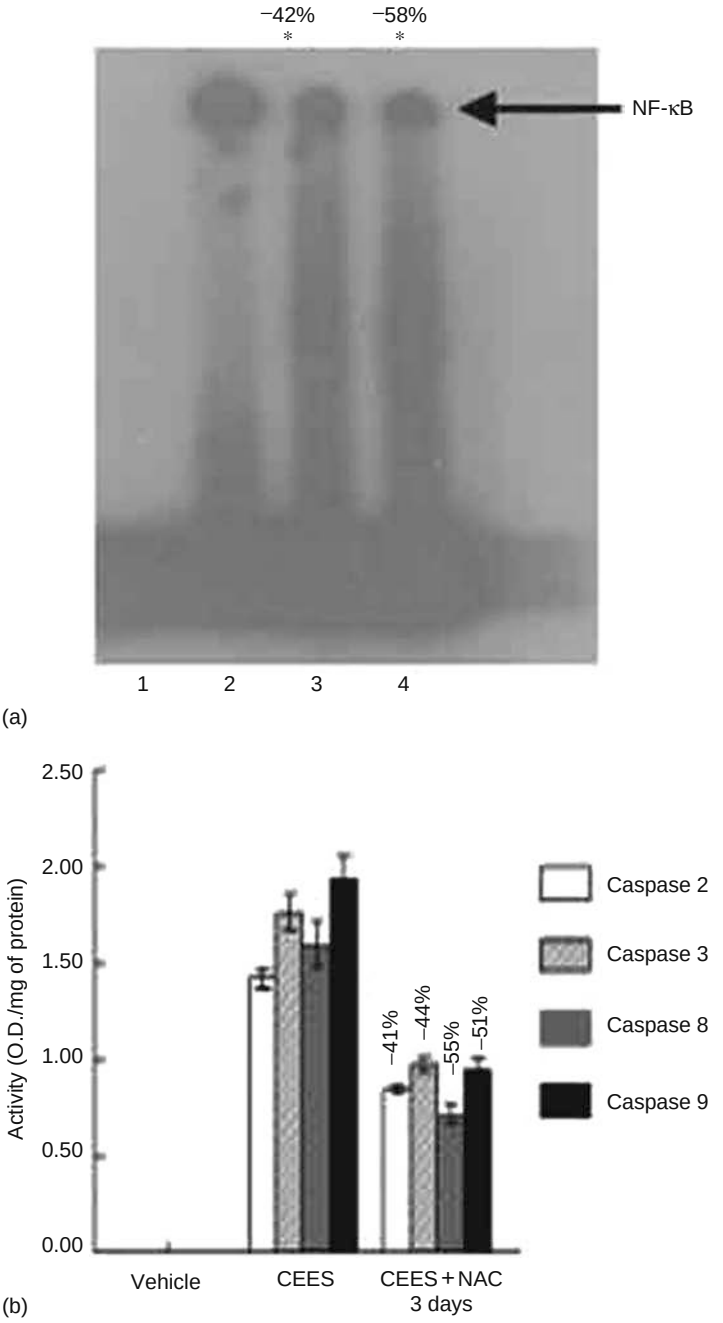

FIGURE 12.14 Inhibition of the activation of NF-κB (a) and different caspases (b) in guinea pig lung after CEES exposure by pretreatment with NAC. (a) Activation of NF-κB after 1 h of intratracheal infusion of CEES (4 mg/kg body weight) was monitored by mobility shift assay. The guinea pigs were treated with NAC either for 3 or for 30 days before CEES exposure. Panels 1, 2, 3, and 4 represent vehicle, CEES, CEES + NAC (3 days), and CEES + NAC (30 days), respectively. *NAC treatment caused a significant inhibition (46% and 58% for 3 and 30 days, respectively, $p < 0.05$) in the accumulation of NF-κB. (b) Accumulation of caspases 2, 3, 8, and 9 at 4 h after exposure to CEES (4 mg/kg body weight) and the prevention of these caspases activation by short-term pretreatment (3 days) with NAC. Values are mean ± SE ($n = 6$). *NAC treatment inhibited the activation of all caspases significantly ($p < 0.05$).

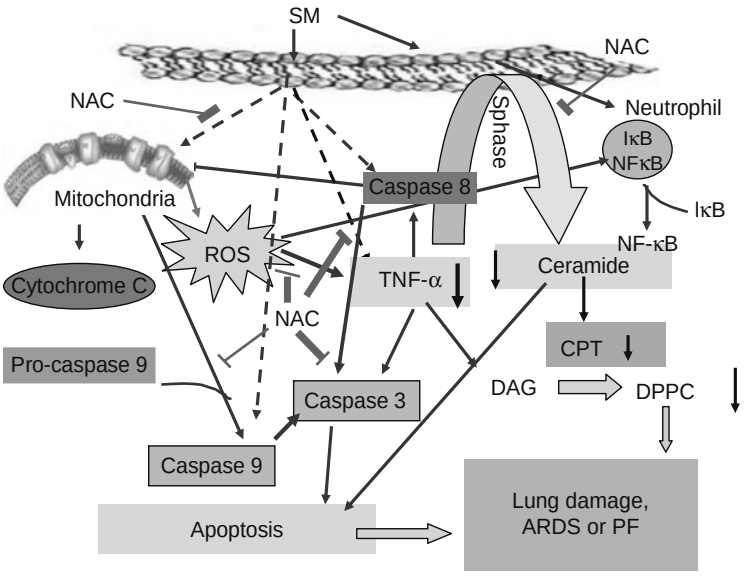

FIGURE 12.15 Proposed mechanism of action of NAC on CEES-induced lung injury.

(3) liposomes are preferentially taken up by the reticuloendothelial system which is an early target of mustards gas toxicity; (4) chemical antioxidants are relatively inexpensive and a wide range of commercial antioxidants are available; and (5) liposomes have low immunogenicity. Furthermore, results from several studies have clearly indicated that the liposomal antioxidant formulations exert a far superior protective effect compared with that of the free (unencapsulated) antioxidants, against OS-induced tissue injuries (Fan et al., 2000).

As shown in Figure 12.17, our preliminary data show that liposomes encapsulated with 5 mM GSH, a major intracellular chemical antioxidant, is effective at preventing CEES cytotoxicity to LPS-stimulated macrophages. We have also found that liposomes encapsulated with either 1 mM NAC (water-soluble antioxidant) or with 13.5 μM α-tocopherol are also effective in preventing CEES toxicity to stimulated macrophages (data not shown). Preliminary results from our ongoing collaborative research efforts have demonstrated that delivery of antioxidants as liposomal formulations were effective in protecting against CEES-induced cellular injury in an animal model.

D. Chlorine

Similar to phosgene, chlorine causes a delayed pulmonary edema. There is no specific antidote for chlorine gas inhalational exposure. Treatment for skin or eye contact is irrigation of the site. Inhalation of the gas can be treated supportively by the use of nebulized sodium bicarbonate and bronchodilators (Aslan et al., 2006). Modest improvement in animal models was obtained with the inhalation therapy using corticosteroids (Gunnarsson et al., 2000). Thiol-containing compounds are able to scavenge HOCl, which is suspected to be a significant metabolite of chlorine gas exposure (McKenzie et al., 1999). Enhancement of systemic levels of thiols by the use of orally administered NAC may be a consideration in life-threatening exposures to chlorine gas.

E. Phosgene

In experiments using agents as a prophylaxis, there is promise. Rats were exposed to phosgene in a whole-body chamber after 23 days of supplementation of vitamin E (α-tocopherol) or *N*-propyl gallate (nPG) (Sciuto and Moran, 2001). The vitamin E-fed rats did not show any survival

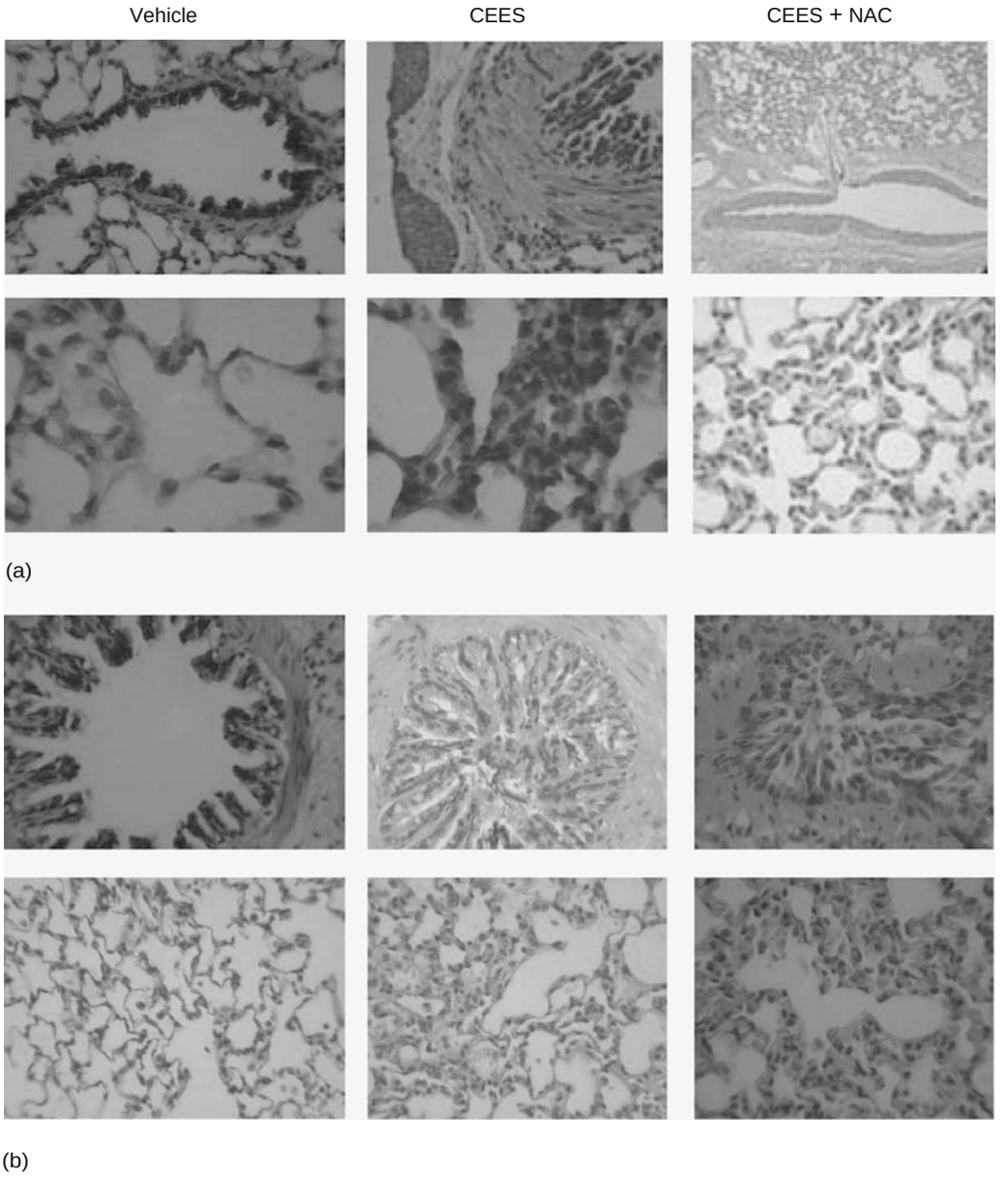

FIGURE 12.16 Histological analysis showing recovery from CEES-induced lung damage by pretreatment of NAC. Guinea pig lungs were examined under light microscope after 1 h (a) and after 21 days (b) of exposure to CEES (0.5 mg/kg body weight). Upper panel represents morphology of bronchi and lower panel represents morphology of the alveoli. Magnifications: 400×.

enhancement. The nPG-fed rats fed the lower doses of nPG (0.75%) showed the greatest increase in survival, and the higher dose of nPG (1.5%) was ineffective. In the lower dose of nPG, there was an obvious decrease in lipid peroxidation and increased lung tissue-reduced GSH. Gamma-tocopherol, in comparison with α-tocopherol, is a more potent anti-inflammatory agent and may have produced a much different result under the same experimental conditions that Sciuto and Moran (2001) performed (Jiang and Ames, 2003).

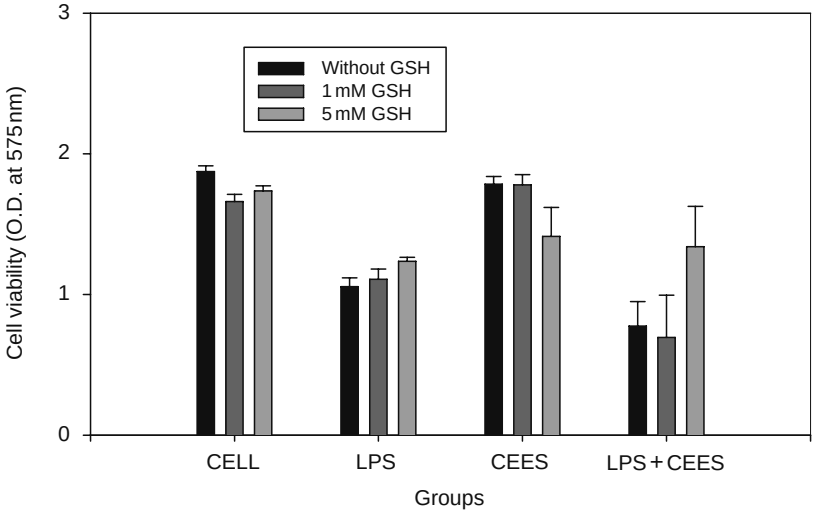

FIGURE 12.17 GSH-liposomes (5 mM) protect simulated macrophages from CEES toxicity.

Postexposure drugs used as a countermeasure to phosgene are more reflective of a clinical application or military theater exposures. The arachidonic acid analog 5-, 8-, 11-, and 14-eicosatetraynoic acid (ETYA) inhibits the release of arachidonic acid metabolites, which produce both leukotrienes and prostaglandins (Farrukh et al., 1988). ETYA used as a postexposure treatment in an in situ lung model resulted in less lung edema, as well as increased gluathione levels and lower lipid peroxidation levels. It is questionable as to whether ETYA possesses any antioxidant properties (Sciuto, 2000).

VIII. OXIDATIVE STRESS IN DIFFERENT ORGAN SYSTEMS

A. Lung

SM inhalation can produce hoarseness, laryngitis, acute airway obstruction, bronchopneumonia, pulmonary edema, and hemorrhage (Calvet et al., 1994). Bronchiolitis and dyspnea are common clinical findings. Examination of the pulmonary tree reveals a mild fibrosis in the parenchyma. Exaggerated fibroblast proliferation and increased collagen synthesis represent two critical events in the pathogenesis of this type of pulmonary fibrosis. The main initial changes are extreme hyperemia, exudation of inflammatory cells, cellular infiltration of the submucosa and detachment, necrosis, and cellular death of the respiratory epithelial lining (Chevillard et al., 1992).

Acute, heavy exposure to SM causes loss of the columnar cells of the upper respiratory tract, peribronchial edema, hyperemia of the blood vessels, cellular infiltrations in the submucosa, and intense vacuolization and disorganization of the cytoplasmic and nuclear structures (Emad and Rezaian, 1997, 1999). Pulmonary hemorrhage, pulmonary edema, and respiratory failure similar to ARDS may also occur. These cytotoxic effects are associated with acute thermal injury sustained by the airway mucosa and lead to scarring and development of stenosis of the tracheobronchial tree as was observed in 9.64% of the SM-exposed patients.

Cross-sectional study on the late pulmonary sequelae of SM-exposed veterans showed airway narrowing or stricture, asthma (10.65%), chronic bronchitis (58.88%), bronchiectasis (8.62%), and pulmonary fibrosis (PF; 12.18%) after 10 years. Crystal et al. (1984) recommended that SM be added to the various causes of interstitial lung diseases and PF (Crystal et al., 1984; DePaso and Winterbauer, 1991; Emad and Rezaian, 1997). The cellular constituents of bronchoalveolar (BAL)

fluid in patients with SM-induced PF are very similar to the cellular constituents seen in patients with idiopathic PF, and this finding indicates the presence of an ongoing active alveolitis in PF (Emad and Rezaian, 1997).

Under normal physiological conditions, airway lining fluids and extracellular spaces are maintained in a highly reduced state. Typically, the levels of antioxidants and oxidants in lung are balanced in favor of a reducing state (redox homeostasis). Decreases in antioxidants or increases in oxidants can disrupt this equilibrium and can cause OS. An imbalance in oxidant–antioxidant system has been recognized as one of the first changes that ultimately lead to inflammatory reactions (Crapo, 2003a).

An increased oxidizing environment can facilitate antigen-presenting cell (APC) maturation and T-cell activation, leading to the activation of the adaptive immune responses and, potentially, a hyperresponsive innate immune system. Thus, OS appears to play an important role in the pathogenesis of the asthmatic reaction. It is not surprising that OS occurs in many forms of lung disorders, such as pneumonia, adult respiratory distress syndrome, idiopathic pulmonary fibrosis, lung transplantation, chronic obstructive pulmonary disease, cystic fibrosis, bronchiectasis ischemia-reperfusion, and lung cancer (Bowler and Crapo, 2002; Crapo, 2003b; Rahman and Kelly, 2003; Rahman et al., 2004).

An oxidizing state in cells can initiate intracellular signaling cascades that lead to the production of inflammatory mediators. Stress kinases (JNK, ERK, p38) and transcription factors, such as NF-κB and AP-1, are known to be redox sensitive. On their activation, these signaling pathways lead to production of TNF-α, IL-1-beta, IL-6, IL-8, IL-12, adhesion molecules (VCAM-1, ICAM-1), and GM-CSF (Mastruzzo et al., 2002). In addition to protein expression, OS favors uncoiling of DNA, thereby increasing accessibility to transcription factor binding (Rahman et al., 2004). The presence of OS can also damage alveolar epithelial cells by the induction of apoptosis. The angiotensin-converting enzyme system is also known to be redox sensitive. OS facilitates the conversion of angiotensinogen into angiotensin II, which is a bioactive peptide with a broad range of activities, including the induction of apoptosis in epithelial cells and the activation of fibroblasts. Angiotensin II can also increase the lung levels of transforming growth factor (TGF)-α, which is a crucial factor in the development of fibrosis (Mastruzzo et al., 2002).

The maintenance of a reducing environment in lung is considered to be crucial in the lung function. A balance between intracellular and extracellular oxidants and antioxidants is a prerequisite for normal lung homeostasis. The lung has highly specialized and compartmentalized antioxidant defenses to protect against reactive oxygen species (ROS) and RNS. These include the following: (1) small molecular weight antioxidants (e.g., GSH, vitamins, uric acid); (2) mucins; (3) metal-binding proteins (transferrin, lactoferrin, metallothionein, etc.); (4) SODs (e.g., mitochondrial manganese SOD [MnSOD], intracellular copper zinc SOD [CuZnSOD], and extracellular SOD [ECSOD]); (5) a group of enzymes that decomposes hydrogen peroxide (H_2O_2) (numerous GSH-associated enzymes and catalase); (6) detoxification enzyme systems (e.g., GST); and (7) other redox-regulatory thiol proteins (e.g., thioredoxin–peroxiredoxin system and glutaredoxins) (Powis et al., 2000; Fattman et al., 2003; Kinnula and Crapo, 2003).

Reduced GSH appears to be one of the most important antioxidant defense systems. In the extravascular lung fluid that coats the alveolar epithelial surfaces, GSH is present in millimolar quantities. A decrease in the ratio between GSH and oxidized glutathione (GSSG) occurring during OS leads to the activation of a variety of cellular redox-sensitive signaling pathways. Antioxidant enzymes such as SOD and catalase also play an important role in the clearance of oxidative radicals in the lung. The primary oxidant-generating enzyme is nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase in phagocytes such as alveolar macrophages and neutrophils. On activation, phagocytes generate superoxide anions, which lead to the production of H_2O_2 for host defense.

It is clear that a better understanding of the oxidative state in the lung is important for the diagnosis and treatment of lung diseases. There are many methods developed for the detection of free radicals from oxygen, ROS, and their by-products to assess the presence of OS. The techniques

include established standard protocols and advanced methodologies using HPLC, mass spectrometry, and electron paramagnetic resonance (EPR). The following sections describe the most frequently used methods to measure lung oxidative status.

1. Monitoring Oxidative Stress in Live Cells

ROS in live cells can be detected by using fluorogenic marker for ROS and observed under fluorescence microscopy. One of the frequently used markers is carboxy-H_2DCFDA, a permeable fluorogenic marker, which is oxidized during OS in live cells and emits bright green fluorescence (Wan et al., 2005).

2. Hydrogen Peroxide and Superoxide Radical Generation in Bronchoalveolar Fluids

H_2O_2 fluids in BAL can be measured by the simple assay for the detection of the presence of peroxides in both aqueous and lipid environments. The basis of these assays is the complexing of ferrous ion (Fe^{+2}) by H_2O_2 in the presence of xylenol orange. Peroxides oxidize Fe^{+2} to Fe^{+3}, and Fe^{+3} forms a colored complex with xylenol orange that can be read at 560 nm (Jones et al., 1995). Superoxide radical generation can be estimated by nitroblue-tetrazolium reduction assay (Libon et al., 1993).

3. Antioxidant Status in Lung

The antioxidant status in the lungs can be evaluated by lung levels of SOD and catalase and their activities. SOD activity can be assessed by the OxyScan SOD-525 assay, which measures the activity of all forms of SOD. The method is based on the SOD-mediated increase in the rate of autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. Catalase activity can be determined by a two-step reaction scheme (Catalase-520 assay). First, catalase reacts with a known quantity of H_2O_2 to generate H_2O and O_2. In the presence of horseradish peroxidase (HRP), the remaining H_2O_2 reacts with 3,5-dichloro-2-hydroxybenzenesulfonic acid and 4-aminophenazone to form a chromophore with a color intensity. Lipid peroxidation levels in the lung can be measured by thiobarbutiric acid reactive substances assay (Erdincler et al., 1997). The GSH/GSSG ratio, a useful measure of OS, can also be determined by a colorimetric method by using Bioxytech GSH/GSSG-412.

4. Hydroxydeoxyguanosine, an Indicator of DNA Damage

The most common type of damage caused by ROS in the body is oxidative damage to DNA. Hydroxydeoxyguanosine (8-OHdG), a product of this type of DNA damage, is used as a biomarker for OS. It can be measured by the immunohistochemical procedure and a high-performance liquid chromatography system equipped with an electrochemical detector (HPLC-ECD) (Mei et al., 2003). More recently, it has been reported that capillary electrophoresis-mass spectrometry (CE/MS) can also be used for the analysis of 8-OHdG to study OS (Weiss and Lunte, 2000).

5. Direct Measurements of Oxygen Free Radicals

It is very difficult to directly measure oxygen-free radicals (OFRs) due to their short half-lives. To study these OFRs, radical spin-trapping agents have to be employed to facilitate the formation of stable radical adducts with the OFRs, for detection by EPR spectroscopy. Trapping agents are generally nitrone or nitroso-containing molecules, such as 5,5-dimethyl-1-pyrroline-*n*-oxide (DMPO), which react with OFRs to form stable nitroxide free radicals. These radical adducts can be analyzed by using EPR (Olea-Azar et al., 2003).

6. Exhaled Breath Condensate

Exhaled breath condensate collected by cooling or freezing the exhaled air is a totally noninvasive procedure. H_2O_2, leukotrienes, isoprostanes, and 3-nitrotyrosine are good candidates for OS assessments (Paredi et al., 2002). These products have been shown to elevate lung inflammation.

7. Analysis of Expired Air for Oxidation Products

Studies have shown that expired NO and CO can serve as biomarkers for OS, and ethane can serve as a marker of lipid peroxidation (Paredi et al., 2002). CO can be detected electrochemically, and it can also be measured by laser spectrophotometer and near-infrared CO analyzers. The levels of exhaled NO can be assessed by chemiluminescence. Ethane content can be detected using gas chromatography.

B. Skin

Skin is one of the major sites of damage after exposure to vesicant chemical weapons. In the case of SM, there is a latent period after exposure, followed by an erythematous rash within 4–8 h, and then blistering some 2–18 h later. Elsayed et al. (1992) found that a nonlethal, s.c. dose of the mustard analog chloroethyl 4-chlorobutyl sulfide (CECBS) in mice caused damage to distal tissues, such as the lung, that was consistent with free radical, mediated OS. These authors suggest that ''antioxidants could potentially modulate the response and reduce the damage'' (Elsayed et al., 1992). Although useful, animal models do not exactly mimic the development of SM injury to human skin. Nevertheless, the mouse ear model, the rabbit, the hairless guinea pig, the nude mouse, and the weanling swine have all been useful for studying the (1) pathophysiology; (2) molecular mechanism of action; and (3) efficacy of countermeasures for SM injury. Similarly, in vitro models have major limitations but have the advantage of being cost-effective and potentially very reproducible.

A synthetic human skin model, EpiDerm, showed considerable promise as an in vitro model. EpiDerm possessed all the main characteristic features of the native skin tissue, including the cuboidal appearance of the basal cell layer, the presence of the stratum spinosum and stratum granulosum with typical stellate-shaped keratohyalin granules, and the presence of numerous lamellar bodies that are extruded at the stratum granulosum–stratum corneum interface. The EpiDerm system has the potential for identifying and developing SM therapeutic agents but does have its limitations as well. In vivo, skin damage can be accompanied by the rapid leakage of serum and leukocyte infiltration but this cannot occur in the in vitro skin models.

The U.S. Army Research Institute of Environmental Medicine has used the EpiDerm model to study CEES-induced skin toxicity (Blaha et al., 2000a, 2000b, 2001). Blaha et al. (2000a), for example, studied the potential role of inflammatory cytokines on CEES-induced toxicity. Since CEES or SM is known to provoke an acute inflammatory response in skin, it is reasonable to assume that inflammatory cytokines are involved in this process and that EpiDerm would mimic the in vivo responses. Blaha et al. (2000a) found, however, that CEES depressed the levels of IL-1α and related cytokines. These authors concluded that the inflammatory responses seen in vivo are promoted by factors from sources other than keratinocytes (Blaha et al., 2000a). Stone et al. (2003, 2004) found that inflammatory cytokines dramatically enhance the toxicity of CEES in a macrophage model. It would be important, therefore, to determine if inflammatory cytokines increase CEES toxicity in the EpiDerm model. EpiDerm tissues in the presence of inflammatory cytokines may prove to be an excellent model to test the efficacy of countermeasures. Blaha et al. (2001) also demonstrated that CEES induces apoptosis in the EpiDerm model. This is a valuable observation since potential countermeasures could easily be tested for their antiapoptotic effects in this model.

1. Role of Skin Mast Cells in Vesicant Toxicity

Rikimaru et al. (1991) have used full-thickness human skin explants to study inflammatory mediators in response to topically applied SM. These investigators found that culture fluids from

the SM-treated skin contained increased levels of histamine, plasminogen-activating activity, and prostaglandin E2 compared with control explants. It was concluded that both mast cells and epidermal cells were apparently involved in early mediation of the inflammatory response to SM (Rikimaru et al., 1991). In contrast, Inoue et al. (1997) found that the inflammatory response of the mouse ear to SM did not differ in mast cell-deficient mice compared with normal mice. At present, there is no obvious explanation for the differences observed between the work of Rikimaru et al. (1991) and that of Inoue et al. (1997). It may well be that the mouse ear is not an optimal model for human skin. It is, however, critically important to determine if SM, or other toxic vesicants, degranulate mast cells, since this process could be a major source of inflammatory mediators.

C. Eyes

The eye is a complex sensory organ, which receives visual information from the environment. It encodes optical information into complex electrical signals, which are transmitted to the cortex for visual imagery through the optical nerve. The visual efficiency primarily depends on the optical clarity of the eye (e.g., cornea, crystalline lens, and intraocular media) and the neural integrity of the visual pathway (e.g., retina, optic nerve, and visual cortex).

The pathogenesis of most age-related eye disorders remain poorly understood. Significant evidence points to oxidative damage as a major factor in the initiation and progression of numerous age-related diseases (Kowluru and Kennedy, 2001; Algvere and Seregard, 2002; Hogg and Chakravarthy, 2004; Shichi, 2004; Ohia et al., 2005; Truscott, 2005). Generally, OS occurs when the level of ROS exceeds the ability of the cell to respond through antioxidant defenses leading to the modification and degradation of carbohydrates, membrane lipids, proteins, and nucleic acids (Gamaley and Klyubin, 1999; Kimura et al., 2005). H_2O_2, a relatively stable oxidant, is present at low concentrations in the normal eye and is found at elevated concentrations in some patients with maturity-onset cataract (Beatty et al., 2001; Ohia et al., 2005; Truscott, 2005). Oxidative damage has also been hypothesized to play a role in the pathogenesis of glaucoma, as the trabecular meshwork is exposed to high levels of OS arising from aerobic metabolism, high aqueous concentrations of H_2O_2, and photochemical reactions in the anterior segment (Shichi, 2004; Ohia et al., 2005). The retina is particularly susceptible to OS because of its high consumption of oxygen, its high proportion of polyunsaturated fatty acids (PUFAs), its abundance of photosensitizers and its exposure to visible light (Sickel, 1972; Beatty et al., 2001). In general, it is well known that the greater the oxygen content of tissues, the more susceptible they are to oxidative and photooxidative damage. The retina is supplied with oxygen by the blood with generally high oxygen content in different portions of the retinal tissues. The lipids present in the membranes of rods contain a high percentage of PUFAs, particularly docosahexanoic acid (DHA) (22:6ω-3), known to be the most highly PUFA in nature, making the retina inherently susceptible to lipid peroxidation (Sickel, 1972; Bazan, 1989; Beatty et al., 2001).

Although the eye is continuously exposed to OS, cells have numerous protective mechanisms to reduce the incidence of severe oxidative damage. Damage to the eye by increases in ROS is typically avoided because of a very efficient antioxidant system. It is further protected by pigments such as the kynurenines and melanin (Roberts, 2001). The major water-soluble antioxidants are GSH and ascorbic acid (Reddy and Giblin, 1984; Reddy, 1990; Delamere, 1996; Rose et al., 1998; Lou, 2003; Rahman and Kelly, 2003). Ascorbic acid, the most effective aqueous-phase antioxidant in human blood, is present in high concentrations in the lens, cornea, retinal pigment epithelium, and aqueous humor of humans (Delamere, 1996; Rose et al., 1998). GSH is a naturally occurring tripeptide and is found mostly concentrated in the lens epithelium; its concentration in the lens is as high as that seen in the liver. GSH acts as a reductant of peroxides either by a nonenzymatic reaction or by a reaction catalyzed by GSH peroxidase. GSH may be especially important in protecting the thiol groups of crystallins, preventing them from aggregating to form opaque clusters. Aging lenses or lenses under OS show an extensively diminished size of GSH pool, with some protein thiols

being S-thiolated by oxidized nonprotein thiols to form protein thiol-mixed disulfides, as protein-S-S-glutathione (PSSG), protein-S-S-cysteine (PSSC), or protein-S-S-gamma-glutamylcysteine (Megaw, 1984; Reddy and Giblin, 1984; Reddy, 1990; Rose et al., 1998; Beatty et al., 2000; Ganea and Harding, 2006).

The principal lipid-soluble antioxidants are vitamin E and the carotenoids (Hunt et al., 1984; Snodderly, 1995; Khachik et al., 1997; Beatty et al., 2001). Vitamin E is the major chain-breaking, lipid-soluble antioxidant in membranes, and is thus expected to play the most important role in minimizing effects of oxidation of PUFAs. Both vitamin E and the carotenoids scavenge free radicals, particularly hydroxyl radical and singlet oxygen. Vitamin E is recycled by redox coupling with vitamin C. The retina contains high quantities of α-tocopherol (outer segments of rod) and rod and retinal pigmented epithelium (RPE), and the concentrations within these tissues are very sensitive to dietary intake of the vitamin E (Hunt et al., 1984; Beatty et al., 2001). Vitamin E deficiency has been shown to result in retinal degeneration, excessive RPE lipofuscin levels, and a decrease in the PUFA content of rod outer segments and the RPE, suggesting that vitamin E protects against retinal oxidative damage (Hayes, 1974; Beatty et al., 2001). The antioxidant properties of the carotenoids are now well established, and they possess the ability to quench singlet oxygen and triplet sensitizers, interact with free radicals, and prevent lipid peroxidation. Of the many carotenoids circulating in human sera, only lutein and zeaxanthin are accumulated throughout the tissues of the eye, where they reach their highest concentration in the central retina (macula lutea). Lutein and zeaxanthin are more commonly referred to as macular pigments (Snodderly et al., 1984; Khachik et al., 1997).

SOD, catalase, and GSH peroxidase are antioxidant enzymes that play a significant role in protecting the retina from oxidative damage. GSH peroxidase is found in the retina and uses GSH as an electron donor to reduce fatty acid hydroperoxides, phospholipid hydroperoxides, cholesterol hydroperoxides, and H_2O_2 (Beatty et al., 2002; Ganea and Harding, 2006). SOD catalyzes the quenching of the superoxide anion to produce H_2O_2 and oxygen (Beatty et al., 2002; Lin et al., 2005). Catalase is an iron (Fe)-dependent enzyme that scavenges H_2O_2 either catalytically or peroxidatively. Catalase has been demonstrated in human neurosensory retina and RPE (Roberts, 2001; Beatty et al., 2002; Ohia et al., 2005).

1. Vesicant-Induced Ocular Injury and Oxidative Stress

The eyes are the organs most sensitive to vesicants, which cause cellular changes within minutes of contact; however, the onset of signs and symptoms to vesicant exposure may become evident several hours later. The time course of symptom development after exposure between SM and the nitrogen analogs is nearly the same. The initial contact of mustard gas with the eye for the most part does not cause pain and discomfort. Mild (12–70 $mg/m^3/min$) to moderate (100–200 $mg/m^3/min$) exposures might result in irritation, pain, swelling, and tearing that may occur within 3–12 h postexposure. Similar symptoms might appear 1–2 h after severe exposure (>200 $mg/m^3/min$) but the symptomatology might also include light sensitivity, severe pain, or temporary blindness. Physical findings include blepharospasm, periorbital edema, conjunctival injection, and inflammation of the anterior chamber (Solberg et al., 1997; Safarinejad et al., 2001; Banin et al., 2003; Javadi et al., 2005).

In the Iran–Iraq conflict, SM was heavily used and even now, about 30,000 victims still suffer from late effects of the agent, for example, chronic obstructive lung disease, lung fibrosis, recurrent corneal ulcer disease, chronic conjunctivitis, abnormal pigmentation of the skin, and several forms of cancer (Kehe and Szinicz, 2005). Evaluation of Iranian survivors with chronic or delayed-onset mustard gas keratitis revealed that mustard gas caused chronic and delayed destructive lesions in the ocular surface and cornea, leading to progressive visual deterioration and ocular irritation. Excised conjunctival and corneal specimens revealed a mixed inflammatory response without any specific features. Light microscopy of conjunctival specimens showed decreased goblet cell density, thickened epithelium, scarring in the substantia propria associated with plasmacytic and lymphocytic

infiltration, and dilated lymphatic vessels. Excised corneal buttons disclosed the absence of epithelium and Bowman's layer, stromal scarring, and vascularization. The pathophysiologic features of these changes are not clearly identified. Based on the clinical appearance of the lesions and the histopathologic findings, an immune-mediated component seems possible (Javadi et al., 2005).

In addition to their alkylating properties, mustards are now being recognized to mediate their toxic actions, at least in part, via the formation and action of ROS (Banin et al., 2003). A dramatic increase in copper levels and a decrease in ascorbic acid within the anterior chamber after ocular exposure to mustard compounds implicates the role of OS in mustard-induced eye injuries (McGahan and Bito, 1982; Kadar et al., 2001; Banin et al., 2003). Mustards are also known to rapidly inactivate sulfhydryl-containing proteins and peptides, such as GSH. These sulfhydryl compounds are critical in maintaining the appropriate oxidation–reduction state of cellular components, and GSH is also thought to be critical in reducing ROS in the cell and preventing peroxidation and loss of membrane integrity (Stadtman, 2001). Furthermore, the amelioration of mustard-induced ocular injuries by antioxidants is also evidence to implicate OS as a potential mechanism of injury (Banin et al., 2003; Morad et al., 2005).

Recognizing the fact that ROS play a role in the pathogenesis of mustard-induced ocular injuries, compounds that inhibit the formation of ROS or prevent their toxic effects would be beneficial in the treatment of mustard-induced ocular injuries. The topical application of low concentrations of Zn/DFO or Ga/DFO after corneal exposure to nitrogen mustards markedly reduced conjunctival, corneal, iris, and anterior chamber injury. In the cornea, the healing of epithelial erosions was faster, the long-term opacification was reduced, and the levels of neovascularization were lowered. In the anterior chamber, decreased inflammation and better maintenance of intraocular pressure were achieved. Cataractous changes were also notably milder (Banin et al., 2003).

A combination of topically applied Zn/DFO and dexamethasone, by virtue of their additive inhibitory effects on free radical formation and inflammation, reduced nitrogen mustard-induced injury to ocular anterior segment structures. Furthermore, the combination treatment of Zn/DFO and dexamethasone resulted in a speedier corneal reepithelization, less-severe corneal neovascularization, and the intraocular pressure was not as severely elevated as in the saline or the Zn/DFO- or dexamethasone-alone groups (Morad et al., 2005).

2. Redox Proteomics

OS induces free radical damage to biomolecules and alterations in redox-sensitive signaling pathways, both of which are key factors in understanding vesicant toxicology. In particular, small thiols, like GSH, are no longer viewed just as protective antioxidants but as redox regulators of proteins via glutathionylation or by oxidation of protein cysteine residue (Ghezzi and Ungheri, 2004). Redox proteomics is rapidly emerging as a very powerful tool for characterizing and identifying proteins based on their redox state (Ghezzi and Ungheri, 2004). This approach has not yet been applied to the study of vesicant toxicity, but studies should soon begin on exploring this area.

IX. OXIDATIVE STRESS: THE CONCEPT AND THE EFFECT ON GENE EXPRESSION

A. Definition of Oxidative Stress

Redox potential is defined by the half cell reduction potential that is created by redox couples that are primarily due to GSH, NAD^+ and nicotinamide dinucleotide phosphate. These couples are in ratios of the oxidized to reduced form of the molecules (NAD^+/NAD, $NADP^+$/NADPH, and GSSG/2GSH). The redox couples can be independent, as well linked to each other to form related couples. The redox environment is a reflection of these couples. These ratios can be measured by the Nernst equation, similar to a voltaic cell.

The Nernst equation is

$$E_h = E_0 + (RT/nF)\ln\{[\text{acceptor}]/[\text{donor}]\}$$

E_h is the electromotive force at a particular pH, its units are in volts or millivolts relative to a standard hydrogen electrode (1 atom H_2, 1M H^+). The electromotive force is a quantitative measurement of a redox-active molecule that donates or accepts electrons.

R is the gas constant, T is the absolute temperature, F is the Faraday's constant, and n is the number of electrons transferred. The steady state of E_h for a redox-active component depends on the kinetic of the transfer of the reduction and oxidation reactions. Under normal homeostatic conditions (absence of OS), the E_h for 2GSH/GSSG relatively reduces because of the NADPH-coupled GSSG reductase. During periods of OS, the E_h becomes more oxidized.

Under normal cellular conditions, the redox potential is reductive. In diseased conditions, wherein OS occurs, the redox potential becomes more oxidative. The redox potential is similar in different cell types, but varies according to the cellular processes: proliferation $E = -240$ mV, differentiation $E = -200$ mV, and apoptosis $E = -170$ mV (Kirlin et al., 1999).

An illustrative experimental example of a cell exposed to an oxidant was carried out by Kirlin et al. (1999). When they used HT29 cells (colon adenocarcinoma) and exposed them to sodium butyrate, it was found that there was an oxidant reaction that caused a drop from -260 to -200 mV in E_h. The 60 mV decrease resulted in a 100-fold change in protein dithiols:disulfide ratio. A correlation was noted between E_h, GST, and NADPH:quinone reductase Kirlin further indicated that the E_h provides two additional pieces of information (1) in reactions that use GSH as a reductant to maintain protein thiol/disulfides in their reduced form, it is an indicator of the reducing power quantitatively and (2) if the redox state is controlled by a GSH redox couple, it is an indication of the functional state of the protein (Kirlin et al., 1999).

B. Molecules that Influence the Redox Potential

GSH synthase, a tripeptide (glutamylcysteinylglycine), is not only a water-soluble antioxidant, but is also part of a redox buffer (Smith et al., 1996). It is found in all cells and is used for a multiplicity of cellular functions, such as protein and prostaglandin synthesis, detoxification, etc. Cytosolic concentrations of GSH range from 1 to 11 mm (Smith et al., 1996) and are 100–1000 times greater than the extracellular levels. Many proteins contain sulfhydryl groups because of their cysteine content. The content of thiols in proteins is greater than that of the pool of GSH (Torchinsky, 1981).

The intracellular compartment exchanges GSH with the cytosol (Griffith and Meister, 1985; Schnellmann et al., 1988; Fernandez-Checa et al., 1998). GSH concentrations within the nucleus are critical for maintaining the redox state of protein sulfhydryls that are necessary for DNA repair and expression (Arrigo, 1999). The endoplasmic reticulum has a more oxidizing environment than the cytosol or nucleus. GSH/GSSH in the endoplasmic reticulum is in the range of 1:1 to 3:1, in comparison with the cytosol (Hwang et al., 1992). The ratio of reduced GSH to GSSG influences a variety of cellular signaling processes, such as activation and phosphorylation of stress kinases (JNK, p38, PI-3K) via sensitive cysteine-rich domains, activation of SHM-ase ceramide pathway, and activation of AP-1 and NF-κB, with subsequent gene transcription (Singh et al., 1998; Arrigo, 1999; Mercurio and Manning, 1999a, 1999b).

1. Nicotinamide Adenosine Dinucleotide Phosphate

NADPH is usually involved in reductive (biosynthetic) reactions and serves as a source of electrons. In contrast, NAD is involved in oxidative reactions and serves as a sink for electrons. In cells and tissues, the ratio of NADPH/$NADP^+$ tends to be 1:10 and 1:1000. NADPH is considered the primary source of reducing equivalents for GSH.

2. Thioredoxin

Thioredoxin (TRX) is a pleiotropic polypeptide also known as T-cell leukemia-derived factor (Tagaya et al., 1989; Yodoi and Uchiyama, 1992). It has two redox-reactive cysteine residues in the reactive center (Cys-32 and Cys-35) (Holmgren, 1972, 1985, 1989; Buchanan et al., 1994). Reductases are used to donate electrons from NADPH to facilitate the reduction oxidation reaction between the dithiol or disulfide forms of TRX (Luthman and Holmgren, 1982; Watson et al., 2004).

TRX is responsible for the reduction of cysteine moieties in the DNA-binding sites of several transcription factors (Mathews, 1992; Okomoto, 1992), facilitates the refolding of disulfide-containing proteins, and regulates the DNA-binding activity of some transcription factors (e.g., NF-κB and Ref-1-dependent AP-1) (Sen, 1998; Arner and Holmgren, 2000). TRX facilitates gene expression in that it enables protein–nucleic acid interactions (Holmgren, 1985); it does so by reducing cysteine in the DNA-binding loop of several transcription factors (Matthews et al., 1992; Okamoto et al., 1992; Xanthoudakis et al., 1992, 1994). The redox state of TRX varies independently to that of GSH/GSSG. GSH forms intermolecular disulfides, whereas TRX is a protein that usually forms intramolecular disulfides. TRX assists in the control of apoptosis signal, regulating kinase-1 (ASK-1) (Saitoh et al., 1998).

C. Apoptosis

Apoptosis is induced, when tissue is exposed to SM by a calmodulin- and caspase-dependent pathway (Rosenthal et al., 1998; Sciuto and Hurt, 2004). Zhang et al. (2001) using Jurkat cells exposed to CEES, analyzed gene expression for death and survival pathways. The Akt (PKB) is an important kinase, which can block apoptosis and promote cell survival. It was downregulated in a dose-dependent manner. The antiapoptotic genes, Bcl family, were decreased: Bcl-2, 90%; bax, 80%; bcl-X_L, 67%; Bak and Mcl-1, 70%; and Bik, 57%. Caspases 3, 4, 6, 8, and 9 were upregulated. Crawford et al. (unpublished data) found that CEES-exposed human dendritic cells released cytochrome *c* before detectable levels of ROS generation. NAC was found to inhibit the release of cytochrome *c* and decrease apoptosis. The release of cytochrome *c* was likely due to the opening of the mitochondrial transport permeability transition pores, which is regulated by two redox couples, NADP and GSH (Dalton et al., 1999). It is well known that a portion of the toxicity of mustard is the depletion of NAD (Mol et al., 1989; Byers et al., 2000).

A depletion of GSH alone can act as an early activation of apoptotic signaling (Coffey et al., 2000; Coppola and Ghibelli, 2000; Armstrong and Jones, 2002). Conversion from the procaspase to an active enzyme requires a reduction of the cysteine residue (Hampton et al., 1998). OS can trigger caspase activity, but can also suppress it (Hampton et al., 1998).

Studies conducted by Celli et al. (1998) used BSO (buthionine sulfoximine) to deplete cells of GSH. There was a significant decrease in the Bcl levels and associated time-dependent increase in the number of cells undergoing apoptosis. Maintenance of GSH levels with GSH ethyl ester in the presence of BSO decreased apoptosis and prevented a decrease in Bcl-2 protein. The Celli study, if contrasted to Zhang et al. (2001), one begins to suspect that there may be a strong correlation between the loss of GSH and gene expression in CEES-exposed tissues.

The suppression of Bcl-2 expression induces the relocalization of GSH to the cytosol, whereas the overexpression of Bcl-2 induces a relocalization of GSH to the nucleus. There was a direct correlation between GSH levels and the Bcl-2 nuclear protein levels. It was concluded by Voehringer et al. (1998) that one of the functions of Bcl-2 is to promote the sequestration of GSH into the nucleus.

D. Gene Expression Controlled by Redox-State Transcription Factors

Redox homeostasis regulates activation and binding by transcription factors at the 5′ end of the target gene. In the transcription factors, cysteine residues are frequently located in their protein

sequence localized in their DNA-binding domain. The cysteine molecules are often essential for recognition of the binding site due to electrostatic interactions with specific DNA bases. Inhibition of the binding factor occurs if the cysteine is oxidized, which may be a consequence of an alteration of the tridimensional structure of the transcription factors. The function of several transcription factors could be impaired or inhibited because of oxidation of cysteine groups that result in inter- or intramolecular disulfide bonds (Arrigo, 1999). Several of the transcription factors undergo redox modification posttranslationally. NF-κB binding with DNA is augmented by TRX in vitro. Ref-1 is also modulated by redox compounds (e.g., TRX) (Hirota et al., 1997).

1. NF-κB

NF-κB regulates many genes, particularly those involved in immune and inflammatory responses (Baeuerle and Baltimore, 1996). Antioxidants, such as NAC, GSH, and cysteine, inhibit the activation of NF-κB (Mihm et al., 1995). TRX regulates the binding of NF-κB to DNA (Matthews et al., 1992).

2. Ref-1

In an oxidizing environment, Ref-1 facilitates the binding transcription factors to DNA by reduction of cysteine residues within the binding domains of these proteins (Xanthoudakis and Curran, 1992; Xanthoudakis et al., 1992; Walker et al., 1993). Under OS, cysteine 65 forms a disulfide bond with cysteine 93, which ceases the stimulatory activity of Rdf-1. Active Ref-1 is recycled by the contribution of hydrogen from TRX (Hirota et al., 1997).

3. Activator Protein-1

Activator protein-1 (ATP-1) binds to an enhancer element (Angel et al., 1987; Lee et al., 1987), and is modified by redox factor 1 (Ref-1), a DNA repair enzyme (Angel et al., 1987; Lee et al., 1987; Arrigo, 1999). It is present in the promoter region of several genes, which are implicated in cell proliferation and tumor promotion. Several growth factors, PKC activators, and intracellular redox activate it (Angel and Karin, 1991). It consists of c-Jun and c-Fos proteins, which are products of the c-jun and c-fos proto-oncogene. Agents that promote intracellular oxidants such as UV irradiation, H_2O_2, and mitogens induce c-fos and c-jun genes (Datta et al., 1992; Lo and Cruz, 1995). Antioxidants that have a phenolic group (e.g., d-α-tocopherol or butylated hydroxyltoluene) induce expression of c-fos and c-jun (Choi and Moore, 1993; Stauble et al., 1994). ATP-1 acts as a secondary antioxidant-responsive transcription factor (Meyer et al., 1993).

4. Heat Shock Transcription Factor

Heat shock transcription factor (HSF) is part of the family of heat shock factors that are activated and bind to DNA induced by OS. Several chemicals, heat shock, or conditions that generate abnormally folded proteins activate HSF1 conversion from a monomer to a trimer state (Liu et al., 1996). Iodoacetamide (IDAM), an alkylating agent, activates the transcription of Hsp 70 gene (Liu et al., 1996). The depletion of GSH induces oxidation of protein thiols, denaturation, and aggregation of proteins (Freeman et al., 1997). Alkylating agents that deplete GSH increase HSF1 (Liu et al., 1996) and induce trimerization of HSF1.

X. SUMMARY

Chlorine, phosgene, Lewisite, and SM all react with thiol groups as well as produce oxidants. The arsenic group in Lewisite has a high affinity to the alpha and gamma thiol groups of lipoic acid found in enzymes (e.g., pyruvate oxidase). Oxidants occur as part of the normal metabolism of cells (redox homeostasis). In the diseased state (e.g., exposure to a chemical agent), there is an acute

inflammatory response that is also inclusive of OS (redox imbalance). During significant OS, the oxidant burden exhausts the redox buffer of the cell (e.g., GSH/GSSH, NADP/NADPH, etc.) that consequently alters redox couples. The mitochondrial permeability transition pores (MPTPs) are directly regulated by the redox state. They are a cyclosporine A-sensitive, Ca^{2+}-dependent, and voltage-gated channel. There are two types. One is dependent on the GSH:GSSH ratio (Petronilli et al., 1994; Costantini et al., 1995); the other is a voltage-sensitive gate, regulated by the ratio of pyridine nucleotides (NAD^{+}NADP):($NADH^{+}$NADPH) and is independent of the reduced GSH (Reed and Savage, 1995; Costantini et al., 1996). MPTPs appear to be involved in the toxicity of several chemicals (Dalton et al., 1999). Opening of the pores results in energy uncoupling by a Ca^{2+}-dependent decrease of mitochondrial inner-membrane potential (Petronilli et al., 1994). The collapse of the membrane potential and the inhibition of oxidative phosphorylation result in diminished intracellular ATP. Consequently, there may be the release of inner-membrane cytochrome *c* to the cytosol, which may signal the initiation of apoptosis (Krippner et al., 1996).

In a comparison of SM to CEES done using a histological grading system, SM was shown to be about six times more potent than CEES (Dana Anderson, unpublished results). The doses that were used by our group in the in vivo models were found to be equivalent to those used at the U.S. Army Medical Research Institute of Chemical Defense. CEES increases the activity of the transcription factor NF-κB, and consequently, there is an increase in proinflammatory cytokine production (e.g., TNF-α). PARP activity increases dramatically in both CEES- (Crawford, unpublished results) and SM-exposed tissues (Bhat et al., 2006). Increased PARP activity affects the energy levels of the cell by oxidizing NADPH, which causes a redox imbalance ($NADP^{+}$/NADPH). The transcription factor NF-κB and caspases are increased in CEES- and SM-exposed tissues. In both CEES and SM exposures, there is a loss of GSH, likely due to direct interaction with thiols and OS. The combined loss of GSH, NADH, and NADPH has far-reaching ramifications on multiple cellular systems, particularly redox-regulated pathways.

A. Antidotes

Lewisite is the only vesicant with a proven antidote—British anti-lewisite (2,3-dimercaptopropanol). Increasing antioxidant levels have been found to be protective against the mustards analog, NAC. NAC, which we have used in our studies with CEES, is immediately clinically available. It is most commonly used for acetaminophen overdose. NAC has a long history of several gram quantities administered in several doses and has minimal adverse reactions. In the case of acetaminophen overdose, it is administered via the oral-gastric route, which increases hepatic GSH levels, and in turn, suppresses inflammatory cytokines (Dambach et al., 2006). Liposome encapsulation of both water- and fat-soluble antioxidants was proven to be more effective in the suppression of OS than the free molecule of NAC.

Antioxidants that are liposome encapsulated are advantageous in that they enhance delivery to sites at which inflammation occurs. In light of the common effect that the vesicants have on redox-regulated pathways and OS, it becomes a compelling reason for additional research. The down-regulation of OS may be a very significant step forward in developing treatment countermeasures against several vesicants and other WMD.

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13 Health Effects of Exposure to Vesicant Agents

Charles G. Hurst and William J. Smith

CONTENTS

The opinions or assertions contained herein are the private views of the authors and are not necessarily the official views of the U.S. Army or the U.S. Department of Defense.

I. BACKGROUND

Chemical warfare agents have been around for at least 4000 years and probably were originally used as poisons on individuals. The use of chemical weapons dates from at least 423 BC when allies of Sparta in the Peloponnesian War took an Athenian-held fort by directing smoke from lighted coals, sulfur, and pitch through a hollowed-out beam into the fort. Other conflicts during the succeeding centuries saw the use of smoke and flame. During the seventh century AD, the Greeks invented ''Greek fire,'' a combination probably of rosin, sulfur, pitch, naphtha, lime, and saltpeter that floated on water and was particularly effective in naval operations. During the fifteenth and sixteenth centuries, Venice employed unspecified poisons in hollow explosive mortar shells and sent poison chests to its enemy to poison wells, crops, and animals (Prentiss, 1937; Heller, 1984; Medema, 1986). Finally, World War I and the Iran–Iraq War saw the advent of modern chemical warfare.

Vesicants are agents that produce chemical burns. Sulfur mustard was the first vesicant used as a chemical weapon on the battlefields of World War I and is still considered a major chemical agent (Prentiss, 1937; Heller, 1984; Medema, 1986; Dacre and Goldman, 1996). In the intervening years between World War I and today, there have been a number of recorded and suspected incidents of mustard use, culminating with the Iran–Iraq War in the 1980s. During this conflict, Iraq made extensive use of mustard against Iran. Popular magazines and television brought the horrors of chemical warfare to the public's attention with graphic images of badly burned Iranian casualties. In the fall of 1990, when the U.S. military joined the United Nations forces in preparation to liberate Kuwait, a major concern was that Iraq would again use mustard. Although mustard is the most important vesicant militarily, the vesicant category includes other agents, such as Lewisite and phosgene oxime (Table 13.1). Because there is no recorded use on the battlefield and very little information on human exposure to phosgene oxime, this chapter will focus on the effects of mustard and Lewisite.

II. MUSTARD

There are two types of mustard agents—sulfur mustard and nitrogen mustard. Both types of mustard exhibit vesicating properties and both have been considered for weaponization. Sulfur mustard has been used in warfare, whereas nitrogen mustards are used in clinical medicine as chemotherapeutic compounds. Subsequent use of the term ''mustard'' in this chapter, unless otherwise specified, will be restricted to sulfur mustard. Mustard has been stockpiled in the arsenals of various countries since it was first used on July 12, 1917, when the Germans fired shells containing mustard at British troops entrenched near Ypres, Belgium. When a single agent was identified as the source of injury, it was estimated that mustard caused about 80% of the chemical casualties in World War I; other agents such as chlorine and phosgene caused the remaining 20%. The British had 180,983 chemical

TABLE 13.1
Clinical Differences among Vesicants

	Onset		
Chemical Agent	**Pain**	**Tissue Damage**	**Blister**
Mustard 2,2″-dicholorethylsulfide (BCES, H, HD)	Hours later	Immediate; onset of clinical effects is hours later	Fluid filled
Lewisite B-chlorovinyldichloroarsine (L)	Immediate	Seconds to minutes	Fluid filled
Phosgene Oxime (CX)	Immediate	Seconds	Solid wheal

casualties; the injuries of 160,970 (88%) were caused solely by mustard. Of these casualties, 4,167 (2.6%) died. Of the 36,765 single-agent U.S. chemical casualties, the injuries of 27,711 (75%) were caused solely by mustard. Of the casualties who reached a medical treatment facility, 599 (2.2%) died. Just as disconcerting was the fact that mustard survivors required lengthy hospitalizations: the average length of stay was 42 days (Dacre and Goldman, 1996).

Since the first use of mustard as a military weapon, there have been a number of isolated incidents in which it was reportedly used. In 1935, Italy probably used mustard against Abyssinia (now Ethiopia); Japan allegedly used mustard against the Chinese from 1937 to 1944; and Egypt was accused of using the agent against Yemen in the mid-1960s.

Chemical agents were not used during World War II. It is thought that Germany did not use mustard because Hitler had been a mustard victim during World War I and was loathe to use it (Dacre and Goldman, 1996). The Iraqi regime in the 1980s had no such reservations and employed mustard against military and civilian targets (Tucker, 2006). Over 45,000 victims of mustard have been documented among Iranian and Kurdish populations (Mauroni, 2003).

A. Properties of Mustard

Mustard is an oily liquid and is generally regarded as a ''persistent'' chemical agent because of its low volatility, usually allowing the liquid to remain on surfaces longer than 24 h. At higher temperatures, such as those in the Middle East during the hot season, 38°C–49°C (100°F–120°F), mustard vapor is a major hazard. The persistency of mustard (in sand) decreases from 100 to 7 h as the temperature rises from 10°C to 38°C (50°F–100°F) (CRDEC, 1990).

World War I data suggest that the warming of the air after sunrise caused significant evaporation of mustard from the ground (Blewett, 1992). Mustard attacks were frequently conducted at night, and the liquid agent did not readily evaporate in the cool night air. Several hours after daybreak, however, the sun-warmed air would cause the mustard to vaporize. Thinking the danger was over, soldiers removed their masks, falling victim to the evaporating mustard. Because of these night shellings, it soon became standard policy not to unmask for many hours after daybreak.

Mustard vapor has a 5.4-fold greater density than that of air, causing it to hug the ground and sink into trenches and gullies. Despite low volatility, more than 80% of the mustard casualties during World War I were caused by vapor, not the liquid form of mustard (Mann and Pullinger, 1944).

The freezing/melting temperature for mustard is 57°F. This high freezing point makes mustard unsuitable for delivery by higher altitude aircraft spraying or winter dispersal. To lower the freezing point, mustard must be mixed with another substance. During World War I, mustard was mixed with chloropicrin, chlorobenzene, or carbon tetrachloride to lower the freezing point (Prentiss, 1937). Mustard has been mixed with Lewisite to increase its volatility in colder weather. The mustard/Lewisite combination has a freezing point close to 10°F.

B. Toxicity of Mustard

The estimation of actual applied dose of an agent, especially when the agent is in the form of a vapor or gas, is often difficult. A term that is commonly used is Ct where Ct = mg min/m^3. Because Ct is a product of concentration and time, the same Ct (exposure/dose) can be reached by variations in concentration and time. The effect produced by an aerosol or vapor exposure to 0.05 mg/m^3/100 min is equal to the effect produced by an exposure to 5 mg/m^3/1 min; in either case, Ct = 5 mg min/m^3.

Eye damage was produced by a Ct of 10 mg min/m^3 or less under laboratory conditions; other estimates for the eye damage threshold under field conditions range from 12 to 70 mg min/m^3 (Papirmeister et al., 1991; Mann, 1994). The estimated Ct for airway injury ranges from 100 to 500 mg min/m^3. The threshold for skin damage is highly dependent on skin site, heat, sweating, and other factors (localized sweating will lower the threshold on the portion of the skin); the threshold is generally in the range of 200–2000 mg min/m^3 (Marshall et al., 1919).

C. Biochemical Mechanism of Sulfur Mustard Toxicity

Sulfur mustard is an alkylating agent with cytotoxic, mutagenic, and vesicating potential. Its chemical reactivity is based on its ability to undergo internal cyclization of an ethylene group to form a highly reactive episulfonium ion. This reactive electrophile is capable of combining with any of the numerous nucleophilic sites present in macromolecules of cells. The products of these reactions are stable adducts which can modify the normal function of the target macromolecule. Because nucleophilic sites exist in peptides, proteins, RNA, DNA, and membrane components, extensive efforts have been underway to identify the most critical biomolecular reactions leading to mustard injury.

Although the chemistry of mustard interactions with cellular components is well defined, the correlation of these interactions with injury has not been made. Over the past few decades, scientists have made major advances in understanding the cellular and biochemical consequences of exposure to mustard and several hypotheses have been put forth to account for the mustard injury.

Three hypotheses, in particular, have been studied for many years and have been extensively reviewed by Papirmeister et al. (1991). Briefly, these are:

1. The poly(ADP-ribose) polymerase (PARP) hypothesis in which DNA strand breaks, induced by mustard, activates the nuclear enzyme PARP culminating in metabolic disruption and protease activation in the region of the basal epidermal cells (Papirmeister et al., 1985).
2. The thiol depletion hypothesis presented by Orrenius et al. (1987) established that menadione-induced depletion of GSH resulted in loss of protein thiols and inactivation of sulfhydryl-containing enzymes. Included in this class of proteins are the Ca^{2+}/Mg^{2+}-ATPases, which regulate calcium homeostasis. Intracellular Ca^{2+} levels would then increase resulting in activation of proteases, phospholipases, and endonucleases, which could give rise to the breakdown of membranes, cytoskeleton, and DNA, resulting in cell death. Whitfield (1987) suggested that this mechanism could be activated by mustards and might be the mechanism of mustard injury. Ray et al. (1997) extended Whitfield's suggestion by demonstrating, in mustard exposed cells, that Ca^{2+}-activated phospholipase causes arachidonic acid release and membrane fluidity decreases.
3. In the lipid peroxidation hypothesis, (Miccadei et al., 1988) depletion of GSH allows for the formation of toxic oxidants through H_2O_2-dependent mechanisms. The oxidizing species thus formed will react with membrane phospholipids to form lipid peroxides, which could in turn lead to membrane alterations and eventual breakdown of cellular membranes.

Although data have been obtained for discrete elements of each of these hypotheses, a fully descriptive mechanism of mustard-induced injury has yet to be developed.

Due to the highly reactive nature of mustard, it is conceivable that the injury following tissue exposure to mustards might result from a combination of effects described in the three hypotheses above or from additional changes not yet described. The pathogenic processes seen in skin, lung, or eye may have common biochemical initiating events, but their distinct pathologies suggest that tissue specific effects may dominate once the biochemical insults develop. Although much effort is being expended in developing therapeutic interventions that will limit the extent of tissue pathology, the best immediate approaches involve prevention of contact between mustard and tissues and medical procedures that ease patient trauma and discomfort.

D. Clinical Manifestations and Diagnosis

The organs most commonly affected by mustard are the skin, eyes, and airways (Table 13.2): the organs with which mustard comes in direct contact. After a significant amount of mustard has been absorbed through the skin or inhaled, the hemopoietic system, gastrointestinal tract, and central

TABLE 13.2
Initial Clinical Effects from Mustard Exposure

Organ	Severity	Effects	Onset of First Effect
Eyes	Mild	Tearing	4–12 h
		Itchy	
		Burning	
		Gritty feeling	
	Moderate	Above effects, plus	3–6 h
		Reddening	
		Lid edema	
		Moderate pain	
	Severe	Marked lid edema	1–2 h
		Possible corneal damage	
		Severe pain	
Airways	Mild	Rhinorrhea	6–24 h
		Sneezing	
		Epistaxis	
		Hoarseness	
		Hacking cough	
	Severe	Above effects, plus	2–6 h
		Productive cough	
		Mild-to-severe dyspnea	
Skin	Mild	Erythema	2–24 h
	Severe	Vesication	

nervous system (CNS) are also damaged. Mustard may also affect other organs but rarely do these produce clinical effects.

During World War I, 80%–90% of U.S. mustard casualties had skin lesions, 86% had eye involvement, and 75% had airway damage; these percentages are not significantly different from those seen in Iranian casualties (Gilchrist, 1928). Of a group of 233 severely injured Iranian soldiers sent to western European hospitals by the Iranian government for treatment during the Iran–Iraq War, 95% had airway involvement, 92% had eye signs and symptoms, and 83% had skin lesions (Balali-Mood and Navaeian, 1986). In a series of 535 Iranian casualties, including civilians, admitted to a dermatology ward, 92% had skin lesions and 85% had conjunctivitis; of the total number of patients, 79% had erythema and 55% had blisters. Casualties with more serious problems, including injury to the pulmonary tract, were admitted to other wards (Momeni et al., 1992).

The slightly higher percentage of airway and eye involvement in Iranian soldiers versus U.S. World War I casualties is perhaps attributable to the higher ambient temperature in the area (compared with Europe), which caused more vaporization. This might also have been because of available Iranian protective equipment or poor mask seal with facial hair. In 1984, the year the first Iranian casualties were treated in Europe, protective clothing and gas masks were not commonly worn by Iranian soldiers (Willems, 1989).

Mustard-related death occurs in about 3% of the casualties who reach a Medical Treatment Facility; of those who die, most die 4 or more days after exposure (Gilchrist, 1928). Of the casualties who died, 84% required at least 4 days of hospitalization. The causes of death are pulmonary insufficiency from airway damage, superimposed infection, and sepsis. Rarely, the amount of mustard will be overwhelming and cause death within 1–2 days; in these circumstances, death will be due to neurological factors or massive airway damage (Graef et al., 1948; Heully and

Gruninger, 1956). Willems' report on Iranian casualties treated in western European hospitals demonstrates the effect of medical advances since World War I. Clinical files of 65 of these casualties were studied in detail (Willems, 1989). Eight patients died between 6 and 15 days after exposure. One patient died 185 days after exposure: he had received ventilatory support for an extended period because of severe bronchiolitis complicated by a series of loculate pneumothoraces. Most patients returned to Iran in fairly good condition after 2–10 weeks of treatment. The duration of hospitalization was determined mainly by the time needed for healing of the deeper skin lesions. Because some of the most severe patients were sent to Europe, there was a 14% mortality rate, compared to the 3% seen overall in World War I.

1. Skin

The threshold amount of mustard vapor required to produce a skin lesion (erythema) is a Ct of about 200 mg min/m^3. This varies greatly depending on a number of factors, including temperature, humidity, skin hydration, and body site. Warm, moist areas with thin skin, such as the perineum, external genitalia, axillae, antecubital fossae, and neck are much more sensitive. As stated earlier, a liquid droplet of about 10 μg will produce vesication. About 80% of this 10 μg evaporates and 10% enters the circulation, leaving about 10%, 1 μg, to cause the vesicle. Evaporation of small droplets is rapid and nearly complete in 2–3 min; amounts larger than several hundred milligrams may remain on the skin for several hours (Renshaw, 1946).

Mustard vapor rapidly penetrates the skin at the rates of 1.4 μg/cm^2/min at 70°F and 2.7 μg/cm^2/min at 88°F (CRDEC, 1990), liquid mustard penetrates the skin at 2.2 μg/cm^2/min at 60°F and at 5.5 μg/cm^2/min at 102°F. Once mustard penetrates the skin, it is ''fixed'' to components of tissue and cannot be extracted (Renshaw, 1946).

The mildest and earliest form of visible skin injury is erythema, which resembles sunburn (Figure 13.1). Erythema begins to appear 1–24 h after the skin is exposed to mustard, although onset can be later. This is usually accompanied by pruritus (itching), burning, or stinging. After a small exposure, this might be the extent of the lesion. More commonly, small vesicles will develop within

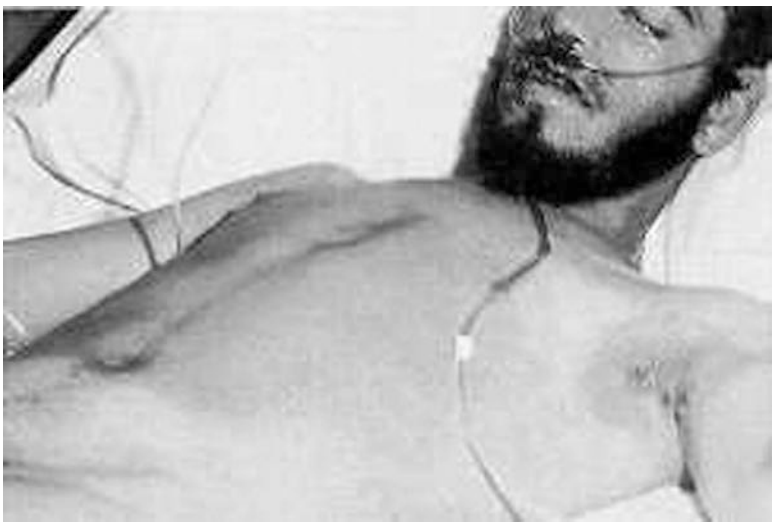

FIGURE 13.1 Erythema of the chest of an Iranian casualty as it appeared 5 days after his exposure to mustard. He also had a pulmonary injury with an associated bronchopneumonia due to infection with *Haemophilus influenzae*. The presence of a nasal oxygen catheter is indicative of the pulmonary insufficiency. (Photograph reprinted from Willems J.L., *Ann. Med. Milit. Belg*., 3S, 13, 1989. With permission.)

or on the periphery of the erythematous areas (like a string of pearls); these vesicles will later coalesce to form larger blisters. The effects from liquid mustard appear more rapidly than the effects from mustard vapor. Characteristically, the onset of erythema is about 4–8 h after mustard exposure. Vesication begins about 2–18 h later and may not be complete for several days.

The typical bulla (large blister) is dome shaped, thin walled, superficial, translucent, yellowish, and surrounded by erythema and can be 5 cm in diameter larger (Figure 13.2). The blister fluid is initially thin and clear or slightly straw colored; later it turns yellowish and tends to coagulate (Reed, 1920; Renshaw, 1946; Willems, 1989). The blister fluid does not contain mustard and is not itself a vesicant. Thiodiglycol, a breakdown product of mustard, has been found in blister fluid and can be used to aid in diagnosis. Vapor injury is generally a first or second-degree burn; liquid mustard may produce deeper damage comparable to a third-degree burn.

After exposure to extremely high doses, such as those resulting from exposure to liquid mustard, lesions may be characterized by a central zone of coagulation necrosis, with blister formation at the periphery. These lesions are more severe, take longer to heal, and are more prone to secondary infection (Mann and Pullinger, 1944).

The healing time for mustard skin lesions depends on the severity of the lesion. Erythema heals within several days, whereas severe lesions may require several weeks to several months to heal, depending on the anatomical site, the total area of skin surface affected, and the depth of the lesion (Warthin, 1926).

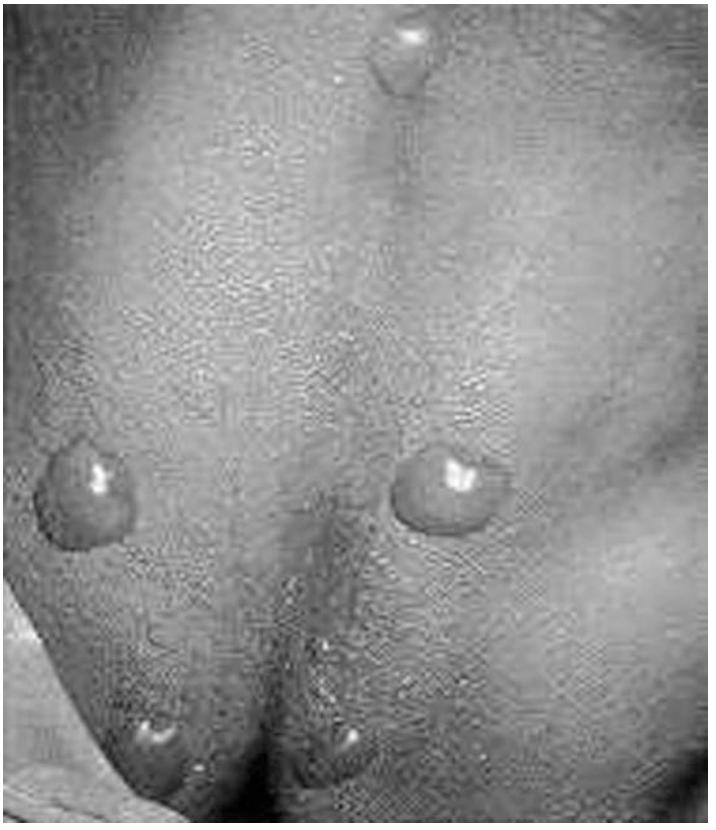

FIGURE 13.2 The back of an Iranian casualty seen 16 h after exposure to mustard. Note the small vesicles in proximity to the large bullae. (Photograph reprinted from Willems J.L., *Ann. Med. Milit. Belg*., 3S, 8, 1989. With permission.)

One of the interesting characteristics of the cutaneous mustard injury that Willems reported in the Iranian casualties was the transient blackening, or hyperpigmentation, of the affected skin (Warthin, 1926). When the hyperpigmented skin exfoliated, epithelium of normal color was exposed. Vesication was not necessary for hyperpigmentation to occur. The syndrome of hyperpigmentation and exfoliation was commonly recognized in World War I casualties, but less commonly in laboratory experiments in which liquid mustard was used (Warthin, 1926). When the initial skin damage, inflammation, only stimulates the melanocyte (pigment cell), increased pigmentation, hyperpigmentation, can be seen. When the melanocyte is destroyed, you see hypopigmentation lasting usually for several months, and occasionally, it may become permanent. This blotchy hyper and hypopigmentation can be extremely distressing to individuals, because similar appearing skin changes are often associated with diseases like leprosy and syphilis. Punctate repigmentation can be seen starting at and around hair follicles where the melanocytes were not destroyed (Figure 13.3).

2. Eye

The eye is the organ most sensitive to mustard. The Ct required to produce an eye lesion under field conditions is 12–70 mg min/m^3 (Papirmeister, 1991). The effective Ct for conjunctivitis, or slightly more severe damage, was just under 10 mg/m^3 in 13 subjects; several subjects had lesions at Cts of 4.8–5.8 mg min/m^3 (Reed, 1920). One subject had no symptoms after several hours; however, by 12 h after the exposure, marked blepharospasm and irritation were apparent.

Generally, the asymptomatic period varies with the concentration of mustard vapor and individual sensitivity. The latent period for eye damage is shorter than that for skin damage. Eye irritation within minutes after exposure has been reported (Warthin, 1926; Geeraets et al., 1977). After a low Ct exposure, a slight irritation with reddening of the eye may be all that occurs. As the Ct increases, the spectrum of injury is characterized by progressively more severe conjunctivitis, blepharospasm, pain, and corneal damage (Warthin, 1926; Papirmeister et al., 1991). Photophobia will appear, and even with mild exposures, may linger for weeks.

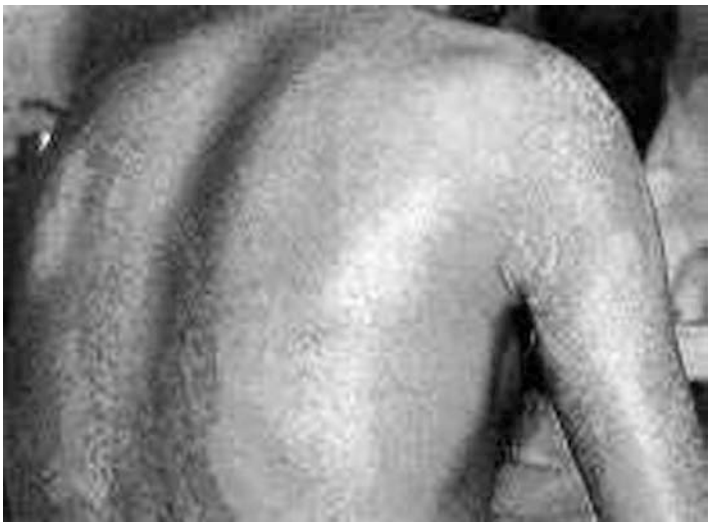

FIGURE 13.3 By 32 days after exposure, this Iranian casualty has punctate hyperpigmentation in a healing deep mustard burn. This condition is perhaps indicative of postinflammatory changes in the epidermis that has regenerated from hair follicles. (Reprinted from Willems J.L., *Ann. Med. Milit. Belg*., 3S, 34, 1989. With permission.)

Corneal damage consists of edema with clouding, swelling, and infiltration of polymorphonuclear cells. Clinical improvement occurs after approximately 7 days with subsiding edema. Corneal vascularization (pannus) with secondary edema may last for weeks. Vision will be lost if the pannus covers the visual axis. Severe effects from mustard exposure may be followed by scarring between the iris and the lens, which restricts pupillary movements and predisposes the individual to glaucoma (Vedder, 1925; Papirmeister et al., 1991).

The most severe eye damage is caused by liquid mustard, which may be delivered by an airborne droplet or by self-contamination (Gilchrist, 1926a). Symptoms may become evident within minutes after exposure (Warthin, 1926). Severe corneal damage with possible perforation of the cornea can occur after extensive eye exposure to liquid mustard. The patient may lose his vision, or even his eye, from panophthalmitis, particularly if drainage of the infection is blocked, such as by adherent lids (Warthin, 1926). Miosis sometimes occurs, probably due to the cholinergic activity of mustard.

During World War I, mild conjunctivitis accounted for 75% of the eye injuries; complete recovery took 1–2 weeks. Severe conjunctivitis with minimal corneal involvement, blepharospasm, edema of the lids and conjunctivae, and orange-peel roughening of the cornea accounted for 15% of the cases; recovery occurred in 2–5 weeks. Mild corneal involvement with areas of corneal erosion, superficial corneal scarring, vascularization, and iritis accounted for 10% of the cases; convalescence took 2–3 months. Lastly, severe corneal involvement with ischemic necrosis of the conjunctivae, dense corneal opacification with deep ulceration, and vascularization accounted for about 0.1% of the injuries; convalescence lasted more than 3 months. Only 1 of 1016 mustard casualties surveyed after World War I received disability payments for defective vision (Gilchrist, 1926b).

Studies conducted on rabbit eyes indicate that mustard injury to the cornea is characterized by initial degeneration of the epithelial cells, with changes ranging from nuclear swelling and nuclear vacuolization, to pyknosis and nuclear fragmentation. Epithelial loosening and sloughing occurs either by separation of the basal cells from the basement membrane, or by shearing of the cell just above its attachment to the basement membrane (Warthin and Weller, 1919; Maumenee and Scholz, 1948).

Mustard initially causes vasodilation and increased vascular permeability in the conjunctiva, which lead to progressive edema. Secretion of mucus occurs within minutes of exposure. Pyknosis of epithelial cells begins concurrently with or shortly after these changes, leading to desquamation of the epithelium. In the later stages, inflammatory infiltration of connective tissue and exudation are present (Warthin and Weller, 1919; Maumenee and Scholz, 1948). Medical personnel have reported seeing delayed keratitis in humans from as little as 8 months to 20 years after mustard exposure (Mann and Pullinger, 1944; Atkinson, 1948). This delayed keratitis, in addition to the chronic inflammation, can lead to erosions and frank ulcerations.

Within approximately 5 min, liquid mustard dropped into the eyes of rabbits was absorbed, had disappeared from the eye's surface, had passed through the cornea and the aqueous, and had produced hyperemia of the iris. Likewise, damage to other structures (e.g., Descemet's membrane) also occurred within a similar length of time (Mann and Pullinger, 1944). Decontamination must be performed immediately after liquid mustard contaminates the eye because absorption and ocular damage occur very rapidly; after a few minutes, there will be no liquid remaining on the surface of the eye to decontaminate.

3. Airways

Mustard produces dose-dependent damage to the mucosa of the respiratory tract, beginning with the upper airways, and descending to the lower airways as the amount of mustard increases. The inflammatory reaction varies from mild to severe, with necrosis of the epithelium. When fully developed, the injury is characterized by an acute inflammation of the upper and lower airways, with

discharge in the upper airway, inflammatory exudate, and pseudomembrane formation in the tracheobronchial tree. The injury develops slowly, intensifying over a period of days.

After a low-dose, single exposure, casualties might notice a variety of irritating symptoms accompanied by a dry cough; on examination, they might have pharyngeal and laryngeal erythema. Hoarseness is almost always present, and the patient often presents with a barking cough. Typically, this hoarseness may progress to a toneless voice, which appears to be particularly characteristic of mustard exposure. Patients characteristically note a sense of chest discomfort. All of these complaints typically commence approximately 4–6 h after exposure, with sinus tenderness appearing hours later. Vapor concentrations sufficient to cause these symptoms typically produce reddened eyes, photophobia, lacrimation, and blepharospasm. There may be loss of taste and smell. Patients occasionally experience mild epistaxis and sore throat. Prominent wheezing and dyspnea (shortness of breath) may be present (Buscher and Conway, 1944).

Exposures to higher concentrations of vapor result in an earlier onset and greater severity of the above effects. Hoarseness rapidly progresses to aphonia. Severe tachypnea and early radiological infiltrates may appear. More severe respiratory exposures create necrotic changes in the respiratory epithelium that result in epithelial sloughing and pseudomembrane formation. There may be substantial airway occlusion from the inflammatory debris or from pseudomembranes, which can obstruct the upper airways as they form or can break off and obstruct lower airways (Vedder, 1925; Buscher and Conway, 1944; Willems, 1989).

The initial bronchitis is nonbacterial. White blood cell elevation, fever, pulmonary infiltrates seen on radiograph, and colored secretions may all be present to mimic the changes of a bacterial process. This process is sterile during the first 3–4 days; bacterial superinfection occurs in about 4–6 days (Vedder, 1925).

Mustard has little effect on lung parenchyma. Its damage usually is confined to the airways and the tissue immediately surrounding the airways, except after an overwhelming exposure to mustard and as a terminal event (Gilchrist, 1926). These changes are most intense in the upper airways and decrease in the trachea, bronchi, and smaller bronchioles, presumably reflecting a differential disposition of vapor on the mucosal surface (Pappenheimer, 1926; Sidell et al., 1997). Pulmonary edema is not a usual feature, except in the case of hemorrhagic pulmonary edema with severe exposures, and it may occur in terminal stages (Vedder, 1925; Gilchrist, 1926).

The lungs of animals exposed to mustard show alternating areas of atelectasis and emphysema. Atelectasis is thought to be caused by the clogging of bronchioles with mucus and the emphysema is compensatory; these findings were confirmed when lungs resected at thoracotomy from Iranian casualties from the Iran–Iraq War showed similar effects (Winternitz and Finney, 1920; Willems, 1989). The lungs showed bronchiectasis and severe chronic inflammation. The bronchiectasis was due to full-thickness injury of the airways. In some casualties, this injury healed by scarring of such intensity that severe and unrelenting tracheobronchial stenosis developed.

4. Gastrointestinal Tract

Nausea and vomiting are common within the first few hours after mustard exposure, beginning at about the time the initial lesions become apparent. The early nausea and vomiting, which are generally transient and not severe, may be caused by the cholinergic activity of mustard, by a general reaction to injury, or because of the unpleasant odor (Fries and West, 1921; Somani and Babu, 1989). Nausea and vomiting occurring 24–36 h later is due to the generalized cytotoxic activity of mustard and damage to the mucosa of the gastrointestinal tract.

Diarrhea is not common, and gastrointestinal bleeding seems to be even less common. Animals that were given approximately 1 LD_{50} of mustard administered either intravenously or subcutaneously had profuse diarrhea, which was frequently bloody; however, this was unusual when mustard was administered percutaneously or by inhalation (Vedder, 1925; Houck et al., 1947). Diarrhea was more common after nitrogen mustard (Graef et al., 1948).

Diarrhea and gastrointestinal bleeding do not seem to be common in humans. None of the 107 autopsied cases had experienced diarrhea; and in the 57 cases in which the gastrointestinal tract was thoroughly examined, none had significant lesions (Pappenheimer, 1926). In several reported series of Iranian casualties, totaling about 700 casualties, few had diarrhea and only a very few who died had bloody diarrhea (Sohrabpour, 1984; Balali-Mood and Navaeian, 1986; Willems, 1989). Constipation was noted in casualties with mild exposure (Vedder, 1925).

5. Central Nervous System

Although the effects are not usually prominent clinically, mustard affects the CNS. Reports of World War I casualties described apathy, depression, intellectual dullness, and languor (Vedder, 1925). Approximately 83% of the 233 Iranian casualties sent to various western European hospitals for medical care during the Iran–Iraq War had CNS complaints; most complaints, however, were mild and nonspecific (Balali-Mood and Navaeian, 1986).

Large amounts of mustard administered to animals via the inhalational, intravenous, subcutaneous, or intramuscular routes caused hyperexcitability, abnormal muscular movements, convulsions, and other neurological manifestations (Vedder, 1925; Marshall, 1926). Animals died a ''neurological death'' a few hours after receiving a lethal amount of mustard (Graef et al., 1948). Autopsies of these animals disclosed few abnormalities (Marshall, 1926).

After three children were accidentally exposed to a large amount of mustard, two of them presented with abnormal muscular activity and the third alternated between coma and agitation. The first two children died 3–4 h after exposure, possibly from neurological mechanisms (Heully and Gruninger, 1956). It is unknown whether these CNS manifestations are from a cholinergic activity of mustard or from other mechanisms.

6. Death

Most casualties die of pulmonary damage complicated by infection bronchopneumonia, immunosuppression, and sepsis. When exposure is not by inhalation, the mechanism of death is less clear. In studies with animals in which mustard was administered via routes other than inhalational, the animals died from 3 to 7 days after the exposure; they had no signs of pulmonary damage and often had no signs of sepsis. The mechanism of death was not clear, but autopsy findings resembled those seen after radiation (Philips, 1950). Mustard is considered radiomimetic because of the delayed onset of signs and symptoms and the accompanying immunosuppression with exposures approaching an LD_{50}.

E. Diagnosis

The differential diagnosis of mustard casualties on the battlefield after a known chemical attack is not difficult. The history of a chemical attack is useful, particularly if the chemical agent is known. Simply questioning the casualty about when the pain started, whether it started immediately after the exposure or hours later, is very helpful. Pain from Lewisite (the other vesicant that causes blistering) begins seconds to minutes after exposure; pain from mustard does not begin until the lesion begins to develop hours later.

Blisters appearing simultaneously in a large number of people, in the absence of a known chemical attack, should alert medical personnel to search the area with a chemical agent detector. The appearance of one or more blisters in an individual does not alone make a diagnosis. Friction, plants, insects, and other diseases also cause blisters.

F. Laboratory Tests

No ''routine'' laboratory test for mustard exposure exists. Investigational studies have demonstrated the presence of significant amounts of thiodiglycol, a major metabolite of mustard, in the urine of mustard casualties. In two studies, Iranian casualties had higher amounts of thiodiglycol in their

urine than did control subjects (Wils et al., 1985, 1988). In a third study, the urinary thiodiglycol secreted by a laboratory worker accidentally exposed to mustard was quantitatively measured for a 2 week period (his postrecovery urine was used as a control); the half-life of thiodiglycol was 1.18 days (Jakubowski et al., 1997). In a more recent accident, thiodiglycol was also demonstrated in the patient's blister fluid. (Except for being a breakdown product from sulfur mustard, thiodiglycol is harmless.) The procedure for analysis of thiodiglycol is described in Technical Bulletin Medical 296 (U.S. Department of the Army, 1996). The procedure for handling urine samples of suspected victims is on the U.S. Army Medical Research Institute of Chemical Defense website (http://ccc.apgea.army.mil).

G. Patient Management

Decontamination within 1 or 2 min after exposure is the only established, effective means of preventing or decreasing tissue damage from mustard. This decontamination is not done by medical personnel; it must be performed by the soldier immediately after the exposure. Generally, a soldier will not seek medical help until the lesions develop hours later. By that time, skin decontamination will not help the soldier. Mustard fixes to the skin within minutes, and tissue damage will already have occurred (Renshaw, 1946).

If any mustard remains on the skin, thorough decontamination later will prevent the further spreading to other areas. After several hours, spreading will have occurred because oily substances flow on warm skin. Decontamination now, however, will prevent mustard from spreading to personnel who handle the casualty and possible contamination of medical treatment facilities (MTFs). By the time skin lesions develop, most mustard will have been absorbed and fixed to tissue. Unless the site was occluded, the remaining unabsorbed agent will have evaporated.

Mustard droplets disappear from the surface of the eye very quickly. Flushing should still be carried out as soon as possible. All mustard casualties must be thoroughly decontaminated before they enter a clean MTF. This should be done with the realization that by the time a contaminated soldier reaches an MTF, this decontamination will rarely help the casualty; it does, however, prevent exposure to medical personnel.

Mustard casualties generally fall into three categories. The first is the return to duty category. These individuals have a small area of erythema or one or more small blisters on noncritical areas of their skin; eye irritation or mild conjunctivitis; and late-onset, mild upper respiratory symptoms, such as hoarseness or throat irritation and a hacking cough. If these casualties are seen long after exposure, there is good reason to believe that the lesion will not progress significantly, they can be given symptomatic therapy and returned to duty.

The second category includes casualties who appear to have non-life-threatening injuries, but who are unable to return to duty. Casualties with the following conditions must be hospitalized for further care:

1. A large area of erythema (with or without blisters)
2. An extremely painful eye lesion or an eye lesion that hinders vision
3. Respiratory injury with moderate symptoms that include a productive cough
4. Dyspnea

Some of these conditions may develop into life-threatening injuries, and these categories, therefore, should be used only to assess a casualty's presenting condition. For example, an area of erythema caused by liquid mustard that covers 50% or more of the body surface area suggests that the individual was exposed to 2 LD_{50} of the agent. Likewise, dyspnea occurring within 4–6 h after the exposure suggests inhalation of a potentially lethal amount of mustard.

The third category comprises those casualties who appear to have life-threatening injuries when they first present at an MTF. Life-threatening injuries include large skin burns caused by liquid

mustard, and early onset of moderate-to-severe pulmonary symptoms. Some of the casualties in this category will die from their injuries.

Many mustard casualties will fall into the first category, the majority will fall into the second category, and only a very small percentage of casualties will fall into the third category. Data from World War I, in which only 3% of mustard injuries were lethal; despite the unsophisticated medical care at that time (e.g., no antibiotics, IV fluids, and electrolytes), suggest that most mustard casualties are not severely injured and that most of them will survive.

Most casualties of mustard exposure will, however, require some form of medical care—from a few days to many weeks. Eye care and airway care will promote healing within weeks; skin lesions take the longest to heal and may necessitate hospitalization for months (Willems, 1989). Casualties with mild to moderate mustard damage will need supportive care. Pain control is extremely important. Fluids and electrolytes should be carefully monitored. Although there is not as great a fluid loss from mustard burns (compared with thermal burns), a casualty will probably be dehydrated when he enters the MTF. Parenteral fluid supplements and vitamins will be of benefit. Casualties who have lost their eyesight because of mustard exposure should be reassured that they will recover their vision.

Casualties who do become critically ill from their exposure to mustard will present with large areas of burns, major pulmonary damage, and immunosuppression. Some of the casualties may die from sepsis or from overwhelming damage to the airways and lungs. Medical officers should remember, however, that even with the limited medical care available in World War I, very few deaths were caused by mustard exposure.

Casualties have been managed from different periods around the world. There are no controlled human studies comparing different treatments; nor have uniform standards of care been developed. The care required for each organ system is described in the section below. Most casualties will have more than one system involved, and many of these casualties will be dehydrated and have other injuries as well.

H. Long-Term Effects

Mustard burns may leave areas of hypopigmentation or hyperpigmentation, sometimes with scarring. Individuals who survive an acute, single mustard exposure with few or no systemic or infectious complications appear to recover fully. Previous cardiopulmonary disorders, severe or inadequately treated bronchitis or pneumonitis, a prior history of smoking, and advanced age all appear to contribute to long-term chronic bronchitis; there is no definitive way to determine whether these conditions are the result of aging, smoking, or a previous mustard exposure. Casualties with severe airway lesions may later have postrecovery scarring and stenosis, which predisposes the individual to bronchiectasis and recurrent pneumonia (Buscher and Conway, 1944).

An important late sequela of mustard inhalation is a tracheal/bronchial stenosis that necessitates bronchoscopy and other procedures (Freitag et al., 1991). Mustard has been reported to create a long-term sensitivity to smoke, dust, and similar airborne particles, probably as a result of clinically unapparent bronchospasm (Morgenstern et al., 1947; Buscher and Conway, 1994).

The relationship between mustard exposure and subsequent cancer has been the subject of much study. It seems clear that individuals who were exposed to mustard daily for long periods (e.g., workers in mustard production plants) have a slightly higher incidence of cancer of the airways, primarily the upper airways (Wada et al., 1968; Manning et al., 1981; Tokuoka et al., 1986). According to two separate reports, the association of one or two exposures on the battlefield with subsequent cancer is not clear; in a third report, the relation between mustard exposure and subsequent cancer is equivocal (Beebe, 1960; Norman, 1975; Pechura and Rall, 1993). Interested readers may consult Watson and associates' 1989 review of the mustard exposure—cancer incidence relation (Watson et al., 1989).

In 1991, the National Academy of Science appointed a committee to survey the health effects of mustard and Lewisite (Pechura and Rall, 1993). Veterans of World War II who, as subjects in test programs had been exposed to mustard and Lewisite, were presenting at Veterans Administration hospitals with complaints of illnesses that they believed were associated with these test programs. The committee was requested to survey the literature to assess the strength of association between these chemical agents and the development of specific diseases. The committee reported finding a causal relationship between exposure and various cancers and chronic diseases of the respiratory system, cancer and certain other problems of the skin, certain chronic eye conditions, psychological disorders, and sexual dysfunction. They found insufficient evidence for a causal relationship between exposure and gastrointestinal diseases, hematological diseases, neurological diseases, and cardiovascular diseases (except those resulting from infection following exposure). Some of these conclusions were not well supported. For example, there were no cases of skin cancer reported, and the alleged psychological disorders were from the trauma of exposure, not from the agent.

Over the past several years, Iranian investigators have provided a number of papers that study the late toxic effects of mustard exposure in patients that are 16–20 years postexposure from the Iran–Iraq Conflicts of the 1980s (Balali-Mood, 1986; Balali, 1992; Afshinniaz and Ghanei, 1996; Ghanei and Vosoghi, 2002; Khateri et al., 2003; Balali-Mood et al., 2005; Hefazi et al., 2005). Balali-Mood and Hefazi (2006) have summarized most of this data in a comparative review of early and late toxic effects of mustard. As a synopsis of this data, the following observations were made:

1. *Carcinogenesis*. Surviving victims of mustard exposure during the Iran–Iraq War are exhibiting carcinoma of the nasopharynx, bronchogenic carcinoma, adenocarcinoma of the stomach, as well as acute myeloblastic and lymphoblastic leukemia (Balali-Mood and Hefazi, 2006). Definitive studies of the nature and types of cancers seen in this patient population and correlations with severity of exposure have yet to be published.
2. *Chronic pulmonary disease*. In the 3 year postexposure time frame, the most severely affected patients demonstrated restrictive pulmonary disease patterns. By 16 years post-exposure, these patterns become obstructive in nature (Balali-Mood and Hefazi, 2006). At 16–20 years after exposure, the main respiratory complications were chronic obstructive pulmonary disease, bronchiectasis, asthma, large airway narrowing, and pulmonary fibrosis (Balali-Mood and Hefazi, 2006).
3. *Skin lesions*. At 16–20 years after exposure, the most common skin lesions, by order of occurrence, were hyperpigmentation, erythematous papular rash, dry skin, multiple cherry angioma, atrophy, and hyperpigmentation (Balali-Mood and Hefazi, 2006).
4. *Nervous system abnormalities*. Balali-Mood et al. (2005) conducted studies on peripheral neuropathic processes in victims exhibiting severe late manifestations of mustard poisoning using electromyography and nerve conduction velocity. Seventy percent of the patients demonstrated disturbances in the peripheral nervous system. Nerve conduction abnormalities were more common in sensory nerves and more prevalent in lower extremities than in upper extremities. Forty percent of the patients exhibited incomplete interference patterns in electromyographic studies.

III. LEWISITE

Lewisite (b-chlorovinyldichloroarsine) is an arsenical vesicant but of secondary importance in the vesicant group of agents. It was synthesized in the early twentieth century and has seen little or no battlefield use (Balali-Mood et al., 2005). Lewisite is similar to mustard in that it damages the skin, eyes, and airways; however, it differs from mustard because its clinical effects appear within seconds of exposure. An antidote, British anti-Lewisite (BAL), can ameliorate the effects of Lewisite if used soon after exposure. Lewisite has some advantages over mustard but also some disadvantages.

A. Military Use

A research team headed by U.S. Army Captain W.L. Lewis is generally credited with the synthesis of Lewisite in 1918, although German scientists had studied this material earlier (Lewis and Stiegler, 1925; Prentiss, 1937; Buscher and Conway, 1944; Harris and Paxman, 1982; Trammel, 1992). Large quantities were manufactured and shipped by the United States for use on the European battlefield; however, World War I ended while the shipment was at sea and the vessel was sunken (Prentiss, 1937; Trammel, 1992).

There has been no verified use of Lewisite on a battlefield, although Japan may have used it against China between 1937 and 1944 (Trammel, 1992). Lewisite is probably in the chemical warfare stockpile of several countries. Lewisite is sometimes mixed with mustard to lower the freezing point of mustard; Russia has this mixture (Madsen, Major, Medical Corps, U.S. Army, 1995, personal communication).

B. Properties

Pure Lewisite is an oily, colorless liquid, and impure Lewisite is amber to black in color. It has a characteristic odor of geraniums. Lewisite is much more volatile and persistent in colder climates than mustard. Lewisite remains fluid at lower temperatures, which makes it perfect for winter dispersal. Lewisite hydrolyzes rapidly, and, on a humid day, maintaining a biologically active concentration of vapor may be difficult (U.S. Department of Defense, 1990).

C. Toxicity

The toxicity of Lewisite vapor is very similar to that of mustard vapor (Houck et al., 1947; Trammel, 1992). Blister fluid from a Lewisite-caused blister is nonirritating; however, it does contain 0.8–1.3 mg/mL of arsenic and in some instances intact Lewisite or equally damaging breakdown products have been found in blister fluid (Buscher and Conway, 1944; Trammel, 1992).

D. Biochemical Mechanisms of Injury

Lewisite shares many biochemical mechanisms of injury with the other arsenical compounds. It inhibits many enzymes: in particular, those with thiol groups, such as pyruvic oxidase, alcohol dehydrogenase, succinic oxidase, hexokinase, and succinic dehydrogenase. As is true with mustard, the exact mechanism by which Lewisite damages cells has not been completely defined. Inactivation of carbohydrate metabolism, primarily because of inhibition of the pyruvate dehydrogenase complex, is thought to be a key factor (Trammel, 1992).

E. Clinical Effects

Lewisite damages skin, eyes, and airways by direct contact and has systemic effects after absorption. Unlike mustard, it does not produce immunosuppression. Data on human exposure are few. Lewisite was applied to human skin in a few studies; however, most information on its clinical effects is based on animal studies (Rovida and Lewisite 1929; Wardell, 1940; Dailey et al., 1941; Buscher and Conway, 1944).

1. Skin

Lewisite liquid or vapor produces pain or irritation within seconds to minutes after contact. Pain caused by a Lewisite lesion is much less severe than that caused by mustard lesions, and it diminishes after blisters form (Buscher and Conway, 1944).

Erythema is evident within 15–30 min after exposure to liquid Lewisite, and blisters start within several hours; these times are somewhat longer after vapor exposure. Lewisite is absorbed by the

skin within 3–5 min (compared with 20–30 min for an equal amount of mustard) and spreads over a wider area than the same amount of mustard. The Lewisite blister begins as a small blister in the center of the erythematous area and expands to include the entire inflamed area, whereas vesication from mustard begins as a ''string of pearls'' at the periphery of the lesion, small blisters that eventually merge (Buscher and Conway, 1944). Other differences between the lesions produced by these two chemical agents are as follows: the inflammatory reaction from Lewisite generally occurs much faster; the lesions from Lewisite heal much faster; secondary infection is less common after Lewisite exposure, and subsequent pigmentation is likewise less common (Buscher and Conway, 1944).

See Goldman and Dacre (1989) for a further review of Lewisite and its toxicology.

2. Eyes

A person is less likely to receive severe eye injury from Lewisite vapor than from mustard vapor because the immediate irritation and pain caused by Lewisite will produce blepharospasm, effectively preventing further exposure. A small droplet of Lewisite (0.001 mL) can cause perforation and loss of an eye (Mann et al., 1946).

In tests performed on rabbits, Lewisite caused almost immediate edema of the lids, conjunctiva, and cornea; as well as, early and severe involvement of the iris and ciliary body followed by gradual depigmentation and shrinkage of the iris stroma (Mann et al., 1946). Miosis appeared early. In this same study, miosis was not noted after mustard exposure. No long-term effects of Lewisite were noted, such as the delayed keratitis seen after mustard.

3. Airways

Lewisite vapor is extremely irritating to the nose and lower airways, causing individuals exposed to it to seek immediate protection, thus limiting further exposure. The airway lesion of Lewisite is very similar to the lesion caused by mustard exposure except that the Lewisite vapor is extremely irritating to the mucous membranes. In large amounts, Lewisite causes pulmonary edema.

After exposure to Lewisite, dogs exhibited massive nasal secretions, lacrimation, retching, vomiting, and labored respiration. These symptoms worsened until death finally occurred. On autopsy, the lungs were edematous, and a pseudomembrane often extended from the nostrils to the bronchi. Tracheal and bronchial mucosa was destroyed and the submucosa was congested and edematous. Bronchopneumonia was commonly mixed with edema (Vedder, 1925).

4. Other Effects

''Lewisite shock'' is seen after exposure to large amounts of Lewisite. This condition is the result of protein and plasma leakage from the capillaries and subsequent hemoconcentration and hypotension.

A small amount of Lewisite on the skin will cause local edema because of the effects of this agent on local capillaries. With a large amount of Lewisite, the pulmonary capillaries are also affected; there is edema at the site of exposure and pulmonary edema. With even larger amounts of Lewisite, all capillaries are affected, and proteins and plasma leak from the circulation into the periphery. Even after small amounts of Lewisite, the fluid loss can be sufficient to cause diminution of renal function and hypotension (Goldman and Dacre, 1989).

Arsines are known to cause hemolytic anemia, but there is little mention of this in reports on Lewisite exposure. A ''true or hemolytic anemia'' was noted with Lewisite shock (Goldman and Dacre, 1989).

F. Diagnosis

Lewisite exposure can be distinguished from mustard exposure by the history of pain on contact with the agent. Phosgene oxime also causes pain on contact, but phosgene oxime does not produce a

liquid-filled blister. If a single individual has an isolated blister, other plant or animal causes of vesication should be sought.

G. Laboratory Tests

There is no specific laboratory test for Lewisite. Urinary arsenic excretion might be helpful. Hemolytic anemia may be seen in Lewisite exposed patients.

H. Patient Management

Medical personnel should follow the same principles for managing Lewisite skin, eye, and airway lesions that they follow for managing mustard lesions. A specific antidote, BAL (dimercaprol), will prevent or greatly decrease the severity of skin and eye lesions if applied topically within minutes after the exposure and decontamination (however, preparations of BAL for use in the eyes and on the skin are no longer available). Given intramuscularly, BAL will reduce the severity of systemic effects. BAL binds to the arsenic of Lewisite more strongly than do tissue enzymes, thereby displacing Lewisite from the cellular receptor sites (Goldman and Dacre, 1989; Trammel, 1992).

BAL reduced the mortality in dogs when it was given within 100 min after they had inhaled a lethal amount of Lewisite (Harrison et al., 1946). Burns of the eyes from Lewisite can be prevented if BAL is applied within 2–5 min of exposure; when it was applied within 1 h after exposure, BAL prevented vesication in humans (Peters et al., 1945; Goldman and Dacre, 1989). BAL has some unpleasant side effects, including hypertension and tachycardia; the user should read the package insert.

I. Long-Term Effects

There are no data on human exposure from which to predict the long-term effects from Lewisite. There is no substantial evidence to suggest that Lewisite is carcinogenic, teratogenic, or mutagenic (Goldman and Dacre, 1989). The committee appointed by the National Academy of Science reported a causal relationship between Lewisite exposure and chronic respiratory diseases, and also that acute, severe injuries to the eye from Lewisite will persist (Pechura and Rall, 1993).

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14 Cyanides: Toxicology, Clinical Presentation, and Medical Management

Bryan Ballantyne and Harry Salem

CONTENTS

I. INTRODUCTION AND BACKGROUND

The most commonly available cyanides are hydrogen cyanide (HCN), a highly volatile liquid, and sodium, potassium, and calcium cyanides, which are solids. They are widely used industrially and commercially; examples are in manufacturing processes (includes dyes, pigments, chelating agents, various nitrile, monomers, resins, and fibers), case hardening, electroplating, extraction of precious metals, and fumigation (Homan, 1987; Ballantyne, 1988; Ballantyne and Salem, 2005). In addition, due to their rapid lethal toxicity, they have been used for suicide, homicide, judicial execution, assassinations, and chemical warfare operations, and there exists a possibility for use by terrorists (Ballantyne, 1987a, 1987b; Gee, 1987; WHO, 2004; Ballantyne et al., 2006). Additionally, cyanide-related toxicity and pathology may result from exposure to man-made and to naturally occurring cyanogens (Ballantyne, 1987b; Brimer, 1988). In addition, HCN is a product of combustion, and inhalation of smoke from fires may cause cyanide (CN) intoxication (Ballantyne, 1987c; Norris and Ballantyne, 1999; Ballantyne and Salem, 2005). As discussed later (Section XI.B), HCN has been employed by the military in chemical warfare operations because of its lethal and incapacitating effects. In addition, because of their known toxicity and comparatively ready availability, cyanides are possible candidates for use as psychological and lethal agents by terrorist organizations. This chapter discusses the experimental and human clinical toxicology of cyanides with particular reference to their potential for application as chemical warfare weapons and use by terrorists.

II. CHEMICAL IDENTITIES AND PHYSICOCHEMICAL PROPERTIES

HCN has the chemical synonyms of formonitrile, prussic acid, and hydrocyanic acid. Some physicochemical properties are compared with those of the sodium, potassium, and calcium salts in Table 14.1. Relevant to the toxicity and hazards of HCN are its liquid state at NTP, poor ionization, low molecular weight (MW), low boiling point, high vapor pressure, low vapor density (0.947 at 31°C), and hence ready diffusibility. The salts are solids that are readily soluble in water, ionize, and in spite of low vapor pressure they hydrolyze in moist conditions with the liberation of HCN, this being markedly increased under acidic conditions.

III. BIOLOGICAL MECHANISMS OF ACUTE TOXICITY

As cyanides inhibit many enzyme systems, and since other biochemical and physiological functions may be adversely affected by cyanides, the overall mechanism and presentation of CN intoxication may be complex. However, it is generally concluded that the major mechanism of the acute toxic action of CN, and which has been extensively investigated, is the inhibition of cytochrome *c* oxidase, the terminal oxidase of the respiratory chain, resulting in a cytotoxic hypoxia. CN causes intracellular hypoxia by complexing with the ferric iron of mitochondrial cytochrome *c* oxidase, inhibiting the electron transport chain and oxidative phosphorylation, resulting in anaerobic metabolism with a decrease in adenosine triphosphate (ATP) production and increased lactic acid production (Beasley and Glass, 1998). Tissues having the greatest O_2 demand are the most markedly and rapidly affected; these include brain and myocardium. CN binds with both the reduced and oxidized forms of the cytochrome a_3 component of cytochrome *c* oxidase (Antonini et al., 1971; Nicholls et al., 1972; Van Burren et al., 1972). The interaction rate of CN with oxidized enzyme is about two orders of magnitude of that for the reduced enzyme, suggesting the disruptive

TABLE 14.1
Physicochemical Properties of Hydrogen Cyanide and Its Salts

Property	HCN	NaCN	KCN	$Ca(CN)_2$
CAS number	74-90-8	143-33-9	151-50-8	592-01-8
Molecular weight	27.04	49.01	65.12	66.10
Density (g cm^{-3})	0.688 (20°C)		1.52 (16°)	
Melting point (°C)	−13.3	563	634	>350[a]
Vapor pressure (Torr)	600 (20°C)			

Source: Data from Ballantyne, B. and Salem, H., *Inhalation Toxicology*, H. Salem and S. Katz, eds, Taylor and Francis, Boca Raton, FL, 2005; Homan, E., *Clinical and Experimental Toxicology of Cyanides*, B. Ballantyne and T.C. Marrs, eds, Wright, Bristol, 1987; Lewis, R.J., *Hawley's Condensed Chemical Dictionary*, Von Nostrand Reinhold Company, New York, NY, 1993, 202, 951, 1053.

[a] Decomposes.

effect of CN on mitochondrial electron transport is at the level of reduced cytochrome a_3 (Yonetani and Ray, 1965). It is probable that CN reacts with the reduced form of cytochrome *c* oxidase, which may subsequently be converted to an oxidized enzyme–CN complex (Way, 1984). The oxidized form is relatively stable, but in the presence of reducing equivalents CN can dissociate from the enzyme–inhibitor complex with reactivation of the enzyme (Ballantyne, 1987a). The reversible nature of the inhibition is the basis for the use of certain antidotes, which reactivate the enzyme by depleting intracellular CN. Thus, moving the equilibrium of CN from the intracellular to the plasma compartment is a significant component of the antidotal effects of cyanmethemoglobin (CNMetHb) formation, chelation of CN, or conversion to thiocyanate (SCN) (see Section XX.10.3.3). As CN toxicity is mediated mainly by an intramitochondrial mechanism causing cytotoxic hypoxia, and since CN is sequestered by erythrocytes (Vesey and Wilson, 1978), it is the plasma concentration of CN that is a prime determinant of cytotoxicity (Vesey, 1976; Ballantyne, 1979, 1987a). A major pathophysiological cause for CN-induced lethality is interference with central regulatory mechanisms for breathing, and cardiotoxicity may also be a significant factor (Susuki, 1968; Ballantyne, 1977; Ballantyne and Bright, 1979).

IV. METABOLISM AND DETOXIFICATION OF CYANIDE

In mammals, the detoxification of CN is rapid; for example, detoxification rates have been estimated at 0.076 mg kg^{-1} min^{-1} for guinea pigs (Lendle, 1964) and 0.017 mg kg^{-1} min^{-1} in humans (McNamara, 1976). The biodetoxification of CN occurs through several pathways, of which the major is enzymic conversion of CN to the less acutely toxic thiocyanate (SCN), and is responsible for conversion of up to 80% of a CN dose. For example, the rat LD_{50} values for NaCN by the per oral (p.o.) and intraperitoneal (i.p.) routes are, respectively, 5.7 and 4.72 mg kg^{-1}, and the corresponding values for NaSCN are 764 and 540 mg kg^{-1}. Thus, the bioconversion of NaCN to NaSCN is associated with a decrease in acute lethal toxicity of about 120-fold in the rat (Ballantyne, 1984). SCN is excreted renally with a half-life ($t_{\frac{1}{2}}$) of 2.7 days in healthy human subjects (Schulz et al., 1979). Two enzymes responsible for this transulfuration process are thiosulfate–cyanide transulferase (EC 2.8.1.1; rhodanese) and β-mercaptopyruvate–cyanide transulferase (EC 2.8.1.2) (Lang, 1933; Sorbo, 1975; Ballantyne, 1987a). Thiosulfate–cyanide transulferase is a mitochondrial enzyme responsible for the transfer of a sulfane sulfur atom from sulfur donors to sulfur acceptors:

$$CN^- + S_2O_3^- \rightarrow SCN^- + SO_3^{2-}$$

The basic reaction involves transfer of sulfane sulfur from the donor (SCN) to the enzyme, forming a persulfide intermediate. The persulfide sulfur is transferred from the enzyme to a nucleophilic receptor (CN) to yield SCN. For most species, this enzyme activity is high in liver, kidney, brain, muscle, and olfactory mucosa (Himwich and Saunders, 1948; Dahl, 1989; Aminlari et al., 1994). The nasal metabolism of CN may have relevance to the toxicity of inhaled HCN. β-Mercaptopyruvate–cyanide transulfurases are present in blood, liver, and kidney, and catalyze the reaction:

$$HS \cdot CH_2 \cdot CO \cdot CO \cdot O^- + CN \rightarrow SCN^- + CH_3 \cdot CO \cdot CO \cdot O^-$$

Since little SCN penetrates the inner mitochondrial membrane, it is generally believed that the thiosulfate sulfurtransferase system may not be the primary detoxification mechanism for CN. A more general concept of the role of sulfur in the detoxification process is that the supply of sulfane sulfur is from a rapidly equilibrating pool of potential sulfane sulfur donors, and these may include per- and polysulfides, thiosulfanates, polythionates, inorganic SCN, and protein-associated elemental sulfur. According to this scheme, the sulfurtransferases catalyze the formation, interconversions, and reactions of compounds containing sulfane sulfur atoms (Westley, 1981; Westley et al., 1983). It is possible that sulfane sulfur is derived from mercaptopyruvate via β-mercaptopyruvate transulferase, and the various forms of sulfane sulfur are interconverted by thiosulfate sulfurtransferase. The sulfane carrier transporting the sulfur formed is albumin; the sulfur sulfane–albumin complex then reacts with CN. Pharmacokinetic studies indicate that the conversion of CN to SCN is mainly in the central compartment with a volume of distribution approximating to that of the blood volume (Way, 1984).

A third sulfurtransferase, cystathionase (cystathionine γ-lyase; EC 4.4.1.1), which is a cytosolic enzyme, may play a role in CN detoxification in the kidney and rhombencephalon (Wróbel et al., 2004). A product of the cystathionase reaction, bis(2-amino-2-carboxylethyl)trisulfide (thiocystine), may serve as a sulfur substrate donor for rhodanese. Another reaction product, 3-(thiosulpheno)-alanine (thiocysteine), may be an additional link between cystathionase and CN biodetoxification. In addition, cystathionase also functions as a sulfane sulfur carrier.

Other, quantitatively more minor, CN biodetoxification pathways are:

1. Exhalation of HCN and of CO_2 from oxidative metabolism (Boxer and Rickards, 1952; Okoh, 1983). Traces of HCN can be detected in expired air of normal humans, but this is not correlated with blood CN. Most HCN in normal breath is derived from the oxidation of SCN by salivary peroxidase in the oropharyx (Lunquist et al., 1988).
2. Reaction with cysteine to produce 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).
3. Combination of CN with hydroxocobalamin yields cyanocobalamin, which is then excreted in urine (Boxer and Rickards, 1951; Herbert, 1975).

In addition to these mechanisms, erythrocytes have a high affinity for CN, and rapidly sequester CN from plasma (Barr, 1966; Ballantyne, 1974; Vesey et al., 1976; Schulz, 1984). The sequestration of CN by erythrocytes may have a protective function in the detoxification of CN.

V. BIOCHEMICAL SEQUELAE OF CYANIDE INTOXICATION

The inhibition of cytochrome *c* oxidase, and resultant disturbance of electron transport, results in decreased mitochondrial O_2 utilization and decreased ATP (Olsen and Klein, 1947). Anaerobic metabolism leads to an accumulation of lactic acid and lactate acidosis, and the combination of lactate acidosis and cytotoxic hypoxia causes severe metabolic disturbances, particularly in the central nervous system (CNS), resulting in disturbances of perception and consciousness.

The endogenous buffering of lactate leads to a progressive fall in plasma bicarbonate. It has been shown experimentally that in the brains of CN-poisoned mice there is an increase in lactate, inorganic phosphate, and ADP, with a decrease in ATP, phosphocreatine, glycogen, and glucose (Estler, 1965; Isom et al., 1975). Rats given an intravenous (i.v.) infusion of 4 mg CN kg^{-1} min^{-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. Lactate acidosis with hyperglycemia has been noted in dogs given KCN (Klimmeck et al., 1979), and rats given NaCN (Salkowski and Penney, 1994). In rats, Isom et al. (1975) found CN increased catabolism of carbohydrate by the pentose phosphate shunt, and decreased utilization by the Embden–Meyerhof–Parnas pathway, tricarboxylic acid cycle, and the glucuronate pathway. It was suggested that increased catabolism of glucose by the pentose phosphate shunt may produce a source of NADPN that can reduce NAD by a transhydrogenase enzyme, and thus compensate for the aberrant redox state produced by CN intoxication. Such 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.

VI. DETERMINANTS FOR CYANIDE TOXICITY

Major determinants for the development of CN toxicity are the rate of accumulation and magnitude of free CN at the cellular target sites. These are achieved by the interaction of many factors, which include bioavailability, biodistribution, detoxification, and bioelimination of CN. The major factors are discussed in the following sections.

A. Implications of Detoxification

Since cyanides are both quickly absorbed and biodistributed, and due to the mechanism of action by inhibition of mitochondrial cytochrome *c* oxidase, cyanides are rapidly acting compounds in biological systems. A major determinant to both latency and severity of toxicity of CN is the balance of the quantitative rates of absorption and endogenous detoxification. The balance is usually such that, after the termination of an exposure, bioaccumulation of CN will not occur. However, during exposure the rate of availability of sulfur substrate is a determinant for the detoxification rate, and a relative decrease in sulfurtransferase detoxification may occur with an increasing available free CN. When the rate of increase in toxicologically available CN is slow, there will be a delay to both time to onset (latency) and progression of toxic effects. Within limits, this relationship between exposure dose and detoxification results in a clear dose–response relationship (Ballantyne, 1987a). With acute exposures to high dosages of CN there may be swamping of endogenous detoxification mechanisms, resulting in the rapid onset of toxicity. However, in general, the effective detoxification of CN prevents its long-term bioaccumulation. Thus, with an acute exposure to a sublethal dose of CN that produces toxic signs during the absorption phase, after exposure, and as the detoxification process continues, the signs ameliorate as CN is metabolized.

B. Rate of Cyanide Absorption

The dose absorbed through a primary absorption route depends on:

1. *Physicochemical properties of the materials*. HCN is of low MW, nonionized, and diffuses readily; in contrast, KCN has a higher MW and is ionized and hence absorbed to a lesser degree. Thus, LD_{50} values for HCN are numerically smaller than those for KCN by a given route (see Table 14.2).
2. *Exposure dose*. In particular, the exposure concentration, exposure time, and number and timing of exposures.

TABLE 14.2
Acute Lethal Toxicity of HCN and Alkali Salts Given by Various Noninhalation Routes of Exposure

Route	Cyanide	Species (Gender)	LD_{50} (95% Confidence Limits) As mg kg^{-1}	LD_{50} (95% Confidence Limits) As mmol kg^{-1}
Intravenous	HCN	Rabbit (F)	0.59 (0.55–0.65)	0.022 (0.020–0.024)
	NaCN	Rabbit (F)	1.23 (1.11–1.1.34)	0.025 (0.023–0.027)
	KCN	Rabbit (F)	1.89 (1.66–2.13)	0.029 (0.026–0.033)
Subcutaneous	NaCN	Hamster (NS)[a]	7.4	0.15
	KCN	Mouse (M)	12.0 (10.8–13.3)	0.25 (0.22–0.27)
Intramuscular	HCN	Rabbit (M)	1.50 (1.27–1.80)	0.056 (0.047–0.068)
	NaCN	Rabbit (M)	1.61 (1.38–1.83)	0.033 (0.028–0.037)
	KCN	Rabbit (M)	3.06 (2.61–3.63)	0.047 (0.040–0.056)
Intraperitoneal	HCN	Rabbit (M)	1.72 (0.85–2.00)	0.064 (0.031–0.074)
	NaCN	Rabbit (M)	2.93 (2.72–3.35)	0.060 (0.056–0.068)
	KCN	Rabbit (M)	3.60 (2.71–4.10)	0.055 (0.042–0.063)
Peroral (US)[b]	HCN	Rat (F)	4.21 (3.76–4.95)	0.156 (0.139–0.183)
(S)	HCN	Rat (F)	3.62 (3.08–3.87)	0.127 (0.114–0.143)
(US)	NaCN	Rat (F)	5.72 (5.23–7.08)	0.117 (0.107–0.144)
(S)	NaCN	Rat (F)	5.09 (4.26–5.83)	0.104 (0.087–0.119)
(US)	KCN	Rat (F)	7.49 (6.68–8.480)	0.115 (0.103–0.130)
(S)	KCN	Rat (F)	9.69 (8.60–11.30)	0.149 (0.132–0.174)
Transocular	HCN	Rabbit (F)	1.04 (0.96–1.13)	0.039 (0.036–0.040)
	NaCN	Rabbit (F)	5.06 (4.44–6.10)	0.103 (0.091–0.124)
	KCN	Rabbit (F)	7.87 (6.51–8.96)	0.121 (0.100–0.138)
Percutaneous (I)[c]	HCN	Rabbit (F)	6.89 (6.43–7.52)	0.260 (0.240–0.280)
(A)	HCN	Rabbit (F)	2.34 (2.02–2.61)	0.087 (0.077–0.097)
(I)	NaCN	Rabbit (F)	14.6 (13.8–15.4)	0.30 (0.28–0.31)
(A)	NaCN	Rabbit (F)	11.3 (9.2–12.7)	0.23 (0.19–0.26)
(I)	KCN	Rabbit (F)	22.3 (20.4–24.0)	0.343 (0.314–0.369)
(A)	KCN	Rabbit (F)	14.3 (13.3–15.1)	0.220 (0.204–0.232)

Source: Data after Ballantyne, B., *Developments in the Science and Practice of Toxicology*, A.W. Hayes, R.C. Schnell, and T.S. Miya, eds, Elsevier, Amsterdam, 1983a; Ballantyne, B., *Cutan. Ocul. Toxicol.*, 2, 119, 1983b; Ballantyne, B., *Clinical and Experimental Toxicology of Cyanides*, B. Ballantyne and T.C. Marrs, eds, Wright, Bristol, 1987a; Ballantyne, B., *Cutan. Ocul. Toxicol.*, 13, 249, 1994a.

[a] NS = not specified.
[b] US = unstarved; S = starved.
[c] I = intact skin; A = abraded skin.

3. *Route of exposure*. For example, HCN is readily absorbed across the pulmonary alveolar membrane, but skin presents a greater barrier to absorption. However, the integrity of the absorbing surface can also be a factor; thus cyanides are more readily absorbed through recently abraded skin than intact skin (Ballantyne, 1994a).

Although the absorbed dose is a primary determinant of the amount of CN available for tissue biodistribution, due to the sequestration of CN by erythrocytes it is the concentration of free (unbound) CN in plasma and tissue fluids that is the major quantitative determinant of both the latency and severity of toxicity.

C. Biodistribution of Cyanide

The biodistribution of CN to various systemic tissues will determine the relative proportions of CN present at detoxification and target tissue or cellular sites. For example, inhaled or percutaneously absorbed CN enters the systemic circulation and only a small proportion of the absorbed dose will be available for first-pass detoxification, particularly in the liver. In contrast, a high proportion of a p.o. dose will pass through the liver and be available for first-pass detoxification. However, hepatic detoxification processes may be complex, since it has been demonstrated that dietary variations that cause alterations in hepatic sulfurtransferase activity do not correlate with CN toxicity (Rutkowski et al., 1985), and extensive chemical or surgical injury to the liver does not increase the susceptibility of the mouse to CN toxicity (Rutkowski et al., 1986). The influence of route on toxicity is probably due to the relative effects of plasma transulfuration, sequestration by erythrocytes, intracellular macromolecular binding, and the differential distribution to all tissues with a detoxification capacity.

D. Miscellaneous Factors

Other factors that influence the development of CN toxicity include:

1. *Diurnal variation in toxicity*. The effect of time of dosing on the lethality of mice to i.p. KCN was investigated by Baftis et al. (1981), who used a 12 h light/dark cycle. Mortality peaked at 16 h (83% deaths) and was least at 8 h (43% deaths). There was also a circadian rhythm in time to death, with the times being shorter at 20 h and longest at 0.8 h.
2. *Age*. Fitzgerald (1954) obtained the following LD_{50} values in mice for s.c. NaCN: adult males, 5 mg kg^{-1}; adult females, 3.5–3.7 mg kg^{-1}; neonatal mice, 2.0–2.5 mg kg^{-1}.
3. *Antidotes*. The presence of antidotes will clearly influence the development toxicity. Thus, if CN is bound to methemoglobin (MetHb), chelated, or transulfurated there will be a shift in the equilibrium of CN from the intracellular to the extracellular compartment, and resultant reactivation of cytochrome *c* oxidase.

VII. ACUTE TOXICITY OF CYANIDES

A. Toxicity to Laboratory Mammals

1. Noninhalation Routes of Exposure

A comparison of typical values for the acute (single dose) lethal toxicity of solutions of cyanides by noninhalation routes is given in Table 14.2. The general order of acute lethal toxicity is HCN > NaCN > KCN, which accords with the more diffusible and unionized form of HCN. Some general comments on the various routes follow.

Intravenous Toxicity. When expressed on a mass basis (mg kg^{-1}) there are difference in LD_{50} values between HCN and its alkali salts, but on a molar basis there is no significant difference in lethal toxicity between HCN and NaCN, but KCN is statistically significantly less lethally toxic. Signs usually appear within 10–30 s and include rapid breathing, ataxia, convulsions, and coma, with death in 2–12 min (Ballantyne, 1983a). If given by slow i.v. injection, rather than bolus injection, there will be a greater proportionate detoxification activity and thus the latency to signs and lethal threshold for total dose will be raised.

Subcutaneous Toxicity. This route has been popular for investigational studies of various types, particularly to assess the effect of therapy given by other routes. Times to onset of signs are usually within minutes, and effects are similar to those seen by the i.v. route. However, as is well known with other chemicals dosed by the s.c. route, absorption may be erratic depending on the tissue stratum into which the injection is given.

Intramuscular Toxicity. On a molar basis, HCN and NaCN are both equitoxic, and more toxic than KCN. Signs appear within minutes, and include rapid breathing weak and uncoordinated movements, ataxia, tremors, convulsions, and respiratory arrest. Postmortem features are few, and include alveolar and subpleural hemorrhages, and congestion of tracheal mucosa (Ballantyne et al., 1972).

Intraperitoneal Toxicity. There are no significant differences in the i.p. acute lethal molar toxicity between HCN, NaCN, and KCN. Time to onset of signs is between 1 and 4 min, and time to death is 1–26 min.

Per Oral Toxicity. Molar toxicities for HCN, NaCN, and KCN are similar. For HCN and NaCN, lethal toxicity is somewhat greater for starved than unstarved animals, but the reverse situation is obtained with KCN (Ballantyne, 1984). Time to onset of signs is around 1–8 min and time to death from 7 to 26 min. When cyanide is given per orally the gastric environment favors the formation of HCN, which facilitates absorption. In addition, favoring the absorption of HCN across the gastrointestinal mucosa is its weak acidity, with a pK_a of 9.2.

Transocular Toxicity. Instillation of HCN and its salts into the inferior conjunctival sac results in the absorption of lethal amounts of CN. HCN is significantly more lethally toxic by this route than either NaCN or KCN. The effect of varying the physical state of local presentation of NaCN to the eye has shown that both solution (10%–20%) and solid present a similar hazard (Ballantyne, 1983b). Signs of toxicity appear in the following order: rapid breathing, weak and ataxic movements, convulsions, tonic spasms, irregular and shallow breathing, coma, and cessation of breathing. For HCN, signs develop within 30–60 s, and times to convulsions from 45 to 90 s. The corresponding times with NaCN and KCN are 2–2.5 min and 2–3 min. Times to death are 3–12 min postinstillation. Thus, following instillation of CN into the conjunctival sac, it is readily absorbed into the systemic circulation. Factors responsible for this are conjunctival hyperemia, drainage through the nasolacrimal duct to the vascular nasal mucosa, and absorption into the systemic circulation with minimal hepatic first-pass detoxification. Local irritant effects on the eye are conjunctival hyperemia and mild chemosis (Ballantyne, 1983b).

Percutaneous Toxicity. With intact skin, HCN solution is slightly more lethally toxic than NaCN, which itself is slightly more toxic than KCN. Abrading the skin enhances the percutaneous penetration of CN and increases lethal toxicity. This is particularly marked with HCN solution for which there was a threefold increase in lethal toxicity. The influence of physical state was investigated with NaCN (Ballantyne, 1994a). Contact of dry solid NaCN with intact skin did not result in the absorption of lethal amounts of CN ($LD_{50} > 200$ mg kg^{-1}). However, when dry NaCN was maintained in contact with abraded skin there was rapid absorption of CN allowing the calculation of a p.c. LD_{50} of 7.7 mg kg^{-1}, which is lower than that resulting from application of NaCN solution to intact skin (14.6 mg kg^{-1}) or to abraded skin (11.3 mg kg^{-1}). Times to onset of signs ranged widely; 5 min–1 h with HCN, and 15 min–4 h with NaCN and KCN.

Examination of LD_{50} data by different routes shows the decreasing order of lethal toxicity:

HCN: i.v. = i.m. > transocular > i.p. > p.o. > p.c.
NaCN: i.v. = i.m. > i.p. > transocular > p.o. > p.c.
KCN: i.v. > i.m. > i.p. > p.o. > transocular > p.c.

By different exposure routes, the comparative molar LD_{50} values for NaCN and KCN are

> HCN for i.v., i.m., transocular, and p.c.
= HCN for i.p. and p.o.

2. Inhalation Route of Exposure

Due to its low MW, poor ionization, and high diffusion characteristics, HCN is rapidly absorbed following inhalation. Some typical lethality data, expressed as timed LC_{50} and $L(CT)_{50}$ values, are given in Table 14.3. This data indicates that over the time period studied there is a disproportionate relation between the exposure time to produce mortality and the exposure concentration; as the lethal concentration is decreased the exposure time necessary to cause death increases, but not in proportion. The relationships between exposure time (T), exposure concentration (C), and the inhalation exposure dosage (CT) causing mortality was investigated in detail by Ballantyne (1984, 1987a, 1994b). For short exposures to HCN vapor (few seconds to few minutes), as the exposure time required to cause mortality decreases, only short increases in exposure time are needed to give 50% mortality; for example, the exposure time for a 1229 mg m^{-3} LC_{50} is 1 min, and that for a 493 mg m^{-3} LC_{50} is 5 min. This accords with the fact that under both of these conditions it is likely that there is saturation of endogenous detoxification processes. However, with exposure times in excess of 5 min proportionately longer times are required to produce decreases in the LC_{50}; for example, a 173 mg m^{-3} LC_{50} requires an exposure time of around 30 min. With higher inspired HCN concentrations there is a steep concentration gradient across the pulmonary alveolar membrane that facilitates the absorption of HCN, which enters the systemic circulation without a significant degree of first-pass hepatic detoxification and thus the rapid attainment of toxic levels. With lower inhaled HCN concentrations there is a slower rate of titration of HCN into the pulmonary circulation and thus a comparatively longer latency to onset of signs and death. Typical signs of intoxication with HCN vapor exposure are rapid breathing, weak and ataxic movements, loss of voluntary movements, convulsions, loss of consciousness, decrease in both the rate and depth of breathing, and breathing irregularities. Necropsy findings are nonspecific and generally consist of congestion of intra-abdominal viscera, pulmonary congestion, and scattered pleural and pulmonary hemorrhages (Ballantyne, 1994b). Early in an exposure to HCN vapor there may be an increase in respiratory minute volume, which results in a higher absorbed dose (Purser et al., 1984). An important feature with inhaled HCN is the development of incapacitating effects, impeding

TABLE 14.3
Acute Lethal Inhalation Toxicity of HCN Vapor to Various Species as Timed LC_{50} and $L(CT)_{50}$ Values

		Median Lethal Toxicity (95% Confidence Limits)	
Species (Gender)	**Exposure Time**	**LC_{50} (mg m^{-3})**	**$L(CT)_{50}$ (mg min m^{-3})**
Mouse (M)	30 min	176 (179–260)	5280 (3870–7880)
Rabbit (F)	45 s	2432 (2304–2532)	1824 (1728–1899)
Rabbit (F)	5 min	409 (321–458)	2044 (1603–2288)
Rabbit (F)	35 min	208 (154–276)	7283 (5408–9650)
Rat (F)	10 s	3778 (3771–4313)	631 (562–719)
Rat (F)	1 min	1129 (664–1471)	1129 (664–1471)
Rat (F)	5 min	493 (372–661)	2463 (1861–3301)
Rat (F)	30 min	173 (159–193)	5070 (4690–5497)
Rat (F)	60 min	158 (144–174)	9441 (8609–1399)

Source: Data from Ballantyne, B., *Proceedings of the Fourth Annual Chemical Defense Bioscience Review*, R.E. Lindstrom, ed, Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD, 1984; Ballantyne, B., *Clinical and Experimental Toxicology of Cyanides*, B. Ballantyne and T.C. Marrs, eds, Wright, Bristol, 1987a; Ballantyne, B., *Toxic Sub. J.*, 13, 249, 1994b; Matijak-Schaper, M. and Alarie, Y., *J. Combust. Toxicol.*, 9, 21, 1982.

mobility. For example, Purser et al. (1984) found that over a HCN concentration range of 114–175 mg m^{-3} there was a linear relation between exposure time and the time to development of hyperventilation and subsequent incapacitating effects. The slope was such that doubling the HCN vapor concentration from 112 to 224 mg m^{-3} reduced the time to incapacitation from 25 to 2 min. Rat studies estimated the HCN vapor concentration producing incapacitating effects is about 65% of the lethal concentration (Levin et al., 1987).

B. Estimates of Acute Human Toxicity

Estimates of the acute lethal p.o. toxicity of cyanides are not too precisely defined because of uncertainties in determining the exact amounts ingested and the absorbed dose. The estimated fatal oral dose for HCN is 50–100 mg as total (DuBois and Geiling, 1959) or 0.7–3.5 mg kg^{-1} (Hallstrom and Moller, 1945); for KCN a total dose of 150–250 mg has been suggested (DuBois and Geiling, 1959).

To estimate percutaneous lethal doses, Dugard and Mawdsley (1978) measured CN transport across human epidermis using a diffusion cell technique, and the data obtained allowed absorption for differing conditions; for example, 10% NaCN at pH 11.4 in contact with skin may lead to symptoms within 25 min and death within 1 h.

A limited number of industrial exposures to HCN vapor have been documented that allow an estimation of the acute lethal toxicity of HCN vapor to humans. In one case, an individual who had intensive medical management survived a 3 min exposure to approximately 560 mg m^{-3} (500 ppm) HCN (Bonsall, 1984), but in other cases 302 mg m^{-3} (270 ppm) caused immediate death, 203 mg m^{-3} (181 ppm) was lethal within 1 min, and 151 mg m^{-3} (135 ppm) was lethal after 30 min (Dudley et al., 1942). Hall and Rumack (1998) stated that inhalation of high HCN vapor concentrations (200–500 ppm) may cause abrupt loss of consciousness after one or two breaths. Based on available LD_{50} values in various species, and assuming a detoxification rate of 0.017 mg kg^{-1} min^{-1}, Moore and Gates (1946) estimated an absorbed lethal inhalation dose for man of HCN at 1.1 mg kg^{-1}. Using this approach, they calculated the timed LC_{50} values shown in Table 14.4 for a 70 kg man having a breathing rate of 25 L min^{-1}. McNamara (1976) re-evaluated the lethal inhalation toxicity of HCN vapor (Table 14.5). Based on available metabolic rate data, Hilado and Cumming (1977) suggested the following LC_{50} values for man, which are somewhat lower than those calculated by McNamara (1976): 5 min LC_{50} of 680 ppm (748 mg m^{-3}) and 30 min LC_{50} of 200 ppm (220 mg m^{-3}). According to WHO (2004), with exposure to HCN vapor concentrations in the range 120–150 mg m^{-3}, death may occur within 0.5–1.0 h, with 150 mg m^{-3} being fatal within 30 min; 200 mg m^{-3} is likely to be fatal after

TABLE 14.4
Estimated Human Acute Inhalation Toxicity of HCN Vapor Based on the Moore and Gates (1946) Analysis

	Calculated Lethal Inhalation Toxicity	
Time (min)	As LC_{50} (mg m^{-3})	As $L(CT)_{50}$ (mg min m^{-3})
1	4400	4400
3	1500	4500
10	504	5040
30	210	6300
60	140	8400

Note: Calculated for a 70 kg man with a breathing rate of 25 L min^{-1} and a detoxification rate for cyanide of 0.017 mg kg^{-1} min^{-1}.

TABLE 14.5
Estimates of the Acute Inhalation Lethal Toxicity of HCN Vapor after McNamara (1976)

Time (min)	LC_{50} (mg m^{-3})	$L(CT)_{50}$ (mg min m^{-3})
0.5	4,064	2,032
1	3,404	3,404
3	1,466	4,400
10	607	6,072
30	688	20,632

Note: Listed as $L(CT)_{50}$ and timed LC_{50} values.

10 min, and 300 mg m^{-3} can be immediately fatal. Overall, the various estimates for the human acute lethal inhalation toxicity of HCN vapor suggest that for an exposure period of 5–10 min a concentration of 500–600 mg m^{-3} (455–546 ppm) would be fatal, but for a 1 min exposure the HCN vapor concentration would require to be around 4000 mg m^{-3} (3640 ppm).

Available evidence indicates that HCN vapor can be absorbed across the skin and thus contribute additively to that resulting from respiratory exposure. Fairley et al. (1934), using guinea pigs and rabbits, demonstrated that exposure to HCN vapor resulted in p.c. absorption sufficient to cause signs of toxicity, and with sustained exposure to cause mortality. Reports of occupationally exposed workers indicate that symptomatic p.c. absorption of HCN can occur in humans. For example, employees wearing respiratory protective equipment who were working for 8–10 min in an atmosphere containing about 20,000 ppm HCN vapor (22,400 mg m^{-3}) developed dizziness, weakness, and headache (Drinker, 1932). Two reports of intoxication due to p.c. HCN vapor absorption, one in a fire fighter wearing self-contained breathing apparatus, were described by Steffens (2003). Using diffusion cells, Dugard (1987) studied the in vitro absorption of CN through human skin from solutions of NaCN and HCN vapor. His findings suggest that the rate of absorption of HCN is proportional to the atmospheric concentration of HCN. He calculated that contact of the total body surface (18,500 cm^2 for a 70 kg individual) with 1 ppm HCN vapor can result in the absorption of 32 μg CN h^{-1}.

VIII. GENERAL CONSIDERATIONS ON REPEATED EXPOSUE TOXICITY

Several studies have been undertaken to assess the general (overall) toxic effects of CN in various species. These are considered briefly below, and have been discussed in detail in Ballantyne and Salem (2005).

No significant effects were found for signs, food consumption, body weight, hematology, or histology in dogs fed with NaCN in the diet (2.5 mg kg^{-1}) for 30–32 days (American Cyanamid, 1959). Dogs dosed p.o. with NaCN (up to 6 mg kg^{-1} day^{-1}) for 15 months had an immediate onset of signs with increased erythrocyte count, decreased blood albumin, and degenerative changes in cerebrocortical neurones and cerebellar Purkinje cells (Herrting et al., 1960). Baboons dosed with KCN (1 mg kg^{-1}) for 9 months developed decreased Hb and mean corpuscular Hb concentrations (Crampton et al., 1979). Male rats dosed with KCN in drinking water for 15 days demonstrated dose-related increases in cytoplasmic resorption vacuoles in the thyroid follicular colloid, but no effects on serum T_3 or T_4. There were hepatocellular cytoplasmic vacuolation and degenerative changes, and vacuolation of renal proximal tubular epithelial cells (Sousa et al., 2002). Feeding KCN in the diet of rabbits (702 ppm) for 40 weeks resulted in increased lactic dehydrogenase activities in serum, liver, and kidney, consistent with a shift to anaerobic metabolism (Okolie and

Osagie, 1999). Increases in serum and decreases in liver sorbitol dehydrogenase, alkaline phosphatase, and glutamate–pyruvate transaminase suggested hepatotoxic lesions, and decreased renal alkaline phosphatase with increased serum activity accompanied by increased serum urea and creatinine suggested nephrotoxicity.

Histopathologically, there were foci of liver, renal tubular, and glomerular necrosis. It was also demonstrated (Okolie and Osagie, 2000) that aspartate transaminase activity in heart and serum was unaffected, but cardiac and pulmonary alkaline phosphatase was decreased. Histology was normal in pancreas and myocardium, but lungs had foci of edema and necrosis. These findings indicate long-term p.o. dosing with CN causes hepatorenal and systemic pulmonary injury, but not cardiac or pancreatic toxicity. In a multispecies (rat, pig, and goat) subchronic p.o. study with KCN, Soto-Blanco et al. (2001) did not find any biochemical or histological evidence for pancreatic exocrine or endocrine toxicity. In a 13 week study, rats and mice received up to 300 ppm in drinking water (Hébert, 1993), which resulted in a slight reduction in cauda epididymal weights (rats and mice) and reduced numbers of spermatid heads per testis (rats). Rats dosed with up to 500 ppm KCN in drinking water had dose-related decreases in hepatic and cardiac respiration and in cardiac, hepatic, and cerebral ATP concentration (Rickwood, 1987). These findings accord with CN producing mitochondrial dysfunction.

IX. SPECIFIC ORGAN, TISSUE, AND FUNCTIONAL END-POINT TOXICITY

Certain specific organ, tissue, and functional end-point toxic effects may be a direct consequence of CN exposure at the target site; others are due to SCN metabolite exposure, and others are the result of pathophysiological consequences resulting from CN toxic effects. The more relevant of these for hazard assessment and clinical purposes are discussed briefly in the following sections.

A. Neurotoxicity

Studies in laboratory animals, in vitro models, and clinical investigations on exposed humans have demonstrated a neurotoxic potential for CN, notably for CNS morphological and functional effects; these include convulsions, loss of consciousness, alteration of perception and central control functions, and longer-term degenerative neuropathology. Some experimental and human aspects are briefly summarized below. The neurotoxicity of CN is reviewed in detail by Ballantyne and Salem (2005) and Ballantyne et al. (2006).

Experimental studies have demonstrated that in CN intoxication the measured CN concentrations are high in brain (Ballantyne, 1974, 1975; Ballantyne and Bright, 1979). CN rapidly equilibrates across the neuronal plasma membrane and then accumulates in the mitochondria and membrane elements of the neurone (Borowitz et al., 1994). Some investigators have stressed the contribution of cytochrome *c* oxidase inhibition to neurotoxicity. For example, CN produces a highly significant inhibition in cerebral cytochrome oxidase activity (Ballantyne, 1977; Ballantyne and Bright, 1979). Rabbit brain homogenate has a measured I_{50} for CN-inhibited cytochrome *c* oxidase of 6.38 μM and a calculated pI_{50} of 5.20 (Ballantyne, 1977). In vivo measurement of cytochrome *c* oxidase activity by reflectance spectrometry showed that in the cerebral cortex of rats given sublethal doses of CN there was a noncumulative, transient, and dose-related inhibition of the respiratory chain (Piantadosi et al., 1983). The decrease in mitochondrial activity was accompanied by increases in regional cerebral HbO_2 saturation and blood volume. Further studies (Piantadosi and Silvia, 1986) demonstrated a dose-related suppression of electroencephalographic (EEG) activity with isoelectric conditions occurring at 50% reduction in cytochrome *c* oxidase activity. Pretreatment with sodium thiosulfate resulted in a fourfold protection of cerebral cytochrome *c* oxidase activity from CN-induced redox changes.

Cerebral enzyme systems other than cytochrome *c* oxidase may contribute to central neurotoxic effects. For example, inhibition of glutamate decarboxylase results in depletion of the inhibitory

neurotransmitter γ-aminobutyric acid (GABA), which predisposes to convulsions (Tursky and Sajter, 1962). NaCN given i.p. (5–20 mg kg^{-1}) increased glutamic acid concentrations in the cerebellum, striatum, and hippocampus, but higher doses decreased both GABA and glutamic acid (Perrson et al., 1985). Cassel et al. (1991) demonstrated that decreased GABA was associated with increased susceptibility to convulsions, and Yamamoto (1990) demonstrated a 31% decrease in GABA in KCN-dosed mice exhibiting convulsions. Decrease in GABA and the associated convulsions were abolished by dosing with α-ketoglutarate.

Patel et al. (1992) showed that NaCN-mediated cytotoxicity in hippocampal cultures was mediated mainly by activation of *N*-methyl-D-aspartate (NMDA) receptors. In addition, Yamamoto and Tang (1998) found that exposure of cerebrocortical neurones to KCN or NMDA increased lactate dehydrogenase efflux into extracellular fluid, which was blocked by coexposure to 2-amino-7-phosphonoheptanoic acid (selective inhibitor of NMDA), melatonin (OH and peroxyl scavenger), or NG-nitro-L-arginine (nitric oxide synthase inhibitor). These findings suggest that activation of NMDA receptors and nitric oxide synthase and free radical formation may contribute to CN- or NMDA-induced neurotoxicity. Yamamoto and Tang (1996a) found the incidence of CN-induced convulsions was reduced by preinjection into the cerebral ventricles of carbatapentane (a glutamate release inhibitor) or s.c. melatonin. Predosing with melatonin abolished cerebral lipid peroxidation (Yamamoto and Tang, 1996b). An increased peroxidized lipid in rat brain cortex slices following incubation with KCN was prevented by omission of Ca^{++} from the incubating medium or treatment with the Ca^{++}-channel blocker diltiazem. The findings suggest that both free radical formation and increased glutamate release may contribute to CN-induced neurotoxicity.

Cassel and Persson (1985) demonstrated that NaCN caused dose-related decreases in rat striatum dopamine (DA), and Kanthasamy et al. (1994) noted that mice dosed with KCN developed central dopaminergic toxicity. Additionally, Kiuchi et al. (1992) showed that perfusion of the striatum with NaCN produced a transient increase in DA release associated with depletion of ATP. In severe CN intoxication there is decreased dopaminergic activity in the nigrostriatal area (Rosenberg et al., 1989), and lethal CN doses rapidly decrease striatal DA and increase l-dihydroxyphenylalanine (DOPA) (Cassel and Persson, 1992). Although high CN doses decrease striatal DA and produce neurone damage, low CN doses increase striatal DA but without significant neuronal cytotoxicity (Cassel, 1995).

In isolated cellular preparations, ionic disruption results in marked cellular acidosis and accumulation of cytosolic Ca^{++} (Li and White, 1977; Nieminen et al., 1988), and this may result in disturbance of Ca^{++}-activated lipolytic enzyme activity, peroxidation of membrane phospholipids, changes in transmitter release, and effects on other Ca^{++}-modulating cell-signaling systems. Johnson et al. (1986) found that CN increased whole brain Ca^{++}, and demonstrated that centrally mediated tremors were correlated with changes in brain Ca^{++}; diltiazem prevented the increase in Ca^{++} and decreased tremors. These and other findings indicate that Ca^{++} may have a significant effect on CN-mediated neurotoxicity. Several other mechanisms have been implicated in CN neurotoxicity. These include increased blood ammonia and brain amino acids (Yamamoto, 1989, 1993); involvement of caspase-3-like activity (Gunaseker et al., 1999); and uncoupling protein-2 activity (Li et al., 2005).

A number of animal studies have provided evidence that acute and repeated exposure to CN produces CNS neuropathological changes. These include gray matter necrosis in dogs (Haymaker et al., 1952); degenerative changes in ganglion cells and Purkinje cells in dogs (Herrting et al., 1960); degenerative changes in cerebrocortical neurones and cerebellar Purkinje cells in rats (Smith et al., 1963); necrosis of the optic nerve and corpus callosum (Hirano et al., 1967; Lessel, 1971); and leucoencephalopathy (Brierley et al., 1977; Funata et al., 1984).

Several human case reports have described the neurological and neuropathological sequelae of acute CN poisoning. Some representative examples of acute and delayed effects are as follows. Finelli (1981) described a 30 year old male who attempted suicide with CN, and 14 months later developed choreiform movements and dysdiadochokinesis of the left hand. Sixteen years after the

incident mental status was normal but he was mildly dysarthric, had limb decreased muscle tone, and mild athetoid movements in the upper limbs. Computerized axial tomography (CAT) showed bilateral infarction of the globus pallidus and left cerebellar hemisphere. Utti et al. (1985) described an 18 year old male who swallowed KCN, and after 4 months developed generalized rigidity and bradykinesia, with intermittent resting and postural tremor in the arms. Autopsy (after an overdose of imipramine and alcohol) at 18 months after the original poisoning incident revealed lesions in the globus pallidus and widespread lacunae in the striatum. Varnell et al. (1987) described two cases of lethal CN poisoning due to ingestion of adulterated Excedrin capsules. CAT scan carried out within 3 h showed diffuse cerebral edema, with diffuse loss of white-gray discrimination. Borgohain et al. (1955) described a 27 year old female who attempted suicide with KCN and subsequently developed persistent generalized dystonia. Cranial CAT demonstrated bilateral putaminal lucencies, and magnetic resonance imaging (MRI) showed sharply delineated lesions corresponding to the two putamina. Grandas et al. (1989) described a man who became comatose after ingesting NaCN, and after regaining consciousness he had reduced speech and loss of balance. During the subsequent years, dystonia and Parkinsonism developed, and CAT revealed lucencies in the putamen and external globus pallidus. Feldman and Feldman (1990) described a 28 year old man who swallowed KCN and subsequently developed Parkinsonian signs, including micrographia and hypersalivation. Lovecchio et al. (2006) described cranial CAT scan findings in a 43 year old man who swallowed CN in a suicide attempt. He collapsed, had a grand mal seizure, became apnoeic, and died within 24 h in spite of sodium nitrite/thiosulphate treatment; blood CN was 167 $\mu g\ dL^{-1}$. The CAT scan showed diffuse hypodensity involving the brain and cerebellum with a small fourth ventricle, cisternal space effacement, and abnormal density of the basal ganglia. The authors regarded the findings as consistent with diffuse cerebral and cerebellar edema with impending brainstem herniation. Further details and additional case reports have been given by Ballantyne and Salem (2005) and Hantson and Duprez (2006).

B. Cardiotoxicity

In experimental acute CN toxicity, high concentrations of CN are found in myocardium (Ballantyne, 1983a, 1984), and there is a significant inhibition of cytochrome oxidase (Ballantyne and Bright, 1979). Direct evidence for cardiotoxic and pathophysiological effects on the myocardium comes from morphological, biochemical, and physiological studies in animals and from human clinical observations (Ballantyne and Salem, 2005; Ballantyne et al., 2005). Several investigators have discussed ECG changes (Leimdorfer, 1950; Susuki, 1968). Ultrastructural changes in cardiac myocytes have been seen following KCN (Susuki, 1968). O'Flaherty and Thomas (1982) found an increase in cardiospecific creatinine phosphokinase following exposure to HCN vapor. Studies by Baskin et al. (1987) suggested that CN exerts an initial response on β-adrenergic receptors and reduces myocardial contractility through inhibition of cytochrome oxidase.

C. Vascular Toxicity

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). Robinson et al. (1985b) found that oubain and verapamil enhanced aortic strip contractions, but atropine, pyrilamine, 2-bromolysergic acid diethylamide, and phentolamine did not alter contractions. In contrast, 4,4′-diisothiocyano-2,2′-stilbenedisulphonic acid (DIDS) or chlorpromazine partially reduced strip contractions. These findings may have the following relevance: (1) CN-induced vascular contractions are probably not a consequence of stimulation of muscarinic, serotoninergic, or α-adrenergic receptors and (2) if effects on the coronary arteries are similar to those on the aorta, then hypoxia-induced depolarization could enhance CN-induced coronary artery vasoconstriction. Paulet (1961) found evidence in animal studies for cardiac failure in acute CN intoxication, and implicated the

following major factors: direct myocardial toxicity, central vagal stimulation, and inhibition of central sympathetic activity. Using intra-aortic injections of NaCN in dogs, Krasney (1971) found that CN produced abrupt increases in cardiac output, cardiac rate, and arterial blood pressure, but systemic vascular resistance was unchanged. These and other findings (Krasney et al., 1966; Krasney, 1970) suggest that the major sites for circulatory responses to CN are outside the sinoaortic reflexogenic zone, and probably lie in the CNS.

D. Respiratory Effects

Typical early respiratory signs in acute CN intoxication are tachypnea and hyperpnea, resulting in an increased tidal volume; this clearly may increase the inhaled dose of HCN vapor. This is believed to result from stimulation of aortic and carotid chemoreceptors following the accumulation of acid metabolites (Comroe, 1974). Glomus cells, secretory cells in apposition with afferent nerve endings in chemoreceptor zones, probably play a role in the hypoxic transduction process (Verna et al., 1975; Ponte and Sadler, 1989). Additionally, several studies such as infusion of CN into the upper aorta indicate that in addition to the aorticocarotid chemoreceptor stimulation, CN may stimulate ventilation by other mechanisms (Levine, 1975). Depression of respiration by CN may be mediated through the brain, notably the ventral medulla. 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 (Haxhiu et al., 1993). The intermediate ventral medullary areas receive inhibitory influences from carotid chemoreceptors (Carroll et al., 1996).

E. Thyroid Gland Toxicity

Animal studies and clinical observations on occupationally exposed workers indicate that CN, or its detoxification product, SCN, may adversely affect thyroid gland function. Most animal studies in which CN is given by the p.o. route show an increase in resorption vacuoles in follicular colloid and sparse colloid, but reported changes in thyroid hormone levels are variable (Philbrick et al., 1979; Kamalu and Agharanya, 1991; Sousa et al., 2002). Several studies in occupationally exposed humans suggest that CN exposure results in impaired thyroid gland function (Blanc et al., 1985; Banerjee et al., 1997). Some investigators believe the effects are mediated by SCN, which inhibits both the uptake and utilization of iodine by the thyroid gland (Ermans et al., 1972; Fukayma et al., 1992).

F. Developmental and Reproductive Toxicity

Relatively few studies have been conducted. Developmental studies have indicated that CN causes maternal toxicity, is embryofetotoxic and teratogenic (Doherty et al., 1982; Singh, 1982; Frakes et al., 1986). The limited numbers of reproductive studies that have been undertaken suggest that CN does not produce significant effects on reproductive performance (Tewe and Maner, 1981a, 1981b; Hébert, 1993).

G. Genotoxicity

There is limited information on genetic toxicology studies of CN. In most studies, CN does not cause reverse mutations in *Salmonella typhimurium* with and without metabolic activation (DeFlora, 1981; Hébert, 1993), and only one study has shown HCN to be marginally mutagenic in *S. typhimurium* strain TA100 (Kushi et al., 1983). DNA repair test in *Escherichia coli* WP67, CM871, and WP2 with KCN was negative (DeFlora et al., 1984). KCN induced both time- and dose-dependent DNA fragmentation with cytotoxicity in rat thymocytes in vitro. CN also induced DNA damage in hamster kidney cells (BHK-21) in vitro, but unlike thymocytes, internucleosome

DNA fragmentation was not observed (Bhattacharya and Rao, 1997). The pathogenesis of DNA fragmentation was studied using an A549 human epithelial-like lung carcinoma cell line treated with KCN (Vock et al., 1998). Induction of double-strand breaks by KCN was only observed after cell viability was reduced to less than 60%, indicating that double-strand breaks were the result of extragenomic damage, as a secondary effect of high toxicity combined with cytolethality. CN did not induce DNA strand breaks in a culture of mouse lymphoma cells (Garberg et al., 1998).

H. Oncogenicity

In a chronic study with rats given diets containing 0.07 or 0.09 mg HCN kg^{-1} for 104 weeks, there were no effects on growth rate and no histopathological findings (Howard and Hanzal, 1955). Rats fed diets containing 1.5 g KCN kg^{-1} for 11.5 months (~30 mg kg^{-1} day^{-1}) did not show any oncogenic pathology (Philbrick et al., 1979).

X. CLINICAL TOXICOLOGY OF HUMAN CYANIDE POISONING

In general medical practice, acute CN intoxication is a rare, potentially fatal, but treatable condition. It may be encountered in several specific situations: these include smoke inhalation, occupational exposure to cyanides, or metabolic release following systemic absorption of laetrile, amygdalin, or cyanogenic glycosides of plant origin and aliphatic nitriles (Hall et al., 1986; Ballantyne, 1987c; Geller et al., 1991; Meyer et al., 1991; Beasley and Glass, 1998). Sodium nitroprusside therapy may also result in CN intoxication (Hall and Rumack, 1987).

A. Clinical Presentation

The initial signs and symptoms are generally nonspecific. Their latency to onset, severity, number present, and sequence of appearance depend on a number of factors, which include: route of exposure, exposure concentration (dose), duration of exposure, physical mode of presentation of CN, rate of absorption and total absorbed dose (Ballantyne and Salem, 2005; Ballantyne et al., 2006). If exposure is to low concentrations (doses) then there may be a slow onset and progressive sequential appearance of signs and symptoms. In contrast, if there is massive exposure then collapse is usually rapid in onset and death may follow promptly (Ballantyne and Salem, 2005). Overall, symptoms that may be encountered include weakness, fatigue, headache, anxiety, restlessness, palpitations confusion, dizziness, vertigo, dyspnea, nausea, nasal irritation (respiratory exposure), and precordial pain. Physical signs may include an initial increase in breathing rate and depth (later becomes slow and gasping), vomiting, diarrhea, facial flushing, transient hypertension followed by hypotension, tachycardia followed by bradycardia, cardiovascular collapse, epistaxis, generalized convulsions, loss of consciousness, urinary and fecal incontinence, cyanosis, areflexia, mydriasis, sluggish or unreactive pupils, apnea, decreased A-V O_2 difference (visible on retinoscopy), non-cardiogenic pulmonary edema, decerebrate rigidity, and cardiac arrest. Due to the mechanism of toxicity by cytochrome *c* oxidase inhibition causing a cytotoxic hypoxia, cyanosis is not usually a presenting sign. However, if present, it indicates that a stage of apnea and circulatory collapse has been reached.

Initial symptoms and signs are generally nonspecific and may include CNS stimulation (headache, giddiness, and anxiety), hyperpnea, slight hypertension, and palpitations. These early signs may be confused with hyperventilation or simple anxiety. In contrast, massive exposure doses may cause rapid collapse and prompt onset of convulsion and coma, and rapid death.

The odor of HCN, likened to that of bitter almonds, has been stated to be an important clinical clue in the recognition of acute CN poisoning. A detection range of 0.5–5.0 ppm has been suggested (Kulig and Ballantyne, 1993). However, it needs to be noted that some individuals are not able to detect the odor of HCN by olfaction. This CN anosmia, present in 2%–45% of different ethnic

populations, is probably a genetically determined trait (see detailed discussion in Ballantyne and Salem [2005] and Ballantyne et al. [2005]).

Late complications of acute CN poisoning may include acute renal failure (Mégarbane and Baud, 2003), rhabdomyolysis (Brivet et al., 1983), CNS degenerative changes and diffuse cerebral edema (Fligner et al., 1987; Varnell et al., 1987), and neuropsychiatric manifestations including paranoid psychosis (Kales et al., 1997).

B. Investigation and Confirmation of Poisoning

Confirmation of suspected acute CN poisoning should include the following investigations: ECG, plasma lactate, serum electrolytes (with calculation of anion gap), blood glucose, arterial ketone body ratio (AKBR), pulse oximetry, arterial blood gas analysis, chest radiography, and blood CN analysis. Morphological neuroimaging may assist in the early detection of central neurotoxicity (Hantson and Duprez, 2006), with MRI giving better definition than CAT scan.

The ECG may show increased T-wave amplitude, shortening of the S-T segment, third-degree heart block, supraventricular or ventricular tachycardias, A-V block, ischemic myocardial changes (DeBush and Seidel, 1969; Lee-Jones et al., 1970; Ballantyne et al., 2006). Plain chest radiography may demonstrate pulmonary edema.

Lactate acidosis is an important biochemical feature of acute CN intoxication, and if marked is accompanied by an elevated anion gap (Graham et al., 1977; Baud et al., 1991; LaPostolle et al., 2006; Peddy et al., 2006). The AKBR (acetoacetate/β-hydroxybutyrate), which reflects the redox state of hepatic mitochondria, is a useful measure for the progress of treatment of CN poisoning (Nakatani et al., 1993). Arterial blood O_2 analysis and A-V O_2 differences often demonstrate high arterial blood PO_2, increased venous blood PO_2, and reduced A-V O_2 difference. Although most clinical pathology laboratories do not have the capability to undertake rapid quantitative analysis for CN, blood samples should be collected into tightly closed tubes for subsequent analysis. Blood should be collected as soon as possible after intoxication and analyzed promptly to reduce potential artifacts (Ballantyne, 1975, 1976, 1987b; Kulig and Ballantyne, 1993).

C. Management of Acute Cyanide Poisoning

Acute CN intoxication is an acute medical emergency. It is important that those who may encounter a suspect case should seek appropriate advice and guidance, ideally initially from the regional Poison Control Center, who may refer the enquirer to a specialist unit for diagnostic criteria and appropriate treatment, including the necessary antidotal treatment.

1. First-Aid Measures

First aid should be conducted by appropriately trained personnel who have the necessary background knowledge to understand the basis for primary care. Treating first-aid and medical staff should wear protective equipment; ideally, skin protection (including impermeable gloves), and an absorbent filter or, preferably, air supplied self-contained positive pressure breathing (Ballantyne and Salem, 2005). The first aider should ensure that the affected individual is decontaminated (water flushing of skin) and transferred to a clean environment. Contaminated clothing should be kept in isolated double plastic bags. If breathing has stopped or is labored, then artificial ventilation may be required; Holger-Nielsen method or using a mask with manual ventilation. To avoid secondary intoxication, mouth-to-mouth ventilation should not be used. If cyanide has been swallowed, a dose of activated charcoal (1 g kg^{-1}) may be useful. If breathing is difficult, cylinder O_2 should be supplied by mask. If ampules of amyl nitrite are available, and the subject is breathing, a vial should be broken and placed under the nose; this may be repeated every 3–5 min if necessary (details in Ballantyne and Salem [2005]). The vasogenic effect of amyl nitrite may be a factor in its antidotal effect. External cardiac massage may be required if cardiac arrest occurs.

2. General Medical Management

A physician should supervise and ensure the following supportive medical management procedures are conducted:

1. A large bore i.v. is inserted for therapeutic purposes.
2. There is a patent airway, and ventilation is adequate. This may necessitate employing mechanical assisted ventilation.
3. Clinical experience indicates that the use of O_2 may be a valuable adjunct to treatment. Normobaric O_2 alone may have minimal effect, but it acts synergistically with other antidotes (Holland and Koslowski, 1986; Litowitz, 1987; Kulig and Ballantyne, 1993; Beasley and Glass, 1998). It is not certain if hyperbaric oxygen (HBO) offers any clinical advantage over normobaric O_2 (Gorman, 1989; Kulig and Ballantyne, 1993; Salkowski and Penney, 1994), but when antidotal treatment is refractory HBO should be considered as a treatment option (Goodhart, 1994). The AKBR is a useful measure of the efficacy of treatment (Nakatani et al., 1993); it is closely correlated with electron transport and O_2 utilization. During recovery from CN intoxication, as the P_vO_2 decreases the AKBR increases.
4. It is important to reverse the acid–base imbalance of lactate acidosis by the i.v. infusion of bicarbonate.
5. Cardiovascular complications may require the administration of atropine, i.v. fluids, and vasopressors.
6. In addition to periodic monitoring of physical signs, blood pressure, and serial measurements of blood chemistry and arterial blood gas analyses, there should be continual monitoring of the ECG and pulse oximetry. However, pulse oximetry may be unreliable following MetHb-inducing antidotes.

3. Antidotes

Acute CN poisoning is one of a few chemical-induced intoxications for which specific antidotes are available. Indeed a large number of antidotes of differencing structure and mode of action have been studied, although only a relatively few have been approved for clinical use. Since there is variability in the efficacy of different antidotes, and since some may have a high risk: benefit ratio, a decision as to what antidote is to be used should only be taken by a general or emergency room-treating physician after consultation with a Poison Control Center. The major antidotes that have been used for the antidotal treatment of human cases of acute CN poisoning are listed in Table 14.6, and briefly reviewed below. Detailed discussion on the antidotal treatment are to be found in Marrs (1987, 1988), Meredith et al. (1993), Mégarbane and Baud (2003), Mégarbane et al. (2003), Gracia and Shepherd (2004), Ballantyne and Salem (2005), and Ballantyne et al. (2005).

Sulfur Donors. These enhance the endogenous sulfurtransferase mechanisms for the detoxification of CN to SCN. Sodium thiosulfate is often used in combination with other antidotes having different modes of antidotal action; for example, with sodium nitrite or 4-dimethylaminophenol (4-DMAP). As a generalization, sodium thiosulfate is used as a supplementary treatment on the basis that it is slow acting, possibly due to limited penetration into mitochondria.

MetHb Generators. These are indirectly acting CN antidotes that cause the formation of MetHb that binds, and sequesters, CN as CNMetHb. MetHb generators include sodium nitrite, 4-DMAP, and *p*-aminopropiophenone (PAPP). A disadvantage of MetHb generation is the impairment of O_2 transport, and a drawback of nitrite MetHb generators is their adverse effects on the cardiovascular system due to induced vasodilation and hypotension. MetHb levels should be kept below 40%. The MetHb generator 4-DMAP probably acts more rapidly in MetHb generation than sodium nitrite (Weger, 1983), but PAPP is slower in antidotal action than 4-DMAP, although both have been experimentally shown to be effective as CN antidotes (Bright and Marrs, 1987).

TABLE 14.6
Antidotes Used Clinically in the Treatment of Human Acute Cyanide Poisoning

Antidote	Mode of Antidotal Action
Sodium thiosulfate	Enhancer of endogenous transulferase activity
Stroma-free MetHb[a]	Direct formation of CNmetHb
Sodium nitrite	MetHb generator
4-Dimethylaminophenol	MetHb generator
Dicobalt edetate	Direct binding agent
Hydroxocobalamin	Binding agent with formation of cyanocobalamin

[a] MetHb = methemoglobin.

Cobalt Compounds. These act as direct CN-binding agents. Dicobalt edetate has been used in some countries, but there have been reports of severe adverse effects: these have included vomiting, facial edema, urticaria, collapse, chest pains, anaphylactic shock, hypotension, cardiac arrhythmias, and convulsions (Hilmann et al., 1974; Naughton, 1974; Tyrer, 1981). It has been recommended that dicobalt edetate should be used only with clearly established cases of CN poisoning, and then with caution (Pontal et al., 1982; Meredith et al., 1993). Hydroxocobalamin binds to CN to form cyanocobalamin, and does not interfere with O_2 transport. Its antidotal efficacy has been confirmed based on animal studies and clinical experience with human acute CN intoxication (Borron and Baud, 1996; Borron et al., 2006; Dart, 2006; Froyshov et al., 2006). The recommended use of hydroxocobalamin is based on its demonstrated antidotal action and on its low toxicity (Pontal et al., 1982; Borron and Baud, 1996). Studies with human volunteers together with postmarketing experience in CN-exposed patients indicate a high-safety profile for hydroxocobalamin (Forsyth et al., 1993; Borron et al., 2005; Forin et al., 2006; Uhl et al., 2006). A few cases of acute allergic reaction have been reported (Branco-Ferreira et al., 1997; Vidal and Lorenzo, 1998; Heyworth-Smith and Hogan, 2002; Uhl, 2006). In some countries, formulations containing several grams of hydroxocobalamin are available. The usual adult antidotal dose is 5 g, and the pediatric dose is 70 mg kg^{-1} (Ballantyne et al., 2006).

We recommend that the treatment of choice based on efficacy and low therapeutic risk should be hydroxocobalamin, which may be used in combination with sodium thiosulfate. On a global basis and for emergency mass use, we stress the need for an international agreement on the management of CN poisoning, particularly with the antidote of choice for stockpiling. The criteria for choice of an anti-CN antidote have been given by Ballantyne et al. (2006).

XI. CYANIDES IN MILITARY AND POTENTIAL TERRORIST SETTINGS

A. Chemical Warfare Considerations

A standard military classification of CW agents refers to cyanides as lethal blood agents; HCN is coded AC (HMSO, 1987; Maynard, 1999). In the context of CW operational situations, cyanide is likely to be dispersed atmospherically and would be used for either low-concentration mental and physical incapacitations or high-concentration lethal objectives. For the former, it is well known that exposure to HCN vapor produces a disturbance of consciousness and perception, and combined with muscle weakness and ataxia would cause mental and physical incapacitation of troops and a

resultant reduction in their ability to conduct military tasks. As a battlefield lethal agent, HCN has been regarded as being of potential strategic usefulness because it exists as a colorless vapor with effects that have a short latency to onset. As noted above, the concentration required to produce lethality within 1–5 min is in the order of 500–4000 mg m^{-3}.

HCN was used in World War I by the French, with the first employment of HCN shells being on the Somme on July 1, 1916 (Prentiss, 1937). During World War II CN was used as an agent of genocide in the German concentration camps, and the Japanese allegedly used CN against China. Iraq also supposedly used CN against the Kurds during the 1980s. HCN has an affinity for oxygen and is flammable, and hence is not efficient when dispersed by artillery shells (RAMC, 2002). Additionally, due to its low vapor density, low molecular weight, and ready diffusibility, there are problems in achieving lethal concentrations of HCN against unprotected troops in open battlefield conditions. In this respect, HCN acts as a nonpersistent CW hazard. HCN is only strategically useful as a lethal agent for localized and circumscribed circumstances. However, less-than-lethal concentrations of atmospheric HCN cause mental and physical incapacitation, which may be of operational significance in reducing efficiency and determination of opposing troops. Concentrations of the order of 100–200 ppm would be necessary to induce such incapacitating conditions.

Cyanogen bromide and cyanogen chloride were also used in World War I to liberate HCN on the battlefield, but additionally these halides are strongly irritant to the eyes and other mucosal surfaces. Medical management is as for HCN, but pulmonary irritation may also need attention.

B. Terrorist Implications

A terrorist incident definitionally covers a situation in which there is a desire on the part of the terrorists to produce physical, chemical, or psychological assault on an organized (law-abiding) society to modify popular attitude, opinion, legislation, or political dictate, by the use of procedures designed to make the terrorist motivation fearfully known to the appropriate members or sectors (Ballantyne et al., 2006). The use of chemicals in terrorist operations has been discussed widely and accepted as being likely to occur in some situations. Generically, the chemicals that could be used include irritant and disorientating materials, psychogenic substances, and lethal agents. It is assumed that the majority of terrorists will be attracted to chemicals that are cheap to purchase or synthesize, can be readily obtained or manufactured, capable of causing a mass incapacitation or mortality, are comparatively easy to handle, have high biological activity with short latency to onset, and can be purchased (along with dispersal systems) without arousing a high degree of suspicion. Specific chemicals that have been used, or are considered likely to be used, include: organophosphate anticholinesterases (including commercially available pesticides) as in the Tokyo subway attack (Okudera et al., 1997; Simon, 1999), commercially available toxic and irritant materials, cyanides, and biotoxins, including ricin and botulinum (Tendler and O'Neill, 2005).

Random small-group or individual terrorist activity has included the repacking of medicinal capsules with CN and returning them to the shelves of shops (Brahams, 1991; Centers for Disease Control, 1991), although this is now made not possible by the use of tamper-proof wrappings. Intramuscular injection of HCN from a concealed syringe was considered as a possibility for assassination (Ballantyne et al., 1972). A notorious example of mass killing with CN was the Jonestown massacre of cult murder (Thompson et al., 1987). There have been several documented accounts of the existence of plans by some international terrorist groups for the possible use of CN in likely major terrorist activities (Ballantyne et al., 2006; DesLauriers et al., 2006). In the United States, various governmental agencies, including the Centers for Disease Control and the Department of Homeland Security, consider cyanides among the most likely of agents to be used for chemical terrorism (Khan et al., 2000; NTARC, 2004). Due to its low molecular weight, low vapor density, and diffusibility, HCN vapor is most likely to be most effective when used in enclosed and confined spaces. It could be generated from cylinders of the liquid or from devices (likely to be

crude) for mixing NaCN or KCN with acidic fluids, one of which was invented by terrorists for possible use of the New York City subway system (Suskind, 2006). Cyanides are also likely to be used as solid salt or concentrated solution for contamination of various domestic, commercial, or other publicly available sources of swallowed materials, such as pharmaceutical preparations, bottled drinks, or by injection into food stuffs or food containers. Attempts to poison public water supplies by dumping cyanide into reservoirs are not feasible because of the massive amounts that would be required at source. Therefore, to be effective, CN would need to be introduced into water supplies at points close to the consumer, such as storage tanks.

Preparedness for major chemical terror incidents with CN has been discussed in detail by Ballantyne et al. (2006), who note the particular need for the following: (1) constant liaison between military, civil, and political ''intelligence'' agencies to keep informed on the likelihood, nature, possible location, and timing of potential incidents, (2) continually updating on methods likely to be encountered in terrorist actions, appropriately revising methodologies to deal with these, (3) maintaining equipment (including protective equipment) and medical management needs, (4) constant training sessions for all scenarios, (5) having the technology and skills for analytical recognition of threat chemicals, in particular for rapid and on-site use, and (6) ongoing discussions and frequent training exercises between security organizations, police, rescue workers, emergency medical responders, and health-care providers.

The U.S. Center for Disease Control (CDC) has prepared strategic recommendations for, and responses to, biological and chemical terrorism (Khan et al., 2000). They identified the following five main activities that should be undertaken by public health organizations to enhance preparedness for terrorist chemical attacks:

(1) Epidemiological capacity should be enhanced for detecting and responding to chemical attacks.
(2) Awareness of chemical terrorism should be increased among emergency medical service personnel, police officers, and firefighters.
(3) Antidotes should be stockpiled.
(4) Bioassays should be developed and provided for the detection and diagnosis of chemical injuries.
(5) Educational materials should be prepared to inform the public during and after a chemical attack.

In respect of potential CN terrorism we believe that the following three critical items should be added to the CDC recommendations. First, before antidotes are stockpiled there should be international agreement on the most appropriate antidote (or combinations) for the treatment of acute CN poisoning. It is our opinion that hydroxocobalamin is the optimum choice. In the context of mass casualty situations with terrorist release of cyanides on the public, the chosen antidote should be readily available, effective, easy to administer (even by responders with limited training), nontoxic, and does not adversely interact with other antidotes (Thompson, 2004). Second, with respect to CN analyses, there is a requirement for a portable equipment that is specific and, at least semiquantitative, that can be used for on-site reliable bioidentification of CN intoxication. There is also a need for a reliable and sensitive environmental method for the instantaneous measurement of HCN concentrations, and ideally continuous monitoring with automatic warning devices for installation in sites with a potential for HCN attack. Third, educational materials should be made immediately available for distribution to the general population so that they can be prepared for what to expect in the event of a CN terrorism event.

Due to the high pressure with routine duties in health-care facilities, it is considered appropriate that hospitals should appoint a small team, representing relevant specializations, who have the responsibility for the development and organization of arrangements in the event there is a terrorist

incident in their catchment area. This planning and response team should develop guidelines for dealing with the immediate and medical management of an incident. These guidelines should cover, across the board (chemical, biological, and nuclear), the following: (1) ensuring the necessary expertise can be summoned promptly, (2) triage procedures, (3) first-aid and medical management for all likely situations, (4) ensuring there are sufficient available supplies of equipments and therapies for use at the incident site and in the hospital area, (5) arranging for decontamination sites and procedures including in the hospital grounds, (6) ensuring immediate and free lines of communications with other advisory groups (e.g., rescue groups, Poison Control Centers), (7) ensuring appropriate protective equipment is available for use by staff who could be exposed, and (8) organizing educational and local training sessions, and arranging for practical joint training sessions with local responsible security and rescue services.

In the context of the possible use of CN in a terrorist incident, specific considerations and urgent needs are as follows (Ballantyne et al., 2006):

1. The development of a specific, accurate, and reliable portable rapid blood test to screen for the presence of toxicologically significant concentrations of CN in blood, to permit rapid on-site diagnosis. Ideally, the method should differentiate between bound and free CN (Lindsay et al., 2004).
2. Protective equipment is required for those who come into contact with exposed victims, and should include impermeable clothing and respiratory equipment (ideally air supplied).
3. Responders should be made aware of the fact that symptoms, incapacitating signs, and death can occur within seconds to a few minutes of the start of an exposure, and thus rapid action is required in the context of a well-planned response; this is critical. Speed of recognition of intoxication and appropriate intervention are highly important and life-saving.
4. First-aid and medical management stockpiles for on-site and hospital use should include masks with manual ventilators; oropharyngeal airways; oxygen cylinders with masks; in-date ampoules of amyl nitrite (kept at, or below, 15°C); sodium bicarbonate for i.v. infusion; recommended antidotes (to be administered by a physician). The choice of antidotes should be made in consultation with relevant experts in a Poison Control Center. As noted earlier, there is an urgent need for an international agreement on a rationalized therapeutic approach for acute CN poisoning.

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15 Chemicals Used for Riot Control and Personal Protection

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CONTENTS

The contents of this chapter are not to be construed as an official Department of the Army position, unless so designated by other authorizing documents.

I. BACKGROUND

The causes and circumstances of civil disturbances are extremely variable regarding the numbers of persons involved, reasons for the disturbance, geographical location, the influence of extremists, confrontation with antagonistic elements (opposing the cause), and the activities of demonstrators, police, and security forces. At one end of the spectrum of civil disturbances is physical assault by one or a few individuals on a member of the public or an officer of the law, and where self-protection is needed against the malefactors. At the other extreme are large-scale demonstrations by protestors in which law enforcement personnel may become involved, and where physical violence occurs that could result in damage to private or public property, and where there is likelihood for injury, or even death, among participants or bystanders who, by chance, are present in the area of the disturbance. If many individuals participate in a civil disturbance involving potentially dangerous physical activity, this constitutes what is popularly described as a riot. Such riotous situations occur at differing locations and are precipitated by numerous and varying factors; for example, civil unrest, dissatisfaction or gang conflict in prisons, escalation of a civil demonstration against political dictates, disputes at sports meetings or social events, and indeed any gathering where there are likely to be conflicts of opinions within groups or where emotions may become heightened or distorted. In these situations, and where security forces are used to restore law and order, there may be multiple injuries, sometimes fatal (see Section VII). Demonstrations having variable degrees of conflict with security personnel and law enforcement agencies and full-scale riots have been and will continue to be an inevitable consequence of dictatorial, demanding, and ethically suspect political regimes and administrations. Indeed on the day that this paragraph is being written there are reports of the following large-scale demonstrations and riots in various parts of the world, with markedly differing causations. A jail riot in Los Angeles due to racial tensions, with one death and in excess of 100 injured, many seriously (BBC, 2006a; USA Today, 2006a). A violent demonstration with burning of Embassy buildings and fatal injuries occurred in Beirut as a consequence of the publication in Danish and Norwegian newspapers of demeaning cartoons (images) of the Muslim prophet which could be construed as sacrilegious; simultaneous and subsequent demonstrations, some also violent with fatalities, occurred in other parts of the world including Muslim communities in London, Pakistan, India, Iran, Afghanistan, Iraq, and Jordan (BBC, 2006b, 2006c; CNN, 2006a, 2006b; USA Today, 2006b). After a ferry sank in the Red Sea resulting in hundreds of deaths, a physical conflict occurred at the Saudi port of Doha between relatives of the ferry passengers and police as a result of the maritime transport company failing to give timely information about the conditions of the incident (CNN, 2006c, 2006d). These examples emphasize the variable causations of civil disturbances and that the outcomes may include widespread publicity, accusations of excessive and unnecessary physical force by security personnel, claims for injury, litigation, public discussions, and official enquiries; such postevent implications are discussed in detail by Ballantyne and Salem (2004).

Peacekeeping operations against individuals or protesting groups may, depending on the nature of the disturbance and whether there is violence between demonstrators or between demonstrators and security forces, necessitate the use by law enforcers of various devices and substances to control and quell activities of those participating in the disturbance. Physical methods involve the use of direct assault procedures (e.g., truncheons, nightsticks) or remote infliction of distracting or painful procedures (e.g., water cannon, bean bags, rubber/plastic bullets, and tasers), and may result in

direct trauma (see Section II.A). Chemicals used in peacekeeping operations, colloquially known as riot control agents (RCAs), are intended to distract, harass, temporarily incapacitate, or otherwise discourage individuals from participation in a disturbance by producing a situation which impairs the conduct of coordinated activities and results in demonstrators not undertaking individual or group activities, causes crowd dispersion, and decreases the likelihood for damage and injury. As discussed in Section III, when chemicals are used to control civilian disturbances, it is necessary to use substances of low health hazard potential and employ delivery methods that carry the minimum potential for injury. This chapter reviews the nature and effects of chemicals used, and proposed for use, in peacekeeping operations. Particular attention is given to their operational uses in various circumstances, pharmacology, toxicology, evaluation of safety-in-use, delivery, effects on humans, consequences and medical management of overexposure and injury, and the need for preparedness planning. Historical aspects of the use of chemicals in peacekeeping operations have been presented in detail elsewhere (Ballantyne, 1977a, 2006a; Salem et al., 2005a, 2006).

II. GENERIC ISSUES RELEVANT TO PROCEDURES USED IN PEACEKEEPING INCIDENTS

A. Chemical and Physical Approaches

Chemicals used in peacekeeping operations are intended to distract, harass, temporarily incapacitate, or otherwise discourage participation in a disturbance. The choice of agent will depend on the desired operational end result. The following characteristics are considered appropriate for chemicals used in peacekeeping operations against civilian populations: (1) have rapid onset of incapacitating effects even with the most motivated; (2) easy to disseminate and subsequently decontaminate; (3) have long shelf life; (4) are of low cost; (5) should not facilitate the escalation of the situation; and (6) do not produce short or long-term adverse effects when used against a heterogeneous population (Maynard, 1999; Ballantyne, 2006a). The physical equipments and chemicals used by law enforcement personnel can be categorized as outlined below.

1. Physical Measures for Close Range and Remote Incapacitation

This category includes physical measures intended to deter or incapacitate; in some cases they are deployed at close range and with other measures at significant distances. They essentially involve ballistic or electrical immobilization of individuals. Included are truncheons, nightsticks, beanbags, plastic or rubber bullets, and tasers. Clearly such procedures are intended to incapacitate by physically causing pain and immobilization, but equally clear is a potential for soft tissue and bone injury, and several deaths have been associated with the resultant trauma. Contrary to statements that the risk of serious and fatal injuries is very low from ''nonlethal'' weapons such as tasers and baton rounds (Cooper, 2004; Buchanan, 2005), there are clear indications that this is not true, and documentation exists of serious injuries and deaths from the use of baton rounds (Metress and Metress, 1987; Yellin et al., 1992; Paret et al., 1996; Jaouni and O'Shea, 1997; Mahajna et al., 2002; BBC, 2003a, 2003b, 2003c; Sutter, 2004). Tasers aimed at the trunk discharge electrode needles that remain attached to the projection device by fine wires that carry high voltage pulses (50,000 V), which cause muscle spasms, weakness, and incapacitation. They have been used against a wide range of ages (BBC, 2006d), and although it is claimed that their use carries a minimum potential for adverse effects, several deaths and injuries have been attributed to tasers (Mehl, 1992; Ng and Chehade, 2005; Lewer and Davison, 2006; Sherman, 2006); lawsuits have been brought by some victims (BBC, 2005). Also, it is difficult to agree with statements that those with hypertension, cardiac diseases, and arrhythmias are free from risk. The working range of tasers may be extended through the use of shotgun shells that combine blunt force trauma with the delivery of high voltage impulses (Myers, 2006).

TABLE 15.1
Local Reflexes Resulting from Sensory Nerve Receptor Stimulation by Peripheral Chemosensory Irritants

Stimulation Site	Reflex
Eye	Excess lacrimation
	Blepharospasm
Respiratory tract	Increased nasal and tracheobronchial secretions
	Cough
	Sneezing
	Bronchospasm
	Change in breathing rate
Oropharynx	Salivation

2. Peripheral Chemosensory Irritant (PCSI) Chemicals

These materials constitute the major group of chemicals that are used to harass and incapacitate individuals in civil disturbances and for personal protection. The pharmacological basis of their use is that they interact with sensory nerve receptors in skin and exposed mucosal surfaces, producing local discomfort and pain at the site of contact together with related local and systemic reflexes; local reflexes are listed in Table 15.1. The uncomfortable sensations coupled with local reflex effects such as excess lacrimation, blepharospasm, and coughing, cause harassment, difficulties in conducting coordinated tasks, and a desire to vacate the contaminated zone (Ballantyne et al., 1999a, 2006a). PCSI effects develop promptly after exposure and generally persist for only a short period (several minutes). PCSI chemicals are sometimes referred to as harassing agents or short-term incapacitants.

3. Obscuring Smokes

Although used mainly in military operations (see Chapter 18), obscuring (screening) smokes could also be used in peacekeeping operations to cause distraction, disorientation, and obscuration. However, this approach has not been frequently used. Several smokes used for military purposes have been associated with adverse health effects, notably to the respiratory tract, and are thus not appropriate for the control of civil disturbances; these include phosphorus, titanium tetrachloride, zinc oxide/hexachloroethane, and zinc chloride. Smokes of significantly lower toxicity are required for peacekeeping operation; for example, cinnamic acid and the dyes Disperse Red 9, Solvent Green 3, and Solvent Yellow 33, although exposure to high concentrations of these dyes may result in pulmonary retention of dye and foreign body reactions (Marrs et al., 1984; Sun et al., 1987).

4. Visible and Occult Markers

Substances in this class are used to contaminate individuals for immediate discouragement and also for subsequent identification purposes. They can be dispersed in solution from pressurized handheld canisters for one-on-one use, or by water canon for large-scale antiriot use. One series of markers are visible dyestuffs, whose use results in immediate staining of clothing and skin for deterrent or recognition purposes. These markers clearly need to be chosen on the basis of their staining properties, lack of local toxicity to skin and eyes, notably irritancy and sensitization, and freedom from environmental problems. Occult markers are colorless substances that will fluoresce under ultraviolet light, and thus are used for identification purposes.

5. Malodorants

Contamination of persons with malodorous substances, such as mercaptans and amines, has been proposed as a means to deter less motivated rioters based on psychological and physiological effects such as olfactory repulsion and nausea (Whitten et al., 1970). They can be delivered by frangible missile, and addition of a thickening agent will prolong adhesiveness.

6. Low-Friction Polymers

Dispersion of low viscosity (slippery) agents in the direction of rioters has been suggested as a means to impair their ability to undertake coordinated activities and make it difficult to carry out malicious tasks. However, in addition to the likelihood for physical accidents due to uncontrolled body movements, there may also be problems in the control of motorized vehicles in the area, including those of security forces. Also, in public areas there would be a need for rapid and thorough street and road decontamination.

7. Centrally Acting Neuropharmacological Agents

There have been proposals that systemic delivery of agents that produce effects on some aspects of central nervous system (CNS) function could find a use in peacekeeping operations, particularly for the induction of tranquillization, narcosis, or emesis (Swearengen, 1966; Conner, 1967; Security Planning Corporation, 1972; Dean-Drummond, 1975). Those substances that lead to alterations in conscious state, or motor control, are sometimes referred to as mental incapacitants. The use of neuropharmacologically active agents requires a delivery system that would ensure absorption into the systemic circulation in order to reach the brain which is the target organ; this includes the use of drug-injecting dart guns for deployment against single individuals, but for use against groups of people, airborne distribution would be necessary. Clearly the use of centrally acting substances (drugs) could result in sociopolitical problems, medical complications, and possible litigation, and their use to date has been limited to hostage situations. The adverse consequences and widespread criticisms that can result from using centrally acting drugs are well illustrated by the use of the narcotic fentanyl by Russian security personnel against rebels who held citizens as hostages (Section V.B).

B. Generation and Dispersal Methods for Agents

Screening smokes are usually generated pyrotechnically, and marking agents can be dispersed in solution from large or small-scale dispensers, and sometimes mixed with PCSI materials. Depending on the operational circumstances, PCSI materials are used either by airborne dispersion or projected as solutions in the form of coherent liquid jets. Airborne PCSI materials can be widely disseminated and result in many individuals being nonselectively affected, depending on how local meteorological conditions affect drift and persistence. Airborne dispersions are generated pyrotechnically from grenades as smokes, or else as aerosols, mists, or vapor generated from pressurized systems containing PCSI material in a volatile solvent. They may also be dispersed as powder clouds from fogging devices. The use of the word ''gas'' to describe airborne PCSIs (e.g., CS gas or tear gas) is technically incorrect, because a gas is a homogeneous dispersion of molecules in the atmosphere and exists in this phase at NTP; this is distinct from a vapor which exists as an atmospheric dispersion of molecules at a temperature below its critical temperature, and is liquid or solid at NTP. Atmospheric dispersions of PCSIs are in the form of aerosols or vapor. Solutions of PCSI materials projected as coherent fluid streams can be used to selectively contaminate specific individuals or groups of persons. Depending on the volume of fluid to be projected, dispersion devices range from small handheld pressurized canisters for use against single or small numbers of individuals, to water cannons to engage larger groups or crowds. Examples of specific dispersal methods are briefly reviewed below.

1. Smoke Generation

Aerosolization by thermal volatilization is the most frequently employed method for generating screening smokes and for dispersion of PCSI materials for military or peacekeeping uses. The active material is mixed with a base such as chlorate or lactate that, on ignition, causes volatilization of the material that subsequently condenses into a cloud of solid or super cooled liquid droplets. The suspended particles or droplets then settle or aggregate into larger agglomerations. Grenades and canisters have an igniting fuse that initiates after a delay of 1–5 s, following which the filler is ignited and pressure builds up until it is sufficient to open gas ports and expel the pyrotechnic mix. Grenades are thrown or propelled by a launcher, and cartridges can be fired from a shotgun, a rifle-mounted launcher, or a gas/air gun. Firing allows for greater accuracy of delivery and increased distance compared with hand-thrown grenades, but as high-velocity projectiles they can cause body injury or lethality if deployed at too close a range. On a calm day a standard canister or grenade will generate a cloud that is some 20–30 ft in diameter. The standard smoke grenade or canister produces a unifocal source of smoke that spreads with air movements from its origin. A variant on this is a grenade that, on ignition, disperses (projects) several burning subunits over a large area, and thus produces a large cloud from the scattered burning subunits, but with a reduced emission of smoke from each focal subunit. One example of such a device is the ''rubber-bursting grenade'' that consists of a rubber cylindrical casing packed with small pellets composed of PCSI material mixed with pyrotechnic composition. The increased internal pressure that results from ignition of the mixture causes the rubber casing to burst and scatter burning pellets over a wide area, thus producing a multifocal source of smoke (Ballantyne and Johnson, 1974a). One disadvantage of pyrotechnically generated smokes is that they contain potentially harmful combustion products formed during the burning of the mixture. Thus, one aspect of a hazard evaluation of pyrotechnically generated smokes is to conduct chemical analyses to determine the nature and relative amounts of the various combustion products (Kluchinsky et al., 2001, 2002). A biological indication of the relative contribution of combustion products to the inhalation toxicity of a smoke can be obtained by comparing the toxicity of the products resulting from igniting the burning mix alone with that resulting from igniting the burning mix containing the active agent. For example, Table 15.2 compares the acute inhalation toxicity of pyrotechnically generated CR smoke with that for smoke generated from ignition of burning mix alone; it can be seen that a high proportion of deaths occurred with burning mix smoke alone, and that this clearly contributed to mortalities from exposure to pyrotechnically generated irritant smoke.

TABLE 15.2
Comparative Acute Lethal Inhalation Toxicity to Four Species of Pyrotechnically Generated CR Smoke with that of Smoke Resulting from the Combustion of the Burning Mix Alone

Exposure Time	Grenade	CR Dosage ($mg\ min\ m^{-3}$)	Mortality (Number Dying/Number Exposed)			
			Rabbit	Rat	Guinea Pig	Mouse
60	Blank[a]	0	9/10	5/5	5/5	9/10
60	CR	148,000	4/10	2/5	1/5	3/10
60	CR	182,000	10/10	5/5	2/5	2/10
120	Blank	0	10/10	4/5	5/5	10/10
120	CR	325,000	—	10/10	10/10	17/20

Source: Data after Ballantyne, B., *Toxicology*, 8, 347, 1977b.

[a] Burning mixture without CR.

2. Powder Clouds

PCSI materials can be blown into the atmosphere in fine particulate form using fogging machines. This means of dissemination of PCSIs avoids toxic effects from inhaled combustion products that are a feature of grenade-generated smokes. For large-scale applications the PCSI can be mixed with a hydrophobic anti-agglomerative material such as Neocil (fumed silica) to improve flow properties. For example, CS formulations (CS1 and CS2) have been produced for use in fogging machines. CS1 is a micronized powder containing 5% hydrophobic silica aerogel, which persists for about 2 weeks; CS2 is a siliconized microencapsulated form of CS1 with improved flow properties and greater weather resistance, and composed of 95% micropulverized CS with silica treated hexamethyldisilazane. Because they persist in the environment, these formulations are not suitable for civilian peacekeeping operations but are used to create a persistent hazard in military operations (Blumenfeld and Meselson, 1971; Ministry of Defence, 1972). On a smaller scale, and mainly for self-defense purposes, ''tear gas pen guns'' or ''tear gas pistols'' are available in some countries. These devices have an explosive charge that propels a cloud of irritant material toward the assailant. They have a high physical injury potential and have caused severe local skin and eye injuries (Rengstorff, 1969).

3. Dispersion as Vapor

Pyrotechnically generated smoke, or even powder clouds, may not be acceptable as safe when based on operational circumstances; for example, situations where fires may be started, or in confined spaces with low ventilation rates where asphyxial effects could develop. To reduce such potential hazards, various approaches have been investigated. One is the use of highly volatile PCSI materials which can be projected into enclosed spaces to produce an irritant vapor, but materials of sufficiently low toxicity have not yet been discovered. For example, 1-methoxycycloheptatriene, although a highly volatile and potent PCSI material, has been experimentally shown to be centrally neurotoxic, especially to Purkinje cells (Marrs et al., 1991). An alternative approach uses PCSI material dissolved in a highly volatile solvent, and the mixture projected into an enclosed space from a pressurized aerosol canister or using a frangible missile, resulting in the development of an airborne vapor or aerosol of irritant material (Ballantyne, 1979).

4. Dispersion in Solution

Solutions of marking agents or PCSI materials can be dispersed as coherent liquid jets, allowing the selective engagement of specific targets. Using such jets on a small scale (e.g., handheld canister) avoids, or limits, the potential for physical, kinetic, or thermal injuries. However, with large-scale use (e.g., water cannon) the potential for kinetic injuries is present, as is the possibility for sustaining injury from slipping on large volumes of fluid. With water cannon, PCSI materials dissolved at low concentration cause skin and eye sensory irritation following drenching, and this avoids the potential complications from inhaling grenade-generated smokes (Ballantyne et al., 1976a). Handheld irritant liquid spray devices of various types have been commercially manufactured for use by the police and other security groups. Additionally, in some countries, including the United States of America, spray canisters may be sold to the general public as self-protection devices, although they are illegal in other countries, including the United Kingdom, and civilian airlines prohibit them from being taken on to airplanes in hand luggage. These handheld devices are designed to permit the user to direct a spray of sensory irritant at the face of an assailant causing a prompt onset of temporary incapacitation from severe eye and facial skin effects, during which time the individual can be overpowered or subdued for arrest. In some cases, notably with solutions of CN, where a highly volatile solvent is used it is recommended that the solution be directed at the upper torso so that irritant vapor is produced which causes harassment to the individual from skin, eye, and respiratory tract sensory irritant effects. Typical canisters have a spray range of aerosol of 2–5 m. Some employ highly volatile propellants such as propane and *iso*butene, although more recently

TABLE 15.3
Examples of Handheld Irritant Liquid Spray Devices Used for Self-Protection

Common Name	PCSI[a]	Solvent	Propellant
MACE	CN	Kerosene hydrocarbons 1,1,1-trichloroethane	Freon 113
Paralyzer	CS	Dichloromethane	Nitrogen
PIS[b]	CS	Methyl *iso*butyl ketone	Nitrogen
SPAD[c]	CR	50% Aqueous PEG300[d]	Nitrogen
Cap-Stun	OC	*iso*Propanol	*iso*Butane/propane
Punch II	OC	*iso*Propanol	*iso*Butene
Sabre 5.5	OC	TS	Dimel 134a/P
Sabre	OC/CS	TS	Dimel 134a/P
Guardian	OC		
Cap-Tor	PAVA	Water/ethanol	Nitrogen

Source: Data from Ballantyne, B., *Chemical Warfare Agents: Toxicology and Treatment, Second Edition*, T.C. Marrs, R.L. Maynard, and F.R. Sidell, eds, John Wiley & Sons, Chichester, 2006a; Ballantyne, B. and Salem, H., *Riot Control Agents: Issues in Toxicology, Safety, and Health*, E.J. Olajos and W. Stopford, eds, CRC Press, Boca Raton, FL, 2004; Olajos, E.J. and Stopford, W., *Riot Control Agents: Issues in Toxicology, Safety, and Health*, E.W. Olajos and W. Stopford, eds, CRC Press, Boca Raton, FL, 2004; Shreenivason, V. and Boese, B.A., *J. Forensic Sci.*, 15, 433, 1979; Stefee, C.H., Lantz, P.A., Flannagan, L.M., Thompson, R.L., and Jason, D.R., *Am. J. Forensic Med. Pathol.*, 16, 185, 1995.

[a] PCSI = peripheral chemosensory irritant; CN = 1-chloroacetophenone; CS = *o*-chlorobenzylidene malononitrile; CO = oleoresin capsicum; TS = trade secret (proprietary).

[b] PIS = personal incapacitant spray (UK police forces).

[c] SPAD = self-protection aid device (developed by UK Ministry of Defence).

[d] PEG = polyethylene glycol.

produced devices use nitrogen pressurization. The latter has the advantage of avoiding problems associated with potential solvent toxicity, but has the disadvantage of reduction in overall pressure resulting in a progressively weaker stream with use. Some devices now use Dymel (1,1,1,2-tetrafluoroethane) as propellant, which has the advantages of being nonozone depleting, maintains canister pressurization, is compatible, and nonflammable (Olajos and Stopford, 2004). Examples of irritant liquid dispersion devices are given in Table 15.3.

The choice of solvent and formulation should meet the following criteria: (1) the physicochemical characteristics do not, and should not, result in any physical hazards during handling or dispersion of the formulation, such as those resulting from volatility or flammability; (2) acceptable shelf life without the formation of deleterious storage products; (3) chemical compatibility of formulation components; (4) formulation should not decrease the physiological potency of the PCSI; (5) no undesirable effects on the general environment, such as odor, persistence, or environmental toxicity; (6) the solvent should not produce local or systemic toxicity; and (7) should not have potentiating or synergistic effects on the known toxicity of PCSI or marker.

The latter factor is particularly important, because it is sometimes neglected in assessing health hazards from formulations (Holopainen et al., 2003; Ballantyne and Salem, 2004; Gray, 2004). The toxicity and potential health hazards from the solvent and formulation should be considered in addition to those from the active constituent. For example, moderate eye injury and increase in intraocular pressure (IOP) are produced by dichloromethane (Ballantyne et al., 1976b), and methyl *iso*butyl ketone (MIBK) causes an irritant dermatitis (Gray, 2004). A UK police irritant liquid spray of CS in MIBK causes erythematous dermatitis with blistering, which persists longer than occurs with CS exposure alone, and thus MIBK probably enhances the local cutaneous effects of CS (Varma and Holt, 2001; BBC, 2004a; Euripidou et al., 2004). Severe blistering with possible permanent scaring of the face has been described following the use of a CS spray device (BBC,

2006e). One UK irritant liquid spray device contains 5% CS (Varma and Holt, 2001), a concentration known to produce keratitis when it is dissolved in the nonirritant solvent PEG300 (Ballantyne et al., 1974); the corneal injury is likely to be more severe using MIBK as solvent. With CS in dichloromethane, both materials contribute to the local ocular toxicity (Ballantyne, 1979). In addition to causing local ocular injury, dichloromethane may also result in systemic toxicity following inhalation of the vapor and its hepatic metabolism to carbon monoxide (Horowitz, 1985; Rioux and Myers, 1988, 1989). For example, Duenas et al. (2000) reported a case of carbon monoxide poisoning in a 39 year old female after exposure to a solution from a personal defense spray containing CS in dichloromethane. Her carboxyhemoglobin (COHb) concentration was 20.4%, and she recovered after treatment with 100% normobaric oxygen.

III. BIOMEDICAL CRITERIA FOR HEALTH HAZARD EVALUATION

Few countries have formal schemes, guidelines, or regulations for approving chemicals, formulations, and delivery systems intended to be used against populations during times of civil unrest. Rappert (2003) has commented on the procedures necessary for the regulation of nonlethal weaponry to lessen uncertainties associated with claims and counterclaims associated with the medical implications of the use of nonlethal weaponry against civilian populations. The first detailed independent public safety evaluation of chemicals used for riot control purposes was in the United Kingdom following the employment of CS by the Royal Ulster constabulary during sectarian riots in Northern Ireland in August 1969 (HMSO, 1969, 1971). The UK government appointed an independent Committee of Enquiry to investigate this use of CS. A major recommendation of the Committee was that when chemical agents are used for the purposes of civilian peacekeeping, they should be studied more akin to that of a new drug rather than that of a weapon. They did, however, qualify this with a statement that with new drugs there is a need for risk–benefit analyses and these are purely professional medical matters. However, with a chemical intended for use against humans for peacekeeping purposes, although most hazard and risk assessments are of a medical nature, additional questions may arise which are concerned with social policy and there may be a need for political considerations. From a medical viewpoint an assessment of the safety-in-use of riot-control agents, and their formulations, requires information derived from several sources and specialties. Hazard evaluation and safety-assessment programs start with a determination of the potential for the agents to cause adverse effects by in vivo and in vitro laboratory studies followed by controlled human volunteer studies and trials, if these are considered necessary and are safe for participants. Also, there may be a need to conduct studies on ecotoxicology and environmental hazards. These aspects of health hazard evaluations are considered in outline below.

A. Laboratory Toxicology Studies

Toxicology studies are designed and conducted to determine the potential of a substance, or mixture of substances, to cause adverse biological effects (toxicity); in this context adverse effects are defined as those that are detrimental to the normal functioning or survival of the organism (Ballantyne, 1984; Ballantyne et al., 1999a). If adverse effects are produced, it is necessary to know their nature, tissue/cells affected, dosage–effect relationship and incidence, mechanism by which they are produced, factors influencing their induction, and reversibility (spontaneous and induced). Toxicology-testing programs should allow for decisions on hazards from, and operational restrictions on, the chemical agent, its formulation, and its delivery system. The program should also allow for the fact that a heterogeneous population will be exposed. Clearly those who participate in a civil disturbance, or who are coincidently present in the area, will be exposed for a single or a few irregularly spaced intervals, and most exposures will be short and exposure dosages low. However, and particularly in confined spaces, some individuals may have higher exposure dosages. Thus, initial studies should concentrate mainly on acute and short-term repeated exposures by the intended

routes, primary irritancy (skin and eye), and sensitization. For those exposed more frequently, including security forces and production workers, studies of longer duration are required to determine if the longer and more frequent exposures are associated with adverse effects not seen with acute or short-term repeated exposures, and also to assist in developing protective and precautionary measures, including the assignment of exposure guidelines; for example, threshold limit values (TLVs) have been recommended for CS (a Ceiling value of 0.05 ppm) and CN (a time-weighed average over 8 h, TWA_8 of 0.05 ppm) (ACGIH, 2006). In the heterogeneous population encountered with a civil disturbance, there will be individual differences in respect of age, gender, reproductive status, and state of health. Thus, there may be testing requirements for developmental, reproductive, and genetic toxicology. The oncogenic potential of agents needs to be investigated with respect to repeated occupational exposures, and also because it may become a concern by those having interests in the sociopolitical aspects of peacekeeping operations and by the investigative media. The following is a brief generic discussion of what is considered as basic to a testing program for agents used in peacekeeping operations. However, every testing program should be developed individually for each specific agent, taking into account the nature and intended use of the material, foreseeable misuse, and how toxicity could be modified by formulation and mode of delivery. Also, special studies may be necessary on a case-by-case basis. Details of the methodologies for in vivo and in vitro toxicology testing are available in several texts (e.g., Anderson and Conning, 1993; Niesink et al., 1996; Ballantyne et al., 1999b; Hayes, 2001).

Acute Toxicity. There is a clear requirement for single-dose studies by relevant routes of exposure to determine lethal toxicity (LD_{50} or timed LC_{50}), and sublethal injuring potential with dose–response relationships, including a no observable adverse effect level (NOAEL). In addition to testing the pure material, it is advisable to study the technical material to include any impurities that may be in the material used for munitions, and also determine the influence of variants on acute toxicity; for example, solvents, additives, and the effect of pyrotechnic decomposition products.

Primary Irritation. Dispersed as smoke, aerosol, or in solution, agents will contaminate skin and eyes and may cause acute inflammatory reactions at these sites. Therefore, there is a requirement to know if contact with the skin and eye will have a local tissue injuring potential and how formulation may affect the reaction. Supplemental to standard eye irritation tests it is of practical value to undertake in vivo studies on the influence of the agent on corneal thickness by pachymetry and on IOP by tonometry (Ballantyne et al., 1977a; Myers et al., 1998; Ballantyne, 1999b).

Sensitization. Because skin and respiratory tract are the usual routes of exposure to peacekeeping chemicals, there is a need to determine, especially with respect to those recurrently exposed, the potential for induction of immunologically mediated cutaneous and/or respiratory tract sensitization. In vivo and in vitro approaches are available for such investigations (Hermansky, 1999; Kimber and Dearman, 1999; Blackwell, 2006).

Repeated Exposure Studies. From both occupational and in-use perspectives, it is necessary to study the effects of agents initially by short-term repeated exposures to determine the potential for cumulative toxicity (Ballantyne, 1999c). The need for subchronic (circa 15%–20% of lifespan) and combined chronic-oncogenicity studies depends on a broad spectrum of considerations with decisions made on a case-by-case basis. The factors to be considered include exposure patterns, nature and biological reactivity of the agent, genetic toxicology, suspect biological reactivity, and sociopolitical demands.

Developmental and Reproductive Toxicology. Because heterogeneous populations may contain females of childbearing age, it is required that information be available on the embryotoxic, teratogenic, and reproductive hazards of agents. These studies require special design considerations; for example, with airborne PCSI materials the design and interpretation of the investigations needs to take into consideration the possibility of stress caused by both sensory irritant exposure and aerosol exposure during gestation (Upshall, 1973, 1977a).

Genetic Toxicology. As a general guide to potentially serious biological hazards, biological reactivity, and to assist in determining the need for chronic toxicology/oncogenicity studies, it is appropriate to conduct investigations to define the mutagenic and clastogenic potential of peacekeeping agents.

Metabolism and Toxicokinetics. Such studies, conducted by an intended route of exposure, can be of considerable value in determining potential hazards, defining systemic, organ, and tissue doses, and permitting quantitative risk assessments. Metabolism studies may give information on the possible contribution of metabolites to the toxicity of the parent material. Investigations on the absorption, distribution, and excretion of parent compound and its metabolites, will assist in defining the potential for cumulative toxicity, possible target organs and tissues, and allow quantitative assessment of hazards. These studies will also be of help in the design and subsequent interpretation of repeated exposure studies.

Additional, Special, and Compound-Specific Studies. Because of the variety of substances and differing chemical structures that are encountered as potential peacekeeping agents, and the different modes of dispersion, special studies may be required on a case-by-case basis for a given agent, formulation, or delivery mode. In some instances, these can be predicted as being required and, as such, incorporated into the toxicology-testing program during the planning phase, but in other cases the need may arise from results of the defined testing program or as a consequence of experience and analysis of in-use situations and incidents. The requirement for additional or nonstandard studies will be determined on a case-by-case basis, and the following are merely two illustrative examples. Because peacekeeping agents are used in situations conducive to the occurrence of open wounds, it can be predicted that additional studies may be necessary to determine if contamination of open wounds by an agent leads to adverse effects on the healing process (Ballantyne and Johnson, 1974b). In contrast, and unpredicted, following the Northern Ireland riots of August 1969, there appeared reports of cases of diarrhea (The Observer, 1969; The Times, 1969). To experimentally clarify the possibility of an association between exposure to CS smoke and the development of diarrhea, several species were dosed with CS over 5 consecutive days; there was no indication of either increased wet stool production or of an inflammatory reaction in the alimentary tract (Ballantyne and Beswick, 1972).

B. Human Volunteer Studies and Trials

Human volunteer studies and subsequent trials may be needed for the following reasons: (1) confirming or otherwise the possibility that toxic or potentially adverse physiological and pharmacological effects may occur based on preliminary in vivo and in vitro laboratory studies; (2) quantitation of a known effect in human volunteers; (3) assessing the potency and usefulness of an agent; (4) conducting small-scale clinical trials on the effectiveness of agents and delivery systems; and (5) conducting full-scale trials to simulate the likely situation to be encountered in use and to determine the effectiveness and usefulness under such conditions. Items (4) and (5), particularly, can combine the major objectives of the trials with routine medical, physiological, and biochemical monitoring to determine the effects of the trials' procedures and conditions on physiological and biochemical homeostasis, and to detect any unsuspected effects from the exposures. The supportive monitoring often includes cardiovascular studies (e.g., blood pressure, ECG), respiratory function tests, peripheral blood hematology, blood clinical chemistry, urinalysis, and ophthalmic investigations (including tonometry and pachymetry). These supportive investigations are not only of value in assessing safety-in-use for a healthy population and defining any operational restrictions on the agent, formulation, and delivery system, but additionally may indicate susceptible populations, including those with ill-health (e.g., cardiovascular disease, respiratory disease, aneurysm, glaucoma, and immunologically compromised). They will clearly complement the laboratory toxicology studies and are of considerable value in hazard evaluations. Human volunteer studies and trials should ideally start at threshold levels,; be conducted with appropriate medical cover; only

involve participation of subjects who have been informed of the nature of the studies and any potential risks involved; and permit withdrawal of participants at any stage in the study or trial. They should be conducted only after review and approval by a local (establishment) medical safety committee and by an appropriately qualified independent review panel (as discussed by Ballantyne, 2005). These oversight groups should be periodically informed of the progress and results of the studies and should confirm that it is acceptable to continue (authorize) any extension of the studies/trials. Written and signed detailed records should be kept of all aspects of the trials and on discussions of the local approval committee and the independent review body.

C. Environment and Decontamination

When chemicals are dispersed over wide areas, and particularly in urban areas, a multiplicity of differing environmental effects need to be considered and appropriate safeguards developed and effected. These will depend on the physicochemical properties of the agent and its formulation; stability and persistence; amount dispersed; and local geography, geology, hydrology, and climatology. As examples, the diversity of considerations should include: effects on sewage organisms, persistence and resultant contamination of public and private properties, any necessary decontamination procedures, effects on domestic animals, phytotoxicity; fresh water aquatic toxicity, and the effects of accidental contamination of food and drinking water.

IV. PERIPHERAL CHEMOSENSORY IRRITANTS

A. General Comments

As noted above, these materials constitute a major group of chemicals used for crowd control and dispersion, and for personal protection purposes. In addition to these uses, PCSI materials also find application in military situations for enemy troop denial situations (e.g., to flush opponents out of caves or tunnels), for training purposes to simulate chemical warfare operations, to test for effectiveness of respirator fit, and to build up confidence in mask use. PCSI materials have also been used in hostage rescue operations and for eviction of barricaded prisoners. There also exists a possibility that terrorists could use irritant materials to create fright and panic in limited or enclosed spaces, or for distraction from nearby locations where more damaging effects have been planned to occur. The disturbance, panic, and resultant injuries that may be caused by the discharge of PCSIs into populated areas are illustrated by the following incident. In February 2003, a security guard attempted to stop a fight on a crowded dance floor in a Chicago nightclub by using an OC spray device. As a consequence, a stampede started with several hundred persons charging to the front door, resulting in the deaths of 23 persons (USA Today, 2003). Robbers have used PCSIs to intimidate and incapacitate cashiers and store clerks (BBC, 2004c).

PCSI materials interact with sensory nerve receptors in skin and exposed mucosal surfaces, producing local sensations (itch, discomfort, and pain) with associated local and systemic reflexes (Table 15.1). Local effects following contact with the eye are burning sensation and marked discomfort, with blepharospasm and excess lacrimation. Contact with nasal mucosa causes stinging and discomfort with rhinorrhea, and in the mouth there is stinging or burning sensation with excess salivation. Inhaled PCSIs cause stinging, pain, discomfort, and constricting sensations in the chest, sneezing, coughing, increased tracheobronchial secretions, difficulties with breathing, and periods of breath holding. The reflex particularly associated with respiratory tract exposures is the Kratschmer reflex (Kratschmer, 1870). This was described in rabbits following exposure to irritants such as chloroform and carbon dioxide, resulting in apnoea. The effect is accompanied by bradycardia and a biphasic rise and fall in aortic blood pressure. The Kratschmer reflex is mediated via the olfactory, trigeminal, and glossopharyngeal nerves, and has been demonstrated to occur in humans (Allen, 1928–1929). From a biological perspective, the effects produced by PCSI materials are

bioprotective in nature, warning of the presence of an irritant material and limiting exposure to the material. Clearly, and with respect to peacekeeping operations, all the effects are harassing and make individuals want to vacate the contaminated area, and also make it difficult for affected individuals to carry out coordinated tasks. In general, PCSI effects appear within seconds of exposure, and subside within 10–60 min. Effects are usually experienced at concentrations significantly lower than those causing adverse effects (toxicity) by acute exposure. This difference in concentrations limits the likelihood for exposure to potentially harmful concentrations (doses) of a PCSI; this is an integral component of the bioprotective nature of peripheral chemosensory irritation. In addition to local reflexes related to the afferent nerve involved, systemic reflexes also occur, of which the most significantly related to possible medical complications are transient hypertension and bradycardia.

Various materials have been used, are currently in use, and are being investigated for future use in peacekeeping operations. Organic arsenicals such as adamsite (DM; 10-chloro-5, 10-dihydrophenarsazine) and diphenylchlorarsine (DA) have been banned in most countries based on toxicity to humans (Ballantyne, 1977a, 1987; Salem et al., 2005b). 1-Chloroacetophenone (CN) has been used widely both as a smoke and in solution for peacekeeping and personal protection purposes. However, several undesirable side effects and a few deaths have made some countries abandon the use of CN against civilians (Ballantyne, 1977a). *o*-Chlorobenzylidene malononitrile (CS) is used extensively as a smoke and in solution, and the likelihood for serious short- or long-term adverse health effects is low, being less than with CN. Oleoresin Capsicum (OC) is a complex mixture obtained from pepper plants (*Capsicum annuum* and *Capsicum frutescens*) and was introduced in the 1970s in the United States as an alternative to CN. It is used by law enforcement agencies in several countries, but not the United Kingdom because of the problems associated with health hazard assessments for complex mixtures of varying composition. Pelargonic acid vanillylamide (PAVA) is a PCSI dispersed from handheld spray devices and has recently been used by the UK and European police. Unlike OC, it is a single substance and is therefore more readily tested for potential adverse health effects. Dibenz(b.f)-1,4-oxazepine (CR) is a potent PCSI of low mammalian toxicity and not injurious to the eye and skin. Reported uses in peacekeeping operations are few (see Section IV.E). One disadvantage of CR is its chemical stability and thus environmental persistence. Some chemicals screened for use as PCSIs have been rejected on the grounds of low efficacy or toxicity. For example, 1-methoxycycloheptatriene is a potent liquid PCSI with a high vapor pressure that would make it suitable for delivery by frangible missile, but the vapor was shown to be centrally neurotoxic, producing cerebellar Purkinje cell injury (Marrs et al., 1991).

B. Criteria and Determinants of Potency and Effectiveness

In a population, the occurrence of a specific response to a PCSI stimulus when plotted as a function of the exposure concentration takes the form of a normal or bell curve (Gaussian distribution), implying the existence of hyposensitive and hypersensitive individuals within that population. However, the majority of individuals respond over a relatively narrow concentration range. From frequency distribution information (exposure concentration–response data), it is possible to calculate median effective concentrations for sensory and local reflex effects in humans and for local reflex effects in laboratory animals (Ballantyne et al., 1977b; Ballantyne, 1999a, 2006b). Thus, for PCSI-induced sensations in humans, it is possible to calculate a 50% response for concentrations causing threshold sensations (TC_{50}) and for incapacitation (IC_{50}); incapacitating is usually defined as that concentration of PCSI that cannot be voluntarily tolerated for >1 min. Incapacitating concentrations are clearly relevant to those required in peacekeeping operations, although in the practical context of a riot it is more meaningful to determine 75% and 90% responses (IC_{75}, IC_{90}). The operational effectiveness of a specific PCSI with respect to its use in peacekeeping needs to be assessed in terms of both the absolute concentration producing incapacitation and the relationship between the incapacitating concentration and the TC_{50}. The closer these values the smaller will be the IC_{50}/TC_{50} ratio and the more rapidly will incapacitating effects develop within a rising

concentration of PCSI. The ratio of IC/TC is referred to as the effectiveness ratio and can be used for comparison of chemically different PCSIs (Ballantyne, 1977a, 1999a). Also to be taken into account when assessing the effectiveness of a PCSI are the time for the onset of effects (latency) and the duration of the induced effects. To a limiting value, both latency and duration are a function of exposure concentration; latency decreases and duration increases with increasing concentration.

Several variables influence latency and duration of PCSI effects. Those of practical relevance are as follows:

1. *Concentration*. Increasing the exposure concentration will decrease latency and increase duration.
2. *Particle Size*. With aerosols, the smaller respirable particles produce both ocular and respiratory tract effects, while larger particles cause predominantly ocular effects (Owens and Punte, 1963).
3. *Vehicle*. With solutions, surface-active materials facilitate the penetration of a PCSI into skin and mucosae, and enhance the response.
4. *Environmental Conditions*. Elevated temperature and increased humidity may decrease tolerance to PCSIs (Punte et al., 1963).
5. *Motivation*. Increased motivation and distracting influences will increase the threshold for the induction of PSI effects and increase tolerance to suprathreshold effects (Beswick et al., 1972).

The following sections concisely review the uses, toxicology, and effects on humans of PCSI materials that are currently in use or have a potential for use as peacekeeping agents. Individual PCSIs have been reviewed in detail by Ballantyne (1977a, 1987, 2006a), Marrs et al. (1996), Olajos and Salem (2001), and Salem et al. (2001, 2005a, 2006).

C. 1-Chloroacetophenone (CN)

CN has been used in the following circumstances; an incapacitating and harassing agent as a smoke in riots and civilian peacekeeping operations; as an incapacitant in solution for one-on-one or small group engagements (personal protection); for enemy denial and dispersion purposes in military exercises; hostage and siege situations. Dissemination has been by hand-thrown grenades, projectile cartridges, aerosol, and coherent liquid stream dispensers; cluster bombs; aircraft dispensers; and portable powder disperser.

Toxicology. CN is an SN_2 alkylating agent, as is CS, both having active halogen groups that react readily at nucleophilic sites. They react with SH-containing enzymes as prime biochemical targets, which may partly explain some aspects of the acute toxicity of CN. It is of moderate acute toxicity by intravenous (i.v.) injection, intraperitoneal (i.p.) injection, and perorally (Table 15.4). By the peroral (p.o.) route deaths occurred within 2–18 h of dosing. Stomachs were markedly congested with hemorrhagic erosions, and the small intestine was congested with necrosis of the tips of the villi. Several animals had acute renal tubular necrosis and a few had hepatic mid- and centrilobular necrosis (Ballantyne and Swanston, 1978). Several species were exposed to aerosols of CN generated from molten CN in a Collison spray. The inhalation dose–mortality data allowed the calculation of the following $L(CT)_{50}$ values in mg min m^{-3}: rat (male) 8,750; rabbit (female) 11,480; guinea pig (female) 13,140; mouse (male) 18,200. Most animals died during the first 2 days postexposure, with the lungs being macroscopically congested and edematous. Histology showed congestion of alveolar capillaries and intrapulmonary veins, with intra-alveolar hemorrhages, and patchy acute inflammatory cell infiltration of the trachea, bronchi, and bronchioles. Animals dying after 48 h had bronchopneumonic changes. Animals with extensive lung injury had scattered areas of acute renal cortical and medullary necrosis, and a few had hepatic centrilobular necrosis. Survivors sacrificed 14 days showed no histopathology (Ballantyne and Swanston, 1978).

TABLE 15.4
Representative Acute LD_{50} Values for Peripheral Chemosensory Irritants (PCSI) to Various Species by the Intravenous (IV), Intraperitoneal (IP), and Peroral Routes

	LD_{50} (mg kg^{-1})		
PCSI[a]	IV (Rat, Male)	IP (Rat, Male)	Oral (Rat, Male)
CN	31	36	127
CS	27	48	1366
CR	68	817	7500
Capsaicin	0.56	7.6	190
PAVA	ND[a]	8.0	161

Source: Data from Ballantyne, B., *Toxicology*, 8, 347, 1977b; Ballantyne, B., *Chemical Warfare Agents: Toxicology and Treatment, Second Edition*, T.C. Marrs, R.L. Maynard, and F.R. Sidell, eds, John Wiley & Sons, Chichester, 2006a; Ballantyne, B. and Swanston, D.W., *Arch. Toxicol.*, 40, 75, 1978; Glinsukon, T. et al., *Toxicon*, 18, 215, 1980; Saito, A. and Yamamoto, M., *J. Toxicol. Sci.*, 21, 195, 1996.

[a] ND = no data.

The ophthalmic toxicology of CN was investigated as a solution in PEG300 in the concentration range 1%–10% (Ballantyne et al., 1975). The severity and duration of effects was concentration related. Mild lacrimation, blepharospasm, conjunctival hyperemia, and chemosis were all seen with 1%, and becoming severe at 10%. Keratitis was not seen with 1% CN, just detectable at 2%, and severe with 10%. The effect of a 15 min exposure to a CN aerosol at a mean atmospheric concentration of 719 mg m^{-3} (CT = 10,800 mg min m^{-3}) was studied; this resulted in just detectable excess lacrimation and conjunctival hyperemia, with mild blepharitis of a few days duration. The effect of solutions of CN in PEG300 (0.01%–0.75%) on corneal thickness was investigated by pachymetry. This showed increases in thickness with solutions of 0.02% and greater, with times to return to control values ranging from 2 days with 0.025% to 14 days with 0.75%. Also, the effect of solutions of CN in PEG300 (0.0625%–1.0%) on IOP was studied, which demonstrated statistically significant increases by 10 min postexposure ranging from 16% (0.125% CN) to 98% (1% CN). IOP decreased to control values by 1 h, except with 1% CN which was still elevated to 13%. CN caused skin sensitization with several animal studies (Rothberg, 1970; Chung and Giles, 1972).

Several repeated exposure studies have been conducted by inhalation exposure. Mice were exposed to CN at 87.6 mg m^{-3} for 15 min day^{-1} for 10 days. After 5 days, lungs had diffuse hemorrhages, perivascular swelling, alveolar capillary congestion, and bronchial inflammatory infiltrates; there were also hepatic centrilobular necrosis and coagulative renal tubular necrosis. After 10 days, more severe lung damage was apparent (Kumar et al., 1994). Rats and mice were exposed to CN aerosol concentrations ranging 4.8–64 mg m^{-3} for 6 h day^{-1} for 14 days. All rats exposed to 19 mg m^{-3} and all mice exposed to 10 mg m^{-3} died during the first week. As an extension, a subchronic study was conducted with rats and mice that were exposed to concentrations in the range 0.25–4.0 mg m^{-3} for 5 h day^{-1}, 5 days $week^{-1}$ for 13 weeks. All rats survived and mortality for mice was 1/10 at 0.5 and 4.0 mg m^{-3}. No clinical chemistry changes or histopathology were found in rats or mice (NTP, 1990a).

CN was investigated for oncogenic potential in a 2 year bioassay, in which rats were exposed to 0, 1, or 2 mg m^{-3}, and mice to 0, 2, or 4 mg m^{-3} (NTP, 1990a). Compound-related nonneoplastic lesions were seen in the respiratory tract of rats and mice. Neoplastic lesions were not seen in male or female mice or male rats, but female rats had an increase in mammary gland fibroadenomas, indicating equivocal evidence for carcinogenicity.

Observations in Humans. Cited PCSI values are eye, aerosol $TC_{50} = 0.3$ mg m^{-3}; respiratory, aerosol $TC_{50} = 0.4$ mg m^{-3} (Ballantyne, 1977a; McNamara et al., 1969). Punte et al. (1962a) exposed volunteers to a maximum inhalation dosage of 350 mg min m^{-3}; median particle size 0.6–1.1 μm. For irritant effects they found the 1 min $E(CT)_{50}$ was 213 mg min m^{-3}; the 2 min $E(CT)_{50}$ was 119 mg min m^{-3}; and the 3 min $E(CT)_{50}$ was 93 mg min m^{-3}. Symptoms included lacrimation, eye discomfort, blurred vision, nasal discharge, burning sensation in the nose and throat, and difficulty in breathing.

Skin contact with CN causes a primary contact dermatitis, exhibited as erythema, edema, vesication, purpura, and necrosis (Jolly and Carpenter, 1968; Penneys, 1971; Holland and White, 1972). Human skin patch testing and clinical experience with subjects exposed to CN have shown it to cause allergic contact dermatitis (Penneys et al., 1969; Penneys, 1971; Marzulli and Maibach, 1974; Fuchs and Weische, 1990).

The maximum safe inhaled dose for humans has been estimated at 50 mg min m^{-3} (Punte et al., 1962a). The human acute lethal inhalation dosage, extrapolated from animal data, has been estimated at 8,500–25,000 mg min m^{-3} (United Nations, 1969; WHO, 1970). Based on a statistical analysis of several studies conducted over a period of 47 years, McNamara et al. (1968) considered the best estimate of the $L(CT)_{50}$ for man is 7,000 mg min m^{-3} for pure aerosol, and 14,000 mg min m^{-3} for smoke from a commercial grenade. Several deaths have been attributed to overexposure to CN, especially with grenade-generated smokes in confined spaces (Gonzales et al., 1954; Stein and Kirwin, 1964; Chapman and White, 1978; Thornburn, 1982). Death was associated with severe airway lesions (necrosis of laryngeal, tracheal, and bronchial epithelium with pseudomembrane formation) and pulmonary edema. Calculations indicated that the exposure dosages in these lethal cases ranged from 41,000 to 145,500 mg min m^{-3}.

D. 2-Chlorobenzylidene Malononitrile (CS)

Also known as *o*-chlorobenzylidene malononitrile, CS has been used in military training operations, respirator testing, and enemy denial and dispersion; hostage and siege situations; civilian peacekeeping operations; personal protection devices. It is dispersed by grenades, projectile cartridges, aerosol, and coherent liquid stream dispensers; cluster bombs; aircraft dispensers; and portable powder disperser. Hydrophobic antiagglomerative powders have been used in fogging machines.

Toxicology. For solutions (in saline) PCSI values for blepharospasm are rabbit $= 5.9 \times 10^{-5}$ M, guinea pig $= 5.3 \times 10^{-5}$ M (Ballantyne and Swanston, 1973). By i.v. and i.p. injection, CS is of moderate lethal toxicity, but less toxic than CN perorally (Table 15.4; Ballantyne and Swanston, 1978). Animals that died following p.o. CS had extensive hemorrhagic erosions of the gastric mucosa, which was also congested and edematous. Survivors sacrificed 3 weeks postdosing had areas of regeneration in the gastric mucosa. Acute inhalation studies have been conducted with both pure aerosols and grenade-generated smokes of CS (Ballantyne and Callaway, 1972; Ballantyne and Swanston, 1978), for which $L(CT)_{50}$ values are given in Table 15.5. Lungs from animals that died after exposure to CS grenade smoke were macroscopically edematous and congested with variable sized hemorrhages. Histology demonstrated congestion of alveolar capillaries and intrapulmonary veins, alveolar hemorrhages, hemorrhagic atelectasis, and pulmonary edema. Survivors sacrificed 14 days postexposure showed no residual pathology. Similar histopathology was seen with the pure CS aerosol exposed animals. Other acute inhalation studies with CS smoke involved exposure of rats and hamsters as follows (Ballantyne and Callaway, 1972):

1. Exposure to 750 mg m^{-3} for 30 min did not cause mortalities. Animals sacrificed 1 day postexposure had minimal lung pathology (alveolar capillary congestion with a few alveolar hemorrhages). There was no histopathology in animals sacrificed at 10 and 28 days postexposure.

TABLE 15.5
Acute Lethal Inhalation Toxicity of CS as Grenade-Generated Smoke and Pure Aerosol to Various Species

	$L(CT)_{50}$ (mg min m^{-3})	
Species (Gender)	**Grenade Smoke**	**Pure Aerosol**
Rat (male)	68,000	88,480
Rabbit (female)	63,000	54,090
Guinea pig (female)	35,000	67,200
Mouse (male)	76,000	50,010

Source: Data from Ballantyne, B. and Callaway, S., *Med. Sci. Law*, 12, 43, 1972; Ballantyne, B. and Swanston, D.W., *Arch. Toxicol.*, 40, 75, 1978.

2. Exposure to 480 mg m^{-3} for 60 min caused mortalities in rats (10% males, 5% females) and hamsters (males 34%, females 25%). Lungs of rats that died had congestion of alveolar capillaries and intrapulmonary veins, intra-alveolar hemorrhages, and patchy pulmonary edema. A few rats showed centrilobular or midzonal hepatic necrosis, and kidneys had extensive tubular necrosis, mainly medullary. Hamsters that died within 48 h had pulmonary congestion, intra-alveolar hemorrhages, and pulmonary edema. Kidneys showed corticomedullary necrosis. Hamsters dying after 48 h showed minimal pulmonary lesions, but renal tubular necrosis was present.
3. Exposure to 150 mg m^{-3} for 120 min caused a few hamster deaths, with bronchopneumonia as the only pathological finding. Animals sacrificed at 1, 10, or 28 days postexposure showed minimal or no histopathology.

Rats and hamsters exposed to CS at 480 mg m^{-3} for 1 h or 150 mg m^{-3} for 2 h were sacrificed 32 months later. There were no effects on survival and no CS-related toxicity (Marrs et al., 1983a).

The skin irritating potential of CS was studied in rats, guinea pigs, and mice that received 12.5% solutions to trunk skin for 6 h. Erythema was more marked than edema, both of which resolved by 7 days postapplication (Ballantyne and Swanston, 1978). Animal studies indicate that CS is a hapten and can cause allergic contact dermatitis (Rothberg, 1970; Chung and Giles, 1972). CS does not modify the rate and mechanism of healing of cutaneous injuries (Ballantyne and Johnson, 1974b). The ophthalmic toxicology of solutions of CS in PEG300 was studied by Ballantyne et al. (1974). The threshold for inflammatory effects was 0.5%, and just detectable keratitis was seen at 1%, but the ocular effects and their duration depend on the solvent employed (Gaskins et al., 1972; Ballantyne, 1979). The effect of CS solutions (0.125%–5.0%) on IOP was investigated in rabbits (Ballantyne et al., 1974). Increases in IOP were concentration related, peaked at 10 min, and returned to control values by 1 h; increases ranged from 7% (0.125%) to 52% (5.0%).

Several repeated exposure studies by the inhalation route have been conducted. Various species were exposed to pyrotechnically generated smoke at concentrations ranging from 34.2 to 56.4 mg m^{-3} for 5 h day^{-1} for 1 to 7 successive days. The following $L(CT)_{50}$ values in mg min m^{-3} were obtained: guinea pig, 49,000; rabbits, 54,000; rats, 25,000; mice, 36,000 (Ballantyne and Callaway, 1972). Using pure CS aerosols that were thermally generated with a Collison spray, rats were exposed for 5 successive days to between 840 and 3,050 mg m^{-3} for 5 min day^{-1} for 5 successive days. There were no mortalities. Animals sacrificed during the first 2 days postexposure had minimal alveolar capillary congestion and scattered alveolar hemorrhages. In another study, rats were exposed to pure CS aerosols between 12.5 and 14.8 mg m^{-3} for 80 min day^{-1} for 9 successive days; there was 10% mortality (Ballantyne and Callaway, 1972). Punte et al. (1962b) exposed dogs to CS aerosol for 1 min day^{-1}, 5 days $week^{-1}$ for 5 weeks up to total cumulative

doses of 17,000 mg min m^{-3}, and rats for 5 min day^{-1}, 5 days $week^{-1}$ for 5 weeks up to cumulative doses of 91,000 mg min m^{-3}. No dogs died but 3/60 rats died during exposure after cumulative exposures doses of 25,000 and 68,000 mg min m^{-3}. There were no effects on electrolytes or creatinine and no gross pathology. Marrs et al. (1983b) exposed mice, rats, and guinea pigs to CS aerosol at concentrations of 3, 30, and 192–236 mg m^{-3} (MMAD 3–4 μm) for 1 h day^{-1} for 120 days, with sacrifice of 1 year from the first exposure. Excess mortality occurred in the high concentration group. Acute alveolitis was seen in guinea pigs that died. There was no pathology in rats or mice surviving 12 months, but guinea pigs had laryngitis and tracheitis. For a subchronic study, rats and mice were exposed to CS2 concentrations in the range 0.4–6.0 mg m^{-3} for 6 h day^{-1}, 5 days $week^{-1}$ for 13 weeks. Rats developed nasal lesions (erosions, hyperplasia, squamous metaplasia, and inflammatory cell infiltration) and laryngotracheal epithelial inflammation and hyperplasia; mice showed nasal mucosal inflammation and squamous metaplasia, and laryngotracheal epithelial inflammation and hyperplasia (NTP, 1990b).

CS, like CN, is an SN_2-alkylating agent that adducts with thiol groups and inhibits SH-containing enzymes, which may form the basis for some aspects of its acute toxicity. Major metabolites of CS are 2-chlorobenzyl malononitrile, *o*-chlorobenzaldehyde, and *o*-chlorohippuric acid (Feinsilver et al., 1971; Leadbeater, 1973). Due to its malononitrile content, when given by parenteral injection (Ballantyne, 1983), cyanogenesis may occur, but when absorbed by the respiratory tract cyanide generation is very limited, and not relevant to the toxicity of inhaled CS to man (see detailed discussion in Ballantyne, 2006a). Conventional developmental toxicology studies in rats and rabbits exposed to CS aerosols have shown the material is neither teratogenic nor embryotoxic (Upshall, 1973). Several *Salmonella typhimurium* reverse mutation assays have shown CS not to be mutagenic with or without metabolic activation (Reitveld et al., 1983; Wild et al., 1983; Zeiger et al., 1987; NTP, 1990b; Meshram et al., 1992). A Chinese hamster ovary (CHO) forward gene mutation test showed that CS produced four to five times more revertants to 6-thioguanine than controls (Ziegler-Skylakakis et al., 1989). CS was weakly mutagenic in a mouse L5178tk^+/tk^- lymphoma forward gene mutation assay (McGregor et al., 1988). Chromosomal aberrations and increased sister chromatid exchanges were seen with exposure of V79 CHO cells to CS (Bauchingher and Schmid, 1992). In a mouse in vivo micronucleus study, CS given i.p. or by gavage was not clastogenic (Wild et al., 1983; Grawe et al., 1997). In a chronic (2 year) toxicity/oncogenicity study, rats were exposed up to 0.75 mg m^{-3} CS and mice up to 1.5 mg m^{-3}. Nonneoplastic lesions were seen in the nasal cavity, but neither species showed any evidence for a carcinogenic response (NTP, 1990b).

Observations on Humans. PCSI values for studies in human volunteer are as follows: aerosol TC_{50}, ocular sensation $= 4.0 \times 10^{-3}$ mg m^{-3}; aerosol TC_{50}, respiratory tract $= 23 \times 10^{-3}$ mg m^{-3}; solution in saline, blepharospasm $EC_{50} = 3.2 \times 10^{-6}$ M; solution, corneal sensation $TC_{50} = 7.3 \times 10^{-7}$ M; solution, tongue sensation $TC_{50} = 6.8 \times 10^{-6}$ M; aerosol $IC_{50} = 3.6$ mg m^{-3} (Ballantyne and Swanston, 1973; Ballantyne, 1977a).

Skin erythema occurs following contact with CS, and two phases have been described; immediate erythema within a few minutes which persists for less than an hour, and after 2 h delayed erythema persisting for 24–72 h (Weigand, 1969). Heavy and sustained exposure of skin may also result in edema and vesication (Hellreich et al., 1967; Zekri et al., 1995; Salem et al., 2005c), and rarely chemical burns (BBC, 2006e), but primary contact dermatitis with CS is less marked than with CN (Holland and White, 1972). Contamination of the eyes of volunteers with 0.1% and 0.25% solutions of CS caused discomfort, blepharospasm, and transient conjunctivitis, but no corneal injury on slit-lamp biomicroscopy (Rengstorff and Mershon, 1969, 1971). Human epidemiological information provides no evidence that CS exposure is linked to teratogenic or embryotoxic outcomes (HMSO, 1971; Lancet, 1971; McElhatton et al., 2004).

Following the use of CS in the Northern Ireland riots during August 1969, newspaper reports appeared describing cases of diarrhea, notably in infants and young children (The Observer, 1969; The Times, 1969). The UK independent Committee of Enquiry into the use of CS believed that there

were certain features about the outbreak, including time to onset, which made them hesitant to ascribe it as being due to CS (HMSO, 1969). In studies involving human volunteer exposures to CS smoke, diarrhea was not a feature of postexposure symptomatology (Punte et al., 1963; Beswick et al., 1972). Also, laboratory studies involving gavage dosing of several species gave no evidence for an irritant effect of CS on the alimentary tract (Ballantyne and Beswick, 1972).

Whole body drenches of human volunteers were conducted with 0.001%–0.005% CS in aqueous 3.3% glyceryl triacetate (Ballantyne et al., 1976a). There was a rapid onset of stinging in the eyes and blepharospasm with increased blood pressure (BP). With 0.005% CS peak rises were measured at 1–2 min; mean SBP (systolic) increase was 31 mm Hg (range 5–50 mm Hg) and mean DBP (diastolic) increase was 19 mm Hg (range 0–40 mm Hg). Pressures returned to predrench values within 2–13 min. Prompt increases in BP have also been measured in volunteers exposed to CS smokes (Beswick et al., 1972).

Estimates for the human acute lethal inhalation dosage of CS vary between 25,000 and 150,000 mg min m^{-3} (WHO, 1970), but there have been no authenticated reports of death from exposure to CS smokes. Also, exposure to CS smokes did not have any effect on peripheral blood hematology, clinical chemistry, chest radiograph, peak airflow, tidal volume, vital capacity (Beswick et al., 1972), lymphocyte chromosomal morphology (Holland and Seabright, 1971), pulmonary gas transfer, and alveolar volume (Cotes et al., 1972a), but a reduction in exercise ventilation volumes was recorded (Cotes et al., 1972b). Reactive airways dysfunction syndrome (RADS) has been described following exposure to CS (Hu and Cristani, 1992; Bayeaux-Dunglas et al., 1999; Worthington and Nee, 1999), and a case of persistent multisystem hypersensitivity reaction was described in a male heavily sprayed with CS solution (Hill et al., 2000).

E. Dibenz(b.f)-1,4-oxazepine (CR)

CR can be used as an incapacitant in solution dispersed as a coherent stream from a handheld canister, as an aerosol, and as grenade-generated smoke. Reported uses in peacekeeping are few but according to papers released under the UK FOI legislation, CR has been used to quell prison riots in Northern Ireland apparently by aerosol spray and in liquid filled frangible capsules dropped from helicopters (Morrison and Bright, 2005).

Toxicology. PCSI studies with CR in saline solution gave the following values from animal studies: blepharospasm EC_{50}, guinea pig $= 3.5 \times 10^{-5}$ M, rabbit $= 7.9 \times 10^{-5}$ M (Ballantyne and Swanston, 1974). Acute toxicology studies indicate that CR has a low order of acute lethal toxicity by i.v., i.p., and p.o. routes (Table 15.4), and doses >1500 mg kg^{-1} applied cutaneously under occluded conditions did not cause any mortalities or signs of systemic toxicity, with mild erythema being the only local sign. Acute inhalation studies have been conducted with pure aerosols of CR generated by blowing air through molten CR (Ballantyne, 1977b). In rats, this caused blepharospasm and nasal secretions during exposure, but no mortalities up to the highest exposure dosage of 428,400 mg min m^{-3}. With rabbits, guinea pigs, and mice exposed to up to 133,170 mg min m^{-3}, only one guinea pig died, and at the highest exposure dose of 169,500 mg min m^{-3} moralities were rabbits, 9/20; guinea pigs, 5/20; and mice, 2/40. Thus, the $L(CT)_{50}$ was >169,500 mg min m^{-3}. With hamsters, there were no mortalities up to and including 233,200 mg min m^{-3}; above this mortalities were as follows: 370,000 mg min m^{-3} (1/10) and 440,400 mg min m^{-3} (2/20). Rats, rabbits, guinea pigs, and mice were exposed to pyrotechnically generated smokes of CR for 20–120 min at concentrations ranging from 2,467 to 4,025 mg m^{-3}. Exposure–mortality data gave the following $L(CT)_{50}$ values in mg min m^{-3}: rats, 139,000; rabbits, 160,000; guinea pigs, 169,000; and mice, 203,600. Animals that died up to 3 days postexposure had congestion of alveolar capillaries and intrapulmonary veins, multiple focal intra- and interalveolar hemorrhages, and pulmonary edema. Animals dying after 4 days had pulmonary congestion, hemorrhages, and edema, with bronchiolitis and neutrophil infiltration of the alveoli. Studies comparing the acute inhalation toxicity of pyrotechnically generated CR smoke with that of smoke from the burning mix alone indicate that the

experimental acute lethal toxicity of CR smoke is in part due to a contribution from the combustion products of the burning mix (see Table 15.2).

The ophthalmic toxicology of CR in solution up to 10% in PEG300 was studied in rabbits (Ballantyne et al., 1975). With 1% and 2%, effects (excess lacrimation, conjunctival hyperemia, chemosis, and blepharitis) were just detectable to mild and of 24 h or less duration. A Concentration of 10% CR caused a just detectable keratitis of a few days duration, and 5% was the threshold concentration for keratitis. Measurement of the effect of CR (0.05%–10.0%) on corneal thickness in vivo showed peak increases between 1 and 6 h postinstillation, with return to normal values by 2–10 days. Tonometry showed concentration-related peak increases in IOP at 10 min, ranging from 20% to 40% for 0.5%–5.0% CR. Rabbits exposed for 30 min to a CR aerosol at 360 and 571 mg m^{-3} showed only just detectable excess lacrimation and conjunctival injection of 1 h duration.

Several repeated exposure studies by various routes of exposure have been conducted. By gavage, rats, rabbits, and guinea pigs received 10% of the species LD_{50} for 5 successive days; there were no mortalities, no clinical signs, no significant effects on feces, and no gross or microscopic pathology; hematology and clinical chemistry conducted in rabbits were not affected (Ballantyne, 1977b). Rabbits had daily ocular contact with 0.05% CR for 10 successive days and a 5 month follow up. Just detectable transient conjunctival hyperemia occurred during the treatment period, but no effects during the 5 month posttreatment were observed (Ballantyne et al., 1975). Repeated exposure of the rabbit eye to 5% CR for 5 days $week^{-1}$ for 4 weeks resulted in transient conjunctivitis but no abnormalities were found on slit-lamp biomicroscopy. Light and electron microscopy of the cornea at 32 days after the final application did not show any injury (Rengstorff et al., 1975). Subchronic (12 weeks) repeated cutaneous contact of mice with CR followed by an 80 week observation period showed only a high incidence of fatty infiltration of the liver, but without other microscopic pathology (Marrs et al., 1982). In a subchronic inhalation exposure study, hamsters and mice were exposed to a CR aerosol (MMAD 2.86 ± 1.17 μm) at concentrations of 204 (5 min), 236 (8.6 min), and 267 (15.8 min) mg m^{-3} for 5 days $week^{-1}$ for 18 weeks, and survivors were sacrificed 1 year after the first exposure (Marrs et al., 1983c). The only exposure-related pathology was an increased incidence of chronic laryngeal inflammation.

CR aerosol is absorbed rapidly by the respiratory tract with a $t_{1/2}$ of about 5 min (Upshall, 1977b). The major metabolic pathway for CR is oxidation to the lactam, subsequent ring hydroxylation, sulfate conjugation, and renal excretion (French et al., 1983a, 1983b). Developmental toxicology studies have shown CR not to be embryotoxic or teratogenic by gavage or aerosol exposure (Upshall, 1974). CR was not genotoxic in *S. typhimurium* reverse mutation tests, CHO forward gene mutation test (HGPRT locus), mouse lymphoma assay, and a micronucleus test (Colgrave et al., 1983).

Observations on Humans. Skin patch tests with 0.001%–1.0% CR solutions for 5–30 min contact caused only erythema of 2–4 h duration (Weigand and Mershon, 1970). Repeated skin patch testing of human volunteer subjects demonstrated that CR does not cause contact sensitization (Holland, 1974). Whole body drenches of human volunteer subjects were conducted using 0.001%–0.0025% CR in 3.3% aqueous dipropylene glycol monomethyl ether (Ballantyne et al., 1976a). This resulted in immediate discomfort and pain in the eye with blepharospasm and increased lacrimation, all of which began to subside by 3–5 min. Mean peak increase in SBP was 45 mm Hg (range, 30–70 mm Hg) after 0.001% CR and 59 mm Hg (range, 30–80 mm Hg) after 0.0025% CR; mean peak increase in DBP was 23 mm Hg (range, 15–30 mm Hg) with 0.001% CR and 29 mm Hg (range, 20–45 mm Hg) with 0.0025% CR. Peak values were attained by 1–3 min postdrenching and decreased to control (predrench) values within 2–13 min. The effect of CR solutions on IOP was studied in volunteer subjects who had 0.04 mL of either 0.05% or 0.1% CR in PEG300 applied to the corneal surface. Peak increases in IOP for the treated eye were measured at 5 min; 40% increase with 0.05% CR and 44% with 0.1% CR. IOP decreased to control values by 15 min with 0.05% CR and 3.5 h with 0.1% CR (Ballantyne, 1977a). The human acute $L(CT)_{50}$ for CR is probably in excess of 100,000 mg min m^{-3} (Ballantyne, 1977a).

F. Oleoresin Capsicum (OC)

Oleoresin capsicum is an oily reddish-brown extract of pepper plants of the genus *Capsicum*, principally *Ca. annuum* and *Ca. frutescens*. Depending on the variety of the plant, OC contains 0.01%–1.0% capsaicinoids (Cooper et al., 1991), but the composition is highly variable depending on factors such as plant growth conditions, maturity, and extraction conditions. Capsaicin and dihydrocapsaicin constitute 80%–90% of the OC extract.

The mechanism by which capsaicin stimulates neurones involves action on a subpopulation of neuropeptide-containing afferent neurones and activation of a specific receptor (the ''vallinoid'' receptor) that recognizes capsaicinoid molecules (Szallasi and Blumberg, 1999). Activation of vallinoid receptors leads to opening of receptor operated cation channels (Marsh et al., 1987; Wood et al., 1988), and the influx of Ca^{++} and Na^{+} leads to depolarization which triggers local release of neuropeptides, including substance P, central protective reflexes, and autonomic motor responses (Martling, 1987; Stjarne, 1991). A transient excitement of primary afferents is followed by a more prolonged refractory period and desensitization. For self-protection and larger scale peacekeeping operations, OC is dispersed as an aerosol ''spray'' from devices of varying capacity. These are used by police and security agencies in many countries, but not in the United Kingdom because of problems associated with toxicology testing and hazard evaluation of complex mixtures of varying composition (Ballantyne, 2006a). The New York State Department of Health has determined that OC poses a lower public health concern than either CN or CS, and it developed a rule that specifies OC as the only active ingredient to be used in self-defense sprays in New York State (Recer et al., 2002). The capsaicinoid content of extracts used in ''pepper spray'' varies with manufacturer; 1.2%–12.6% being a typical range (Smith and Stopford, 1999). Such sprays cause severe ocular pain, lacrimation, blepharospasm, nasal irritation, bronchospasm, coughing, sneezing, shortness of breath, and burning sensations in skin. The effectiveness of OC sprays varies with total concentration of extract used, and the variability of the capsaicinoids composition of the extract used. Commercially available OC aerosol spray devices include Cap-Stun and Punch II (Table 15.3). Much of the testing and research has been conducted on capsaicin, and not on OC.

Toxicology. Capsaicin is of high acute i.v. and i.p. toxicity, and moderate acute p.o. toxicity (Table 15.4). Animals given OC perorally show gastric mucosal hyperemia, edema, focal hemorrhages, and focal necrosis. Acute inhalation $L(CT)_{50}$ values for OC are 835,000 mg min m^{-3} for rats and 270,000 mg min m^{-3} for mice. Capsaicin (1%) applied to the rat eye causes neurogenic inflammation and loss of reaction to mechanical and chemical stimuli for up to a week. It is well appreciated that repeated dosing with capsaicin produces systemic desensitization to chemogenic and thermal nociceptive stimulation (Hayes et al., 1981; Miller et al., 1981). In addition to stimulating afferent receptors and causing a PCSI effect, OC also causes the release of tachykinins and neuropeptides, including substance P, which induce neurogenic inflammatory effects of the airway blood vessels, epithelial cells, glands, and smooth muscle, leading to vasodilation, increased vascular permeability, neutrophil chemotaxis, mucus secretion, and bronchospasm (Smith and Stopford, 1999). Studies in rodents on the effects of capsaicin on pulmonary inflammation associated with infections demonstrated that during *Mycoplasma pulmonis* infection a 30-fold increase in neurogenic plasma extravasation occurred that persisted for several weeks (McDonald, 1992). Capsaicinoids undergo hepatic phase I metabolism involving oxidative and nonoxidative paths (Kawada and Awai, 1985; Oi et al., 1992; Reilly et al., 2003).

Pregnant rats dosed with OC (50 mg kg^{-1}) on gestation days 15–16 and 16–17 produced pups with reduced crown–rump length but no other indications of reproductive and developmental abnormalities (Kirby et al., 1982). Pregnant rats and rabbits dosed continuously with pure trans-capsaicin produced no evidence for developmental toxicity (Chanda et al., 2005). Male mice given capsaicin i.p. at doses of 0.4, 0.8, and 1.6 mg kg^{-1} for 5 consecutive days had no alterations in epididymal weights, caudal sperm counts, sperm morphology, testicular weights of testicular histology (Narasimhamurty and Narasimhamurty, 1988). Some studies on the genotoxicity of

capsaicin are equivocal due to variability in purity or to metabolism. Bacterial mutagenicity tests have given variable results, but generally are only weakly positive (reviewed by Ballantyne, 2006a). A mouse bone marrow micronucleus test was positive with fractionated capsaicinoids (Villesansor and de Campo, 1994) and with purified capsaicin (Arceo et al., 1995). Capsaicin is metabolized by hepatic P-450-dependent mono-oxygenases to a phenoxy radical intermediate that can bind to proteins and nucleic acids. The activated metabolite can produce toxic, mutagenic, and carcinogenic events, or interactions with other factors that may inhibit such activity. Capsaicin and dihydrocapsaicin inactivate cytochrome P450 IIE1 and other microsomal mono-oxygenases, and this can result in chemoprotective effects against other chemicals that require metabolic activation (Suhr and Lee, 1995).

Observations on Humans. Studies involving the topical application of several products containing various capsaicinoids analogs at various concentrations to the skin of Caucasians and Asians showed that mean skin surface temperature and transepidermal water loss were highly variable and did not demonstrate a dose responsive behavior to increasing capsaicinoid concentrations. Erythema, measured by increase in reflectance colorimetry (reflected light in the red to green color spectrum), correlated well with relative and total capsaicinoids, and this approach may be valuable in evaluating the efficacy of pepper spray products in humans (Pershing et al., 2006). Ten volunteer subjects were sprayed with OC (5.5%) and followed for up to 1 month. They reported mild-to-moderate facial stinging (mean duration 24 min; range, 4–50 min) and development of facial erythema with nasal congestion (Vesaluoma et al., 2000). There was associated tachycardia, with rate increase from 80 to 116 bpm. By 20 min after spray, 4 subjects developed focal corneal epithelial damage, which resolved within 24 h. The investigators concluded that acute exposure to OC may produce minor corneal effects, but caution should be exercised with repeated exposures, which could be associated with prolonged changes in corneal sensitivity. Nasorespiratory responses to capsaicin and OC include sneezing, rhinorrhea, wheezing, dry cough, shortness of breath (Stefee et al., 1995), and tachyphylaxis (Blanc et al., 1991). Capsaicin may cause transient dose-dependent bronchoconstriction, and a 20%–50% increase in airways resistance has been demonstrated at doses that did not induce cough (Fuller, 1990). Observations on persons cutaneously exposed to capsaicinoids reveal that they experience local tingling, burning sensations, erythema, edema, and sometimes vesiculation. Capsaicinoids given orally to volunteers cause burning sensations in the mouth, throat, chest, and abdomen; nausea; vomiting; and diarrhea; with gastric hyperemia, edema, and focal petechiae (Forrester and Stanley, 2003; Ballantyne, 2006a).

A review of 100 cases collected over a 3 year period of prison inmates treated at an emergency ward following being sprayed with OC showed the major presenting features were burning sensation in the eyes and conjunctival hyperemia. They also complained, less commonly, of local skin burning with erythema, shortness of breath, wheezing, and cough. Corneal abrasions were occasionally identified (Watson et al., 1996). However, the following clinically relevant effects have been reported following exposure to capsaicin and OC: acute hypertension, headache, augmentation of allergic reactions, bronchospasm, and laryngospasm (Stefee et al., 1995; Smith and Stopford, 1999). Several deaths have been described when OC was used in conjunction with physical restraint or adverse health factors, including positional asphyxia, cocaine/phencyclidine/amphetamine intoxication, and neuroleptic malignant syndrome (Granfield et al., 1994; Stefee et al., 1995; Busker and van Helden, 1998; McLaughlin and Siddle, 1998). Although a causal relationship with OC has not been established, a contributory role cannot be excluded (Ballantyne, 2006a).

G. Pelargonic Acid Vanillylamide (PAVA)

Pelargonic acid vanillylamide, also known as nonivamide, is a synthetic equivalent of capsaicin (synthetic capsaicin). It is available in a commercial spray product known as Cap-Tor, which is being increasingly used by UK police authorities and in European countries to replace CS irritant liquid sprays (BBC, 2001, 2002, 2004b). It is advised that the device should be aimed at the face, particularly the eyes. Most spray devices contain 0.3% PAVA in 50% ethanol with nitrogen as the propellant. The

maximum effective range is 8–15 ft (COT, 2002), but devices should not be discharged at less than a distance of 3 ft because of risk of pressure injury to the eye. Particle sizes are mainly >100 μm, with a small proportion in the range 2–10 μm, and a trace <2 μm. The toxicology and clinical experience for PAVA and its formulation, as summarized below, is not yet sufficient enough to permit a complete assessment of the potential human health effects of the material.

Toxicology. The EC_{50} for PAVA in a guinea pig blepharospasm test was 0.15 μM (Battensby et al., 1981). PAVA is of moderate acute i.p. and p.o. toxicity (Table 15.4). By 4 h occluded application to rabbit skin, 3.3% PAVA in polyethylene glycol produced no signs of local irritation during a 3 day postapplication inspection period (COT, 2002). A rabbit eye irritation study with 0.3% PAVA in 50% aqueous ethanol showed significant irritation, including some corneal opacity, until 3 days postinstillation; all eyes were normal by 7 days (COT, 2002). It is thus possible that people wearing contact lenses who are sprayed with PAVA solutions could be at risk for more exaggerated ocular response. A 90 day dietary feeding study with PAVA at a dosage of 10 mg kg^{-1} day^{-1} produced no evidence for cumulative toxicity, and was a NOAEL under these limited conditions (Postemak et al., 1969).

Pelargonic acid vanillylamide has a moderate degree of percutaneous absorption in the rabbit; 50%–70% over 14 h when applied occlusively as an ointment in oil–water emulsion (Fang et al., 1996). More limited absorption was reported for the rat (12% over 72 h) using phosphate buffered saline under nonoccluded conditions (Kasting et al., 1997). However, in vitro rat skin permeability studies demonstrated that PAVA in 50% ethanol was absorbed more rapidly than when dissolved in phosphate buffered saline (Kasting et al., 1997). Following absorption, PAVA is extensively metabolized and rapidly excreted; the main route of metabolism is by hydrolytic cleavage of the amide bond with some aliphatic hydroxylation (Suhr et al., 1995; Kasting et al., 1997). There are no detailed published reports on the reproductive toxicity of PAVA. A developmental toxicity study was conducted by gavage in rats (Knox and McKenzie, 2003). Time-mated animals were given doses of 0, 100, 500, and 1000 mg kg^{-1} day^{-1} over gestational days 5–19. Maternal toxicity was not noted. Fetal body weights were statistically significantly reduced at 100 mg kg^{-1} day^{-1}. There were no effects on viability, gender ratio, or of visceral and skeletal abnormalities. Studies on the genotoxicity of PAVA are as follows (COT, 2002). In vitro, bacterial reverse mutation assays were negative, a mouse lymphoma test was weakly positive, and a CHO chromosome aberration test was positive at concentrations that were not cytotoxic. In vivo, a bone marrow micronucleus test showed no evidence for a clastogenic potential.

Observations on Humans. It has been calculated that for a 1 s burst from a standard PAVA, handheld canister, cutaneous exposure would be of the order of 30 mg PAVA, of which about 3 mg would be absorbed resulting in a low systemic dosage of 0.04 mg kg^{-1}. The inhalation effects of a PAVA respirable aerosol generated from a nebulizer were studied in control (normal) and ''mildly asthmatic'' subjects (10 per group; COT, 2002). The controls were exposed to aerosols generated from solutions up to 0.3% PAVA in 50% aqueous ethanol, and the asthmatic subjects were exposed to aerosols generated from solutions up to 0.1% PAVA. Transient coughing was noted in normal exposed subjects. Minimal effects were seen on FEV_1 (1% decrease), cardiac rate (15% increase), and blood pressure (8% increase in SBP). Similar effects were seen with asthmatics: 3% increase in FEV_1, 5% increase in cardiac rate, and 5% increase in SBP; however, a clinically significant reduction in FEV_1 (>0.5 L) was noted in two subjects who had a more severe asthma based on greater methacholine response. The investigators considered the findings to indicate that aerosols generated from 0.3% PAVA in 50% aqueous ethanol are unlikely to have significant adverse respiratory tract effects in healthy humans, but that bronchospasm could be induced in asthmatics and this might compound with the effects from the stress of the situation.

In a limited number of instances, there is the possibility for coexposure to both PAVA and CS aerosols sprays. It has been stated that the information base on which to express an opinion about combined exposure with CS and PAVA is very limited, but it is likely that no more than additive

effects will occur on skin, eyes, and respiratory tract, although there is the possibility that a lower response might occur in some individuals due to desensitization by PAVA; there is uncertainty about the effects of coexposure on the asthmatic subpopulation (COT, 2006).

V. MENTAL (CENTRAL) INCAPACITANTS

Under this classification are pharmacological agents that impair the ability of a subject to perform coordinated tasks by decreasing motor activity or conscious state or producing tranquilization through mechanisms in the CNS. Depending on the effects produced, they are also referred to as central incapacitants or psychomimetic agents. Also in this group can be included centrally acting emetics. Unlike PCSI materials, central incapacitants produce a disabling condition that persists for hours or days after exposure. Clearly, in order for these neuropharmacological agents to act they must be delivered systemically to attain the CNS; for example, by dart gun for use against a single or a few individuals or dispersed in the atmosphere for larger groups. Equally clear is the fact that because these are agents that can modify physical, conscious, and mental state through a central neuropharmacological effect, their use requires that authorization is given at the highest medical and political levels, and the absolute need for their use is confirmed and documented. To use these agents, security forces would require additional training, they should be employed only where there is no likelihood for exposure of noninvolved bystanders, and local emergency and medical services need to be given advanced warning of their use. Misuse could, and as with the Chechen affair at the Moscow Opera, result in international adverse comments and discussion in news media and other political arenas. Therefore, it is not envisaged that central incapacitants would be used in civil uprisings, but more likely in, (1) hostage and terrorist situations where there has been a clear indication of lethal danger to hostages or members of the general population, and (2) as a CW agent to impair the performance and motivation of enemy soldiers. Agents of choice should have the following characteristics: effective at low doses (i.e., high pharmacological potency); have a high ratio of toxic/effective dose; not produce permanent or long-term neurotoxic or other adverse effects; have a sufficient duration of action to meet the operational needs; and ideally have an antidote for reversal of effects. Neuropharmacological agents that have been suggested for use as incapacitants include cannabinols, amphetamines, phenothiazines, *N,N*-diethyl lysergamide (LSD), quinuclidinyl benzylate (BZ), and fentanyls (WHO, 1970). Two are reviewed briefly below, one potentially useful in severe situations and one that has been used in practice with disastrous results.

A. 3-Quinuclidinyl Benzylate (BZ)

BZ, a CNS depressant, is an atropine-like drug that blocks the muscarinic action of acetylcholine at central and peripheral cholinergic synapses. It is a solid at NTP and sufficiently stable to be disseminated as a smoke from pyrotechnic devices. Percutaneous absorption is a possible mode of exposure with a suitable formulation, but the outcomes are likely to be more variable than with aerosol inhalation with respect to time to onset, severity, and overall sign/symptom complex (WHO, 1970). The $E(CT)_{50}$ producing a significant degree of military incapacitation has been stated to be 110 mg min m^{-3} (WHO, 1970). The human safety margin (lethal/incapacitating dose) is estimated at around 30 (RAMC, 2002).

Within 1–2 h of exposure to BZ, there is mydriasis, dry mouth and skin, tachycardia, drowsiness, and after about 4 h, CNS anticholinergic effects develop, including a confusional state with delusions, hallucinations, erratic behavior pattern, and interference with cognitive functions that include problem solving, attention, and comprehension. These effects may persist for several days, during which time the subject is susceptible to injury. Memory for this period of confusion may be lost or be fragmentary. The induced delusional state makes it questionable whether BZ should be used in hostage situations.

Management of those exposed to BZ should include temperature monitoring to detect hyperpyrexia resulting from the peripheral anticholinergic effects of BZ causing hyphidrosis or anhidrosis.

This may call for maintenance of fluid intake, and if a marked increase in temperature occurs, cooling is indicated. If there is continued mental aberration then the antidote physostigmine can be given as 2–3 mg IM for alleviation, followed by repeat injections at 15–60 min intervals to prevent relapse, and if required a slow i.v. infusion. The dose should be determined by the clinical condition of the subject. Another antidote is 7-methoxytocrine (7-MEOTA). For mild intoxication an oral dose of 100 mg can be given. For more severe poisoning, IM dosing (50 mg) is more effective.

B. Fentanyl and Analogs

Fentanyl, *N*-phenyl-*N*-[1-(2-phenylethyl)-4-piperidinyl] propanamide, and its analogs are narcotic analgesics and anesthetics, which act as opiate agonists. They have high lipid solubility and penetrate the blood–brain barrier easily, rapidly producing analgesia, euphoria, miosis, and respiratory depression. The depression of breathing is caused by direct inhibition of rhythm-generating respiratory neurones in the preBoetzinger complex of the brain stem. Selective activation of 5-hydroxytryptamine 4(a) receptors in this area by 5-HT4 receptor agonists abolishes the opiate-induced respiratory depression without loss of analgesic effect. Dissociation of the respiratory depression effect from the opiate-induced sedative effect can be accomplished by the use of antagonists such as naloxone, naltrexone, and nalmefene (Salem et al., 2005a). Fentanyls have been used as drugs of abuse. Human volunteer studies using low i.v. doses have shown that fentanyl produces impaired coordination, dysphoria, and somatic symptomatology, with a transient liking for the experience (Zacny et al., 1992).

The adverse medical and political consequences of using fentanyl as a mental incapacitant have been well documented in respect of its use by Russian security forces in a hostage situation. This occurred as a result of Chechen rebels holding about 800 people as hostages in a Moscow theatre during late October 2002. Apparently, pumping fentanyl vapor into the theatre for about 30 min resulted in the deaths of 119 hostages at the scene of the incident and over 200 required hospital admissions. The situation was complicated by the fact that neither the security forces nor responsible government officials informed local hospitals or health personnel what was the nature of the agent used, and it was some 5 days after the incident that there was an open declaration of what substances were used (Ballantyne and Salem, 2004; Salem et al., 2005a). However, there is still uncertainty about the conditions of use of fentanyl; some have suggested that a mixture of fentanyl and halothane was used, and others believe that massive doses of carfentanil (a potent opioid) were used to saturate the theatre space so that a maximum effect by inhalation could be achieved (Salem et al., 2005a). It has also been considered that remifentanil may have been used because of its high potency, brief latency to onset, and short duration of action.

VI. MALODORANTS

The use of unpleasant malodorous compounds has been suggested as a means of deterring the less motivated of participants in a civil disturbance because of the resultant psychological and physiological effects, such as olfactory repulsion and nausea (Whitten et al., 1970; National Academy of Sciences, 2003). They can be employed for deliberate contamination of individuals, clearing of areas, or for nonlethal terrain denial purposes. Applications could include control of civil unrest, personal protection, hostage rescue, counterinsurgency, and urban combat in enclosed areas. Malodorants can be delivered by using frangible missiles, and addition of thickening agents will prolong adhesiveness. One advantage is that perception of malodors and associated psychological and behavioral effects usually occurs at air concentrations considerably much lower than those that cause a PCSI response or acute toxicity. In this manner, malodorous substances can be used for dispersal and avoidance purposes without the complications of peripheral sensory irritation that produce harassment and a potential for self-accidents. Thus, malodorants can be used as nonlethal weapons where injury to the targeted subjects and surrounding environment must be minimal.

Malodorants can deter individuals based on a variety of physiological and psychological responses, including the following: (1) Offensive odors are often perceived as being associated with health risks (e.g., rotting food, feces) and as such result in an avoidance reaction (Dalton, 1996, 1999). (2) Odors perceived as particularly offensive can induce feelings of ill-health such as nausea, headache, dizziness, and cause gagging or vomiting, all associated with a desire to vacate the area of exposure (Bickford et al., 2000). The choice of malodorant agents should take the following into account: (1) appropriate physical properties such as liquid of high vapor pressure at ambient temperature; (2) do not produce adverse local skin or eye effects; (3) do not cause acute or repeated exposure systemic toxicity; (4) are not detrimental to the environment; and (5) capable of ready decontamination. A variety of chemicals have been considered for use as malodorants or in malodorant mixtures (Pinney, 2001): these are organic sulfur compounds (Table 15.6), carbonyl

TABLE 15.6
Organic Sulfur Compounds Considered for Malodorant Formulations

Methyl mercaptan
Ethyl mercaptan
Propyl mercaptan
iso-Propyl mercaptan
Butyl mercaptan.
iso-Butyl mercaptan
sec-Butyl mercaptan
tert-Butyl mercaptan
Amyl mercaptan
sec-Amyl mercaptan
iso-Amyl mercaptan
tert-Amyl mercaptan
n-Hexyl mercaptan
n-Heptyl mercaptan
n-Octyl mercaptan
sec-Octyl mercaptan
n-Nonyl mercaptan
tert-Nonyl mercaptan
n-Decyl mercaptan
Undecyl mercaptan
n-Dodecyl mercaptan
tert-Dodecyl mercaptan
tri-iso-Butyl mercaptan
Phenyl mercaptan
Benzyl mercaptan
d-Limonene mercaptan
Perchloromethyl mercaptan
3-Methyl-1-butanethiol
2-Mercaptoethanol
2-Ethanedithiol
3-Methyl-1-butanethiol
Methyl sulfide
Dimethyl sulfide
Ethyl sulfide
Diethyl sulfide

TABLE 15.6 (Continued)
Organic Sulfur Compounds Considered for Malodorant Formulations

Propyl sulfide
iso-Propyl sulfide
di-n-Propyl sulfide
Ethyl methyl sulfide
Dimethyl disulfide
Butyl sulfide
Dibuytyl sulfide
Trimethylene sulfide
Ethylene sulfide
Propylene sulfide
Allyl sulfide
cyclo-Propyl phenyl sulfide
Dimethyl trisulfide
Dimethyl sulfoxide
Propyl allyl sulfoxide
3-Chloropropyl *n*-octyl sulfoxide
Allyl *n*-octyl sulfoxide
2-Methanallyl *n*-octyl sulfoxide
Methanesulfonioc acid
Methanesulfonyl chloride
Ethenesulfonyl chloride
Propanesulfonyl chloride
Benzothiazol
2-Mercaptobenzothiazol
N-iso-Propyl-3-benzothiazolesulenamide
N,N-di-iso-Propyl-2-benzothiazaolesulfenamide
N-tert-Butylmercapto-2-benzothiazolesulfenamide
N-cyclo-Hexyl-2-benzothiazolesulenamide
N-N-dicyclo-Hexylbenzothaizolesulfenamide

compounds (Table 15.7), and organic nitrogen or phosphorus compounds (Table 15.8). Examples of malodorant formulations are given in Table 15.9. For descriptive and definitional purposes the following terms are used: *odor detection threshold* is the concentration of gas or vapor that can be discriminated from fresh air; *recognition threshold* is that concentration at which the identity of the odor can be specified; and *odor intolerance threshold* is the concentration at which the physiological and psychological effects are produced in an exposed individual causing a desire for avoidance.

The major (50%–75%) components of the malodorants in the skunk (*Mephitis mephitis*, *Spilopgale gracilis*, and *Conepatus mesoleucus*) defensive secretions are (E)-2-buten-1-thiol and 3-methyl-1-butanethiol. Other disulfides, thioacetates, and thiols are present in lesser amounts, and vary with species. In addition to the S-containing compounds, the alkaloid 2-methylquinoline has been identified in skunk secretions (Wood, 1990; Wood et al., 1991, 1993). Brief notes on examples of representative malodorants are given below.

n-Butyl mercaptan. Also known as 1-butanethiol, it is a colorless flammable liquid having a strong and obnoxious garlic-like odor. It has a high vapor pressure (83 mm Hg at 38°C; MSDS, 2005a). The odor threshold is 0.1–1 ppb, and it is stated to have a ''readily noticeable'' odor at concentrations between 0.1 and 1 ppm. The ACGIH TWA_8 TLV is 0.5 ppm based on irritation, CNS effects, and reproductive toxicity (ACGIH, 2006). Oral LD_{50} values (rat) are cited as 1500 and 1800 mg kg^{-1} and the rat i.p. LD_{50} is 399 mg kg^{-1} (Fairchild and Stokinger, 1958; MSDS, 2005a). Published

TABLE 15.7
Organic Nitrogen Compounds and Organic Phosphorus Compounds Considered for Malodorant Formulations

1,4-Diaminobutane [putrescine]
1,5-Diaminopentane [cadaverine]
4-Fluoro-α-methylbenzylamine
2-Ethylpyridine
3-Ethylpyridine
4-Ethylpyridine
2-Ethyl-3-methylpyrazine
3-Ethyl-2-methylpyrazine
2,3-Diethylpyrazine
1-Methylpyrrolidine
1-(Dimethylamino)pyrrole
3-Methylindole
4-Methylindole
6-Methylindole
2,3-Diethyl-1-methylindole
Ethyl 1-azetidepropionate
S-(+)-2-Methylbutyronitrile
Butyl *iso*cyanide
1,1,3,3-Tetramethylbutyl *iso*cyanide
1H-Benzotriazol-1-yl methyl *iso*cyanide
iso-Phorone di-*iso*-cyanate terminated poly(neopentyl glycol adipate)
Trimethylphosphine
Trimethylphosphine–silver iodide complex
Triethylphophine
Tripropylphosphine
Dichloro *iso*propylphosphine
Dimethylphenyl phenylphosphine
Ethyldiphenylphosphine
Dimethyl phosphate
Trimethyl phosphate
Dimethyl methylphosphonate
1,2-*bis*(Dichlorophosphine)ethane
1,3-Dimethyl-2-phenyl-1,3,2-diazaphospholidine

vapor 4 h LC_{50} values are 4020 ppm (rat) and 2500 ppm (mice); signs included increased breathing rate, incoordination, staggering gait, weakness, partial muscle paralysis, and sedation (Fairchild and Stokinger, 1958). Rabbit eye irritation is mild to moderate, but skin irritation is very slight (NIOSH, 1978). In a developmental study, rats and mice were exposed to 0, 10, 68, or 152 ppm *n*-butyl mercaptan for 6 h day^{-1} over gestation days 6–19 (rats) and 6–16 (mice). With mice there was maternal mortality and embryotoxicity at 68 and 152 ppm, and an increased incidence of cleft palate was noted at 10 and 68 ppm. Rats showed decreased body weight gain at 68 and 152 ppm, but there were no indications of embryotoxicity or teratogenic effects (Thomas et al., 1987). Accidental exposure of workers for 1 h to about 50–500 ppm caused muscle weakness, malaise, sweating, nausea, headache, and confusion. They showed facial flushing, increased breathing rate, and mydriasis (NIOSH, 1978). The majority of workers recovered in 1 day.

tert-Butyl mercaptan. Also referred to as 2-methyl-2-butanethiol, it has a strong offensive odor, and respective p.o. and i.p. LD_{50} values in rats of 4729 and 590 mg kg^{-1} (MSDS, 2005b).

TABLE 15.8
Carbonyl Compounds Considered for Malodorant Formulations

Allyl trifluoro acetate
cis-4-Decanol
Pentafluoropentanoic acid
6,6-Dimethylfulvine
Methyl 4-methylbenzoate
*cyclo*Propanecarboxlic acid
*cyclo*Butane carboxylic acid
tert-Butyl acetic acid
2-Methyl butyric acid
Heptafluoro butyric acid
4-Bromobutyric acid
Pentafluoropropionic aldehyde
Butyric anhydride
Heptafluorobutyric anhydride
*cyclo*Propanecarboxaldehyde
Butyraldehyde
iso-Butyraldehyde
iso-Valeraldehyde
2-Chloropropionyl chloride
3-Chloropropionyl chloride
3-Phenylpropionyl chloride
Valeryl chloride
iso-Valeryl chloride
2-Methylvaleryl chloride
Butyryl chloride
2-Ethylbutyryl chloride
4-Bromobutyryl chloride
4-Chlorobutyryl chloride
*cyclo*Butanecarbonyl chloride
tert-Butylacetyl chloride

TABLE 15.9
Compositions of Three Malodorant Formulations

	Formulation Content		
Component	**1**	**2**	**3**
n-Butyl mercaptan	750 mL	750 mL	
tert-Butyl mercaptan	50 mL		
Mercaptoethyl sulfide		10 g	
2-Mercaptoethanol			800 mL
3-Methyl-1-butanethiol		10 g	
3-Methylindole	10 g	10 g	
Cottonseed oil	200 mL	220 mL	
Water			200 mL

2-Mercaptoethanol. Also known as thioglycol, this is a colorless combustible liquid having a very unpleasant disagreeable odor and vapor pressure of 1 mm Hg (20°C) (Lewis, 1993). A workplace environmental exposure level (WEEL) of 0.2 ppm as a TWA_8 has been proposed by the American Industrial Hygiene Association. Acute lethal toxicity data are as follows: p.o. LD_{50} (rat) 131 mg kg^{-1}; percutaneous LD_{50} (rabbit) 251 mg kg^{-1}, (guinea pig) 300 mg kg^{-1}; 4 h LC_{50} (rat) 2 mg L^{-1}. It is a skin and eye irritant (Mallinckrodt Baker, 2004; Chevron Phillips, 2005).

3-Methylindole. Also known as scatol or skatole, it is a solid with a foul fecal odor. It is a skin and eye irritant (MSDS, 2005c).

1,4-Diaminobutane. Also known as putrescine, it is a colorless liquid of flash point 51°C. It has an acute p.o. LD_{50} (rat) of 463 mg kg^{-1}, i.p. LD_{50} (mouse) of 1750 mg kg^{-1}, and percutaneous LD_{50} (rabbit) of 251 mg kg^{-1} (MSDS, 2005d).

1,5-Diaminopentane. Also known as cadaverine, it is a colorless to pale yellow liquid of flash point 63°C. It is formed postmortem in the decay of animal proteins. Cadaverine is a moderately severe skin and eye irritant (MSDS, 2006).

VII. INJURIES AND MORTALITIES IN PEACEKEEPING OPERATIONS

A. Circumstances

The possible circumstances of a peacekeeping operation are multiple and varied, ranging from a one-on-one encounter to violent conflicts between crowds of demonstrators and security forces. Thus, injuries of variable types, severity, and extent may occur and involve either single or multiple persons, who may require treatment in local hospital accident/emergency departments or offices of medical practitioners. Although covering a continuum of activities and circumstances, it is useful to consider peacekeeping confrontations under the following groupings:

1. *Small Group Activity*. This covers encounters between security personnel (mainly police) during arrests or the quelling of disturbances involving a single or a few individuals. In these circumstances, the equipments and agents that have been used are truncheons, nightsticks, tasers, and handheld irritant liquid spray devices. In the United States, the latter may contain CN, CS, or OC, and in the United Kingdom, CS or PAVA. In most instances where irritant sprays have been employed, there has been appropriate use and justification, but in some cases there has been disregard for situations and likely consequences, resulting in public complaints, and possible litigation because of apparent unnecessary and excessive use of sprays. Thus, there have been documented uses against young, old, disabled, ill, and mentally disturbed individuals (summarized by Ballantyne, 2006a,c).
2. *Full-Scale Civil Disturbances*. In the control of full-scale riots (e.g., for politically motivated reasons) or clashes between rival groups (e.g., at sporting events, a variety of procedures may be used for dispersion and control purposes). These include truncheons, nightsticks, projectiles (e.g., bean bags, plastic bullets), water cannon, PCSIs dispersed as pyrotechnically generated smokes or in liquid spray devices.
3. *Vandalization*. There have been many reports about the release of PCSIs in public places by vandals or antisocial individuals to deliberately create stressful and frightening situations. These usually involve the use of irritant liquid spray devices that have been stolen or purchased in countries where this is not prohibited.
4. *Hostage Situations*. These situations may present special circumstances in respect of limited information that can be divulged and the wide range of physical and chemical approaches that may be used to free the hostages. In addition to screening smokes, irritant smokes, and irritant solutions, there may be the use of systemically active neuropharmacological agents, noise producing stun grenades, and live ammunition.

It is also possible that terrorists could obtain PCSI materials and use them for primary or diversionary purposes. Used in public areas, and particularly if employed in enclosed spaces, they would intentionally create fright and panic which could also result in injuries secondary to attempted vacation of the area. It is also conceivable that PCSIs could be used for diversionary purposes, distracting attention from activities being conducted elsewhere for more serious and dangerous reasons.

The above situations indicate the variability in size and activities of peacekeeping operations, and hence the differences in the magnitude and nature of subsequent health care that may be required. Thus, emergency medical services and hospitals can expect disturbances of the peace to result in casualties requiring medical attention ranging from single injured individuals to a multiplicity of persons with a variety of differing trauma. The latter are likely to be a heterogeneous population with respect to age, reproductive status, state of general health, and mobility. The injuries that may be seen are likely to include: (1) direct (primary) chemical injury from the agents used; (2) ballistic, thermal, and secondary physical injury from delivery systems used, restraint procedures, and incidental accidents resulting from attempted escape of the area; and (3) emotional/psychological reactions from the experiences of being involved in a civil disturbance.

B. Physical Injuries

Major categories of physical injuries that may be encountered are as follows:

Blunt and Projectile Injuries. These may result from the deliberate use of physical force by security personnel or be secondary to kinetic forces from ballistic dispersion methods used to deliver projectiles. These may be due to the use of truncheons or other close range direct assault procedures, from projectiles such as rubber/plastic bullets, and body strikes from smoke grenades and canisters remotely launched from dischargers.

Thermal and Explosive Injuries. These may be caused if grenades or canisters are picked before, or as, they detonate. Also, if they are projected into an enclosed area they may cause burns secondary to ignition of flammable contents in the space. Pellets of rubber-bursting grenades may cause burns if they lodge between clothing and skin. ''Tear gas'' pistols or guns discharged close to the body have caused severe and occasionally fatal injuries (Adams et al., 1966; Stahl et al., 1968; Hopping, 1969; Stahl and Davis, 1969; Ayers and Stahl, 1972; Smialek et al., 1975).

Panic Injuries. The circumstances of a riot, including chemical and physical methods used for control purposes, may result in panic situations causing injuries of varying severity.

Incidental Physical Injuries. Water cannon injuries may result from the effect of the kinetic energy of the projected water, and also from difficulty in maintaining balance on the wet ground. If PCSI smoke drifts into an area where motorized vehicles are present, this could lead to drivers having visual difficulties and resultant accidents. Arrest and in-custody deaths may result from restraint techniques, positional asphyxia, and restraint of those having alcoholic intoxication, cocaine intoxication, and neuroleptic malignant syndrome (Bell et al., 1992; Reay et al., 1992; Granfield et al., 1994; Stefee et al., 1995). Problems may be encountered in the use of tasers, especially with those individuals having established cardiovascular disease. According to a recent report, there have been 156 deaths in the United States associated with the use of tasers (Sherman, 2005).

C. Chemical Injuries and Complications

Major structural and functional injuries of clinical relevance are as follows:

Eye Injuries. Although exposure to most PCSI aerosols results in ocular effects that resolve promptly, prolonged exposure to CN aerosols may cause more persistent and severe conjunctoblepharitis and possible corneal injury (Thornburn, 1982; Grant and Schuman, 1993). Structural injury to the eyes has been described with irritant liquid spray devices, notably those containing CN (Kling, 1969; MacLeod, 1969; Macrae et al., 1970; Ballantyne et al., 1975; Oksala and Salminen,

1975). Also discharge of particulate material from CN-based pen guns has been associated with severe eye injury, including chemosis, corneal edema, necrotizing keratitis, and iridocyclytis (Levine and Stahl, 1968; Hopping, 1969; Oaks et al., 1969; Laibson and Oconor, 1979). Initial injury is probably from blast and heat, during which time explosive deposition of solid irritant particles in the wound leads to chemical necrosis. Corneal abrasions have been described following exposure to OC sprays (Brown et al., 2000).

One medically significant functional effect of local contact of the eye with PCSI materials is a transient increase in IOP. For example, following contamination of the eye of volunteer subjects with solutions of CR, there are increases in IOP in the treated eye and, to lesser degree, in the contralateral eye. For example, with 0.05% CR in PEG300 peak IOP increases occurred at 5 min postapplication of 40% in the contaminated eye and 16% in the contralateral eye; IOP returned to control values by 15 min (Ballantyne et al., 1977a). The increased IOP in the untreated eye is probably mainly a consequence of increased intrathoracic and central venous pressure resulting from the pain of the experience. In the CR-contaminated eye, the increase in pressure is further exaggerated by a local effect of CR causing conjunctival hyperemia with the resultant vascular congestion impeding drainage of aqueous humor. Although these briefly sustained effects are not of long-term significance for most individuals, they can be relevant to precipitating an attack of glaucoma in those with incipient narrow angle glaucoma or exacerbating established glaucoma (Ballantyne et al., 1973).

Cutaneous Injuries. With exposure to low concentrations or doses of PCSI materials, the only visible cutaneous effect is normally erythema. However, sustained exposure to high CN concentration in solution or in the atmosphere may cause severe erythema, edema, and chemical burns of the skin (Thornburn, 1982). Both CN and CS may cause allergic contact dermatitis (Fisher, 1970; Rothberg, 1970; Penneys, 1971; Holland and White, 1972; Frazier, 1976; Pfeiff, 1984; Fuchs and Weische, 1990; King et al., 1995).

Respiratory Tract Injuries. Those with pre-existing respiratory tract diseases, such as chronic obstructive pulmonary disease or asthma, may be at risk from exposure to PCSI materials and chemicals, such as OC, which have pulmonary neurogenic effects (National Institute of Justice, 1994). Sustained exposure to high concentrations of irritant smokes, as in enclosed poorly ventilated spaces, may cause laryngotracheobronchitis (Thornburn, 1982). Susceptible individuals may experience laryngospasm (Ballantyne, 2006a). Lung injury is most likely to occur when there is exposure to irritant smokes in confined and poorly ventilated areas from which escape may be impeded (Thornburn, 1982; Greaves, 2000). Fatal respiratory tract injury has been described from acute overexposure to DM and CN, and is pathologically characterized by laryngotracheobronchial necrosis, pseudomembrane formation, pulmonary edema, and alveolar hemorrhages (Stein and Kirwin, 1964; Ministry of Defence, 1972; Chapman and White, 1978; Krapf, 1981). One possible complication of exposure to high and potentially tissue injuring concentrations of a PCSI is reactive airways dysfunction syndrome (RADS) (Ballantyne and Salem, 2004). A few cases of RADS have been described following exposure to CS.

Cardiovascular Effect. Studies on human volunteer subjects have demonstrated that within a short period of exposure to a PCSI material, in solution or airborne, there are abrupt increases in SBP and DBP often with an associated reflex bradycardia. In general, the increase in BP occurs within minutes of exposure and resolves within about 30 min (Beswick et al., 1972; Ballantyne et al., 1976a; Ballantyne, 1987, 2006a). The magnitude and duration of the pressure changes can be tolerated without significant medical hazards in healthy individuals. However, as with other stressful situations, some susceptible individuals may be at risk from the transient hypertensive episode, including those with essential hypertension, myocardial infarction, coronary artery disease, cardiac arrhythmias, and arterial aneurysms (Ballantyne, 1977a, 1987; Ballantyne and Salem, 2004).

D. Immediate and Medical Management of Casualties

Emergency medical services and physicians who are warned of the occurrence of a peacekeeping event should anticipate the arrival of casualties, which will vary from simple irritant and emotional

events to severe chemical or physical injury, and that hospitalization may be necessary for the more severe injuries. With a large-scale riot those seeking medical attention will be part of a heterogeneous group: old, young, male, female, and in various states of previous health, all of which may affect decisions on the most appropriate management. For larger scale, nonpeaceful civil unrest events (riots) in addition to the clinical management of cases, it is necessary to consider decontamination and triage of casualties; all three aspects of these health care related issues are considered as follows.

Decontamination. To relieve casualties of the discomfort and distress resulting from exposure to a PCSI, and also to facilitate the unimpeded work of those treating casualties, it is important that affected individuals be decontaminated promptly and without secondary contamination of health care workers (Horton et al., 2006). Ideally, a separate well-ventilated area remote from the hospital general buildings should be available to receive, examine, and decontaminate patients (Rosenbaum et al., 2004). It is preferable that casualty departments and receiving rooms should not be contaminated to the inconvenience of other patients, and to prevent PCSI materials from entering the general hospital atmosphere through closed circulation air conditioning systems. In some cases where there has been only brief exposure to a PCSI material, then decontamination in a fresh air stream may be all that is required (Lee et al., 1996; Blaho and Stark, 2000). Contaminated clothing should be removed and stored in a plastic bag. Those undertaking the decontamination should wear gowns, gloves, impermeable goggles, and, with heavy contamination, a respirator. If decontamination requires showering, the affected individual should be advised that this might result in a temporary reprise of symptom due to PCSI being leached out of hair. If surgery is required, then the decontamination and protective measures should be adequate enough to ensure that there is no secondary contamination of anesthetists, because this can be a problem during tracheal intubation (Bhattacharya and Haywood, 1993; Barlow, 2000).

Triage. When multiple casualties are expected to arrive at the health care facility, there should be arrangements for suitably qualified emergency medical staff to conduct triage on arriving patients. Triage staff should be protected against secondary contamination. Triage will assign priorities for clinical examination based on the presence and degree of physical injury, chemical injury, and decisions on whether an individual is ''at risk'' and may develop secondary complications from medical and psychological consequences of being present at a riot. These include, but are not restricted to, individuals with coronary artery disease, myocardial infraction, cardiac arrhythmias, hypertension, arterial aneurysm, chronic pulmonary disease, asthma, RADS, glaucoma, and convulsions.

Psychological Effects. For the majority of persons, being a participant in a civil disturbance can be an emotionally disturbing experience, particularly if there has been exposure to physical or irritant insults. Therefore, some casualties may present in a state of depression, fright, anxiety, or even hysteria, and delayed psychological reactions may occur. Mental reactions may also develop in those living in an area where the threat of violence exists (Frazer, 1971; Lyons, 1971). Emotionally affected individuals may require sedation and reassurance, and with the more severe and sustained reactions might need psychiatric care.

Ocular Effects. Acute chemically induced conjunctoblepharitis may result from exposure to PCSIs in smoke, aerosol, or liquid. With mild cases of smoke or aerosol exposure, aeration in the open air or using an electrical fan may be all that is necessary for decontamination and relief (Gray, 1995; Yih, 1995; Lee et al., 1996; Blaho and Stark, 2000). With more severe cases, irrigation of the eyes with cool water or saline may be necessary. Reports indicate that Diphoterine, a polyvalent, amphoteric, hypertonic chelating agent with six binding sites, can prevent or rapidly ameliorate the ocular and cutaneous effects of irritant compounds, including CS (Hall et al., 2002; Viala et al., 2005). Visual acuity may be slightly reduced immediately after eye contamination, but rapidly returns to normal (Rengstorff, 1969; Yih, 1995). If ocular discomfort is marked or persists, the use of local anesthetic eye drops (e.g., propoxymetacaine hydrochloride) may give relief, but local anesthesia should be approved by an ophthalmologist because it may impair the regeneration of injured corneal epithelium and retard healing (Leopald and Lieberman, 1971). Those with marked

and persistent eye discomfort should have a detailed ophthalmoscopic examination, including fluorescein staining and slit-lamp biomicroscopy, to exclude corneal and anterior segment injury (Balho and Stark, 2000; Brown et al., 2000; Wier, 2001). As discussed earlier, PCSI materials cause a transient increase in IOP that is not of pathophysiological significance in normal eyes but may be a risk factor for those having incipient or established glaucoma, and who may require ophthalmic consultation. With OC, superficial anesthesia and loss of blink reflex may lead to corneal abrasions from contact lenses or foreign bodies. Because capsaicin disrupts the corneal epithelium, those with impaired corneal integrity may be more susceptible (Smith and Stopford, 1999). There are conflicting opinions about the relative advantages/disadvantages of wearing contact lenses during exposure to PCSI materials. For example, in trials with CS aerosols it was found that individuals wearing contact lenses kept their eyes open more easily and longer and have better orientation (Aalphen et al., 1985). Others, however, believe that wearing contact lenses could result in visual impairment due to the PCSI and the formulation constituents (Ballantyne and Salem, 2004). Soft contact lenses may cause entrapment of material between lens and corneal surface and increase contact time with fluid, which may facilitate injury from PCSI or formulation. Thus, it is generally advised that contact lenses be removed to ensure adequate eye irrigation. Hydrophilic soft contact lenses, due to their network structure, may absorb chemicals and thus be a source of sustained exposure (Loriot and Tourte, 1990). Soft contact lenses contaminated with OC may be difficult to decontaminate (Lee et al., 1996). Some solvents in irritant solutions may lead to solubilization, fragmentation, or hardening of soft lenses (Holopainen et al., 2003; Ballantyne, 2006a). This may result in further irritation and possibly superficial corneal injury, which may be enhanced by digital rubbing of the eye that characteristically occurs with ocular irritation.

Cutaneous Effects. Cutaneous erythema is all that is normally seen after PCSI exposure, and the skin should be decontaminated with a copious soap and cool water wash. With CS, and if symptoms and signs are marked, advantage may be taken of its rapid hydrolysis in alkaline solution. Weigand (1969) recommended use of the following solution for rapid hydrolysis and relief of symptoms: aqueous 7% sodium bicarbonate, 3% sodium carbonate, and 1% benzalkonium chloride. Also, as noted above, Diphoterine can prevent or rapidly ameliorate the effects of irritant compounds on the skin. Sustained exposure to high concentration of PCSI in smokes or solutions, particularly of CN, may cause severe primary irritant dermatitis, necessitating therapeutic measures such as topical corticosteroids. Also, a hypersensitivity reaction presenting as allergic contact dermatitis may occur, notably with CS and CN. Perforating injuries with embedded PCSI, particularly CN, may provoke suppurative and necrotizing reactions, resulting in degenerative changes and fibrosis affecting skin, muscle, and nerve (Stahl et al., 1968).

Respiratory Tract. Respiratory effects from PCSI materials normally subside rapidly and completely if exposure has been in open air. If high concentration exposure has been sustained, particularly in an enclosed space, then the individual should be kept under observation and, as dictated by the clinical condition, radiological, and pulmonary function studies undertaken (Park and Giammona, 1972; Sanford, 1976). Patients with gross overexposure to grenade-generated smokes may develop laryngotracheobronchitis, possibly requiring treatment with bronchodilators, postural drainage, corticosteroids, and prophylactic antibiotics (Thornburn, 1982). Those with established pulmonary disease, including asthma, may have an exacerbation of signs and symptoms. The development of RADS in a few individuals who have had acute high dosage inhalation exposure is a possibility.

Cardiovascular Status. Persons exposed to aerosols, smokes, or solutions of PCSI materials may have transient increases in SBP and DBP, often with reflex bradycardia. This could be compounded because of the emotional experience of being involved in a civil disturbance. Those with a history of cardiovascular disease may require hospital admission and further investigation.

Gastrointestinal Effects. Those swallowing PCSI materials may experience a stinging or burning sensation in the mouth and throat with increased salivation, and in a few cases there may be nausea

and vomiting; very rarely hemoptysis and hematemesis have been reported (Anderson et al., 1996). Swallowed OC may cause variable degrees of gastrointestinal irritation, notably mouth and throat irritation with nausea and possibly vomiting (Forrester and Stanley, 2003; Ballantyne, 2006a).

Miscellaneous. A case of multisystem hypersensitivity reaction was described in a man heavily exposed to a CS spray (Hill et al., 2000). This was characterized by cutaneous rash, pneumonitis, hypoxemia, hepatitis, and hypereosinophilia, with rapid response to corticosteroids; a patch test confirmed sensitization to CS. The possible use and abuse of illicit drugs, and the resultant complications, should be considered in the context of some civil disturbances (Hayman and Berkely, 1971). Physicians should be aware of the possible contributions to injury and toxicity of formulation constituents; some may exacerbate the effects produced by PCSI materials (e.g., eye injury and skin irritation), and others may introduce additional toxicity (e.g., carbon monoxide poisoning from the absorption and metabolism of dichloromethane as a formulation solvent).

VIII. PREPARATION AND READINESS FOR PEACEKEEPING OPERATIONS

For anticipated civil disturbances, an advance warning, from the security force or local relevant government agency, of the nature and likely size of the disturbances will greatly help in staff/facility/equipment preparations by local hospitals and health care/emergency services. If a peaceful demonstration erupts into a violent event, this will enormously increase the requirements for health care services, and therefore there should be ongoing communications between those controlling the disturbance (law enforcement officers) and the emergency services and involved health care institutions. With the current international sociopolitical structure and its extreme legislation, domination by self-interest groups, untrustworthy elected representatives in government, and polarized viewpoints on issues such as capitalization, multiculturalization, globalization, immigration, and unfair labor problems, it is inevitable that there will be continuing and perhaps increasing expressions of dissatisfaction by demonstrations and counter demonstrations as the only pointed and public manner by which to convey opinion and objection to specific issues (Ballantyne, 2006). It is thus clear that there is a need for advance planning by health care facilities with respect to staffing, facility requirements, equipment, expertise, education, training, and communication needs for conditions of civil unrest. Some health care facilities have already undertaken meetings and training sessions against the possibility of terrorist activities. This activity could perhaps also be used as a starting point for discussions on the need to preplan for civil unrest. Although the natures of these two generic issues have some commonalities, there may be working differences with respect to legal, sociopolitical, acceptable, and workable approaches to terrorism versus civil unrest. A local permanent advisory and functional group should be formed to act as a focus for discussing and dealing with practical issues relevant to the wide health-related issues related to civil disturbances. Such a group should ideally be centralized at an appropriately located and equipped health care institution. This regional development team should include at least the following among its members: trauma surgeon, ophthalmologist, psychiatrist (ideally experienced in crowd psychology), general physician, anesthetist, clinical toxicologist (or representative from regional Poison Control Center), senior law enforcement officers, emergency and support services representatives, and access to communications experts. They should have responsibility for the planning, organization, preparation, and initiation of the following essential elements, policies, procedures, and strategies with respect to potential local civil unrest activities.

- Ensure the existence of an adequate reference (information) base on the agents and equipments that may be employed in a civil disturbance and on the management of casualties from such disturbances. This could involve the preparation of a manual for training purposes.
- Ensure all necessary medical equipments and treatment needs are readily available and stocked for emergency mobilization and use.

- Decide on suitable sites for reception, decontamination, and triage functions, and ensure their working appropriateness and readiness for an emergency.
- Establish a rapid access integrated communications system with law enforcement, transport, ''on call'' medical/nursing/support staff, and emergency services.
- Organization and conduct of joint educational and training sessions with emergency and support services.

Ideally, there should be centralized coordinated national plans for workable and acceptable low-risk methods that can be used to control or restore law during a civil disturbance, and also for coping with health-related and environmental issues subsequent to nonpeaceful demonstrations. Such a centralized function could (1) ensure consistency of planning, approaches, and actions between itself and local (regional) groups, (2) encourage education, forward planning, and training sessions between regional groups through a network system, and (3) perform random audits of regional centers with respect to preparedness.

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16 Mechanism of Action of Botulinum Neurotoxin and Overview of Medical Countermeasures for Intoxication

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CONTENTS

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I. INTRODUCTION

The botulinum neurotoxins (BoNTs)* comprise a family of seven distinct neurotoxic proteins (A–G) produced by immunologically discrete strains of the anaerobic bacterium *Clostridium botulinum* and in rare cases by *Clostridium baratii* and *Clostridium butyricum* (Habermann and Dreyer, 1986; Harvey et al., 2002; Simpson, 2004). These toxins act on peripheral cholinergic synapses to inhibit spontaneous and impulse-dependent release of acetylcholine (ACh) (Brooks, 1956; Kao et al., 1976). Intoxication by BoNT results in muscle weakness, which can be fatal when the diaphragm and intercostal muscles become sufficiently compromised to impair ventilation (Dickson and Shevky, 1923). The BoNTs are the most potent substances in nature, and exposure to as little as 1–3 ng/kg may be sufficient to cause human lethality (Gill, 1982; Middlebrook and Franz, 1997; Arnon et al., 2001).

The purpose of this chapter is to use the insights gained in our understanding of the mechanism of BoNT action to establish a conceptual framework within which to develop effective treatment strategies for intoxication. The chapter is organized into three major topics: (1) an overview of BoNT action, (2) a description of the manifestations of botulism, and (3) an evaluation of conventional and emergent treatment options. From the first description of botulism in 1793 until the mid-1950s, BoNT was primarily viewed as a public health problem because of its association with food poisoning (Gill, 1982; Hatheway, 1988; Shapiro et al., 1998). Although implicated in only a small fraction of foodborne illnesses ($<0.1\%$), the severity of the clinical syndrome produced by BoNT and the potential for numerous casualties led each outbreak to be considered as a potential health crisis (Hatheway, 1988; Smith and Sugiyama, 1988; Snydman, 1989; Shapiro et al., 1998). Due to its selective targeting of peripheral cholinergic synapses, BoNT has also been used as a tool to study cholinergic pathways, especially to explore the influence of synaptic inactivity and ACh deprivation on muscle function (Drachman and Johnston, 1975; Kao et al., 1976; Thesleff, 1989; Thesleff et al., 1990).

During World War II, BoNT was developed as a biological weapon, because of its potential to create mass casualties on the battlefield (Franz, 1997; Grace, 2003). The battlefield use of BoNT is now viewed as less likely following adoption of the 1972 Biological and Toxin Weapons Convention and the dissolution of the Soviet Union. However, the Iraqi stockpiling of BoNT before the Persian Gulf War of 1991 reveals the ability of a determined nation to acquire biological weapons in relative secrecy (Shoham, 2000; Arnon et al., 2001).

With the rise of global terrorism, exemplified by organizations such as the Japanese Aum Shinrikyo cult and al Qaeda, the potential use of BoNT as a bioterrorist weapon has become a more immediate threat (Franz et al., 1997; Middlebrook and Franz, 1997; Arnon et al., 2001; Grace, 2003). BoNT is well suited for this role because of its extraordinary lethality, capacity to elicit panic, and potential to disrupt the public health system (Atlas, 1998). Additional attributes of BoNT include widespread availability, low cost, and ease of production, transport, and concealment (Arnon et al., 2001). These considerations have led BoNT to be classified as a Category A biothreat agent by the Centers for Disease Control and Prevention (CDC) (Lohenry and Foulke, 2006). Bioterrorist attacks are generally thought to involve dispersal of BoNT as an aerosol, but the toxin can also be used to contaminate the food supply (Wein and Liu, 2005).

Systematic research on the mechanism of action of BoNT began with Emile Pierre van Ermengem's historic isolation and characterization of *C. botulinum* following a large outbreak in Ellezelles, Belgium, in 1895 and has continued with increasing interest and enthusiasm to the present time (Simpson, 2004; Grumelli et al., 2005; Rossetto et al., 2006; Singh, 2006). Early work on BoNT intoxication revealed the existence of multiple serotypes, localized the site of action to peripheral cholinergic synapses, proposed the mechanism of impaired ACh release, and ruled out noncholinergic, sensory, and central nervous system (CNS) involvement (Dickson and Shevky,

* In this chapter, BoNT is used to designate both pure botulinum neurotoxin as well as the neurotoxin complex. Some authors prefer the designation of BoTx for the latter.

1923; Guyton and MacDonald, 1947; Burgen et al., 1949; Ambache, 1951; Brooks, 1956). Most of these findings were established by the mid-1950s and refined during the next three decades (Lundh et al., 1977; Simpson, 1981; Thesleff, 1989).

The remarkable specificity for peripheral cholinergic synapses and long duration of action led to the use of BoNT/A for a growing number of focal dystonias and movement disorders following its approval in 1989 as an ''orphan drug'' by the U.S. Food and Drug Administration (FDA) for the treatment of strabismus, blepharospasm, and hemifacial spasm (Jankovic and Brin, 1997; Schantz and Johnson, 1997). The attributes that render BoNT a deadly poison also make the neurotoxin an ideal therapeutic agent to treat diseases of muscle hyperactivity. In addition to its original indications, BoNTs are also used for treatment of spasticity following brain and spinal cord injuries, stroke, multiple sclerosis, cerebral palsy, and numerous other disorders. Expansion and refinement in its clinical use constitute the most active focus of current BoNT research, and a number of excellent reviews have been published (Jankovic and Brin, 1997; Schantz and Johnson, 1997; Tugnoli et al., 1997; Johnson, 1999; Aoki, 2002; Charles, 2004; Chaddock and Marks, 2006; Eleopra et al., 2006; Dutton and Fowler, 2007).

In addition to discovering additional indications for the native neurotoxin, a promising new approach has been to alter the BoNT-binding domain to retarget the modified toxins to noncholinergic sites. Notable examples include a novel conjugate of BoNT/A, whose binding domain was replaced by *Erythrina cristagalli* lectin for targeting to pain fibers (Chaddock et al., 2004), and a modified BoNT/C1 in which the binding domain was replaced by epidermal growth factor for targeting to epithelial cells to inhibit excess mucus secretion (Foster et al., 2006). The former has potential for relief of chronic pain, whereas the latter may be useful for treatment of asthma and chronic obstructive pulmonary disease.

During the last two decades, enormous progress has been made in understanding the action of BoNT at the molecular level. This was spurred by a number of crucial developments: (1) elucidation of the amino acid sequence leading to recognition of the zinc-binding motif (Jongeneel et al., 1989), (2) demonstration of zinc metalloprotease activity with identification of substrates and cleavage sites (Schiavo et al., 1992a, 1992b, 1993, 1994; Blasi et al., 1993a, 1993b; Montecucco et al., 1994; Yamasaki et al., 1994), (3) solution of the crystal structure for BoNT beginning with serotypes A and B (Lacy et al., 1998; Swaminathan and Eswaramoorthy, 2000), and (4) elucidation of the protein receptor for BoNT/B, BoNT/G, and BoNT/A (Nishiki et al., 1994; Dong et al., 2003, 2006; Rummel et al., 2004, 2007; Chai et al., 2006; Jin et al., 2006). These developments provided a detailed understanding of the mechanisms of actions of BoNT and opened the possibility for rational studies of pharmacological antagonists for BoNT toxicity.

A. Characteristics of BoNT Intoxication

The typical manifestation of botulism is a flaccid paralysis that is bilateral and descending, involving skeletal muscle and structures innervated by autonomic ganglia (Habermann and Dreyer, 1986; Smith and Sugiyama, 1988; Merz et al., 2003). Human intoxication is caused by serotypes A, B, E, and, to a much lesser extent, F, and is generally manifested as foodborne, wound, and intestinal (infant) botulism (Simpson, 1981). Wound and infant botulism are usually mediated by serotypes A and B (Pickett et al., 1976; Arnon, 1995). Two additional forms of botulism have been observed that do not occur in nature: inhalation botulism and iatrogenic botulism. The former is so rare in humans that only one occurrence, a laboratory accident, has ever been reported (Holzer, 1962). An outbreak of inhalation botulism would be suspected as a terrorist incident unless other causes were found (Arnon et al., 2001; Park and Simpson, 2003; Adler, 2006). Iatrogenic botulism stems from overdose of clinically or cosmetically used BoNT (Klein, 2004). A recent case in which four individuals were injected with multiple lethal doses of a nonapproved preparation of BoNT/A during a cosmetic procedure illustrates the potential hazard of this otherwise safe use of BoNT (Chertow et al., 2006; Souayah et al., 2006).

C. botulinum spores are widely distributed in soils, sea sediments, decaying vegetation, animal carcasses, and sewage (Smith, 1978). The intestinal tracts of birds, mammals, and fish may also acquire *C. botulinum* as a transient member of their intestinal flora. The hosts do not exhibit botulism since growth of these anaerobic bacteria is suppressed when there is competition from other organisms and a functional immune system (Smith, 1978; Smith and Sugiyama, 1988; Snydman, 1989). The resistance of clostridial spores to harsh environmental conditions enables their dissemination by air currents and dust particles, leading to surface contamination of exposed food products (Stinger et al., 2005). Botulism is not contagious, however, and contact with spores does not usually lead to disease except in young infants under 1 year in age (infant botulism), in adults with altered gastrointestinal (GI) anatomy and microflora (adult intestinal botulism), or following germination in wounds (wound botulism) (Mershon and Dowell, 1973; MacKenzie et al., 1982; Arnon, 1995; Shapiro et al., 1998).

B. Symptomology

The clinical syndrome of botulism reflects toxin-induced blockade of ACh release from neuromuscular and neuroeffector junctions (Burgen et al., 1949; Ambache, 1951). The basic syndrome of BoNT intoxication is similar for foodborne, intestinal, and wound botulism and does not vary appreciably among toxin serotypes (Sobel, 2005). The earliest symptoms generally include visual disturbances (diplopia, blurred vision) and xerostomia (Hughes and Tacket, 1983). With low-level exposure, these symptoms may gradually resolve, even in the absence of medical intervention. In more severe cases, the initial symptoms are followed by dysphasia, dysphonia, and dysarthria, reflecting an especially high susceptibility of cranial efferent terminals to BoNT action (Shapiro et al., 1998). A descending generalized skeletal muscle weakness may then develop, progressing from the upper to the lower extremities. Involvement of the diaphragm and intercostal muscles can lead to ventilatory failure and death, unless appropriate supportive care is provided (Cherington, 1998; Robinson and Nahata, 2003; Sobel, 2005). Although motor function is severely impaired, there is little or no sensory alteration or CNS involvement in botulinum intoxication (Simpson, 1981).

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 (Robinson and Nahata, 2003; Sobel, 2005). The preponderance of symptoms, including the potentially lethal respiratory collapse, stems from inhibition of neuromuscular transmission (Burgen et al., 1949; Brooks, 1956; Kao et al., 1976; Simpson, 1981). Parasympathetic dysfunction is responsible for blurred vision, xerostomia, constipation, and urinary retention (Ambache, 1951; MacKenzie et al., 1982; Merz et al., 2003).

C. Functional Domains of BoNT

The BoNTs are synthesized as ~150 kDa single-chain protoxins (range, 140–167 kDa). They are proteolytically activated (nicked) to form dichain molecules consisting of a ~50 kDa light chain (LC) and a ~100 kDa heavy chain (HC) (DasGupta and Sugiyama, 1972; Bandyopadhyay et al., 1987). The two chains are coupled by a single disulfide bond and by noncovalent forces. In their natural state, BoNTs exist as complexes consisting of ~150 kDa neurotoxin associated with a group of nontoxic proteins. The latter are designated as neurotoxin-associated proteins (NAPs), some of which possess hemagglutinin activity (Sakaguchi, 1982; Sharma et al., 2006). NAPs associate with BoNT in the bacterial culture medium by noncovalent interactions and protect the neurotoxin from proteolytic and low pH-mediated inactivation. They have also been suggested to facilitate absorption of BoNT from the GI tract into the bloodstream (Sharma and Singh, 1998). The ability of BoNT to manifest oral toxicity has generally been attributed to the presence of these proteins; conversely, the inability of the related tetanus neurotoxin (TeNT) to produce foodborne intoxication has been ascribed to the absence of such NAPs (Singh et al., 1995). Maksymowych et al. (1999) have raised some questions on the importance of NAPs in BoNT toxicity, especially

with regard to their role in transcytosis of the neurotoxin. These investigators demonstrated that pure BoNT/A lacking NAPs was still toxic to mice following intragastric administration, although to a lesser extent than the toxin complex. When examined at elevated concentrations, the differences in efficacy between pure and NAP-containing neurotoxin were progressively reduced. These results indicate that pure neurotoxin does not require accessory proteins for absorption from the GI tract. Moreover, even though the NAPs are clearly protective, sufficient pure neurotoxin can survive the inhospitable environment of the GI tract to produce lethality.

In conformity with the sequential processing of bacterial protein toxins such as diphtheria or cholera toxin, the action of BoNT involves multiple discrete steps: binding to surface receptors, internalization via receptor-mediated endocytosis, and translocation from endosome to cytosol. For BONT, the final step is cleavage of soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNAREs) in the cytosol (Simpson, 1981, 2004; Montecucco et al., 1994). Binding and translocation are mediated by the C- and N-terminal domains of the BoNT HC, respectively (Daniels-Holgate and Dolly, 1996; Koriazova and Montal, 2003; Simpson, 2004; Fisher and Montal, 2006). The LC has zinc metalloprotease activity, targeted to one of the three SNARE proteins (SNAP-25, synaptobrevin, or syntaxin) that are required for the docking and fusion of synaptic vesicles with active zones at the cytoplasmic surface of the nerve terminal (Schiavo et al., 1992a; Montecucco and Schiavo, 1993; Montecucco et al., 1994; Schiavo et al., 2000).

Serotypes B, D, F, and G cleave different sites on the synaptic vesicle protein, synaptobrevin (VAMP), whereas serotypes A and E cleave the presynaptic membrane-associated protein SNAP-25 (Schiavo et al., 2000; Simpson, 2004). Serotype C1 is unique in that it cleaves two cytoplasmic proteins, syntaxin and SNAP-25 (Williamson et al., 1996). Interaction of these SNAREs on the surface of synaptic vesicles and active zone membranes is required for voltage- and Ca^{2+}-dependent release of neurotransmitter; cleavage by BoNT inhibits this process, leading to muscle weakness and paralysis (Sutton et al., 1998; Schiavo et al., 2000). Cleavage of SNARE proteins appears to be sufficient to account for all actions of the BoNTs, and the SNARE hypothesis has received near universal acceptance since its introduction in the early 1990s.

For each BoNT serotype, the dichain form constitutes the active configuration of the neurotoxin; the isolated LC and HC are devoid of systemic toxicity. The absence of toxicity is consistent with findings that the LC cannot gain access to the cytosol unless it is coupled to the HC and that the HC lacks the ability to inhibit neurotransmitter release (Stecher et al., 1989; Goodnough et al., 2002). The isolated LC does, however, remain enzymatically active as evidenced by its ability to inhibit exocytosis from permeabilized chromaffin cells (Stecher et al., 1989), by its ability to cleave SNARE proteins in cell-free assays (Adler et al., 1998), and by its capacity to inhibit ACh release in skeletal muscle when delivered by liposomes (de Paiva and Dolly, 1990). It is not clear whether any portion of the HC is translocated along with the LC, and if so, whether it exerts a role in enhancing the catalytic activity or stability of the LC.

All BoNT serotypes suppress ACh release, show high specificity for cholinergic synapses, and share the same overall mode of action; they differ, however, in potency and in duration of action. Type A neurotoxin exhibits the highest potency (Gill, 1982), and types A and C1 produce the longest intoxication times (Eleopra et al., 1998; Keller et al., 1999; Adler et al., 2001; Keller and Neale, 2001; Foran et al., 2003; Keller, 2006). Other differences include targeting of different functional surface receptors on the motor nerve terminal (Black and Dolly, 1986; Montecucco, 1986; Daniels-Holgate and Dolly, 1996; Rummel et al., 2007), and cleaving unique peptide bonds in the appropriate SNARE proteins (Schiavo et al., 2000; Simpson, 2004; Rossetto et al., 2006).

II. MANIFESTATIONS OF BOTULISM

Botulinum intoxication generally results from ingestion of preformed toxin elaborated in contaminated foods (foodborne) or from colonization by *C. botulinum* of deep wounds with subsequent production of toxin (wound botulism) (Mershon and Dowell, 1973; Snydman, 1989). A third form,

termed intestinal botulism, is observed in young infants (infant botulism), or less commonly in adults with altered GI anatomy or microflora (adult intestinal botulism), and originates from colonization of the large intestine by *C. botulinum* with subsequent production and absorption of toxin (Pickett et al., 1976; Arnon, 1995). Two additional forms of botulism are iatrogenic botulism, from accidental overdoses following clinical or cosmetic procedures (Klein, 2004), and inhalation botulism (Franz et al., 1993).

A. Foodborne Botulism

Elaboration of BoNT in foods requires contact with *C. botulinum* spores under conditions that allow bacterial cell proliferation and toxin production. These consist of an anaerobic environment, temperatures between 4°C and 40°C, pH above 4.6, water activity greater than 0.94 (<10% NaCl), and lack of adequate preservatives (Baird-Parker and Freame, 1967; Stinger et al., 2005). The requirements for growth of *C. botulinum* are stringent, especially anaerobiosis, making outbreaks relatively rare; nevertheless, episodes of foodborne botulism constitute a persistent public health threat (Sobel et al., 2004). In fact, food-related botulism outbreaks in the United States have shown no significant reduction during the past century, with an average of approximately 24 cases/year (Shapiro et al., 1998).

The primary vehicle for foodborne botulism presently and during most of the twentieth century has been improperly prepared home-preserved food products, often involving vegetables with a low acid content (Smith and Sugiyama, 1988; Snydman, 1989). Other sources are food consumed in restaurants that use unsafe procedures and contaminated commercially canned food products; the latter has become rare since the introduction of modern methods (O'Mahoney et al., 1990). Data compiled for foodborne botulism during the decade 1990–2000 in the United States indicate that serotype A was responsible for 50% of all cases, whereas serotypes B and E accounted for 10% and 37%, respectively, of intoxications in which serotype involvement was established (Sobel et al., 2004). Human foodborne intoxication by BoNT/F is exceedingly rare; between 1981 and 2002, only a single case was reported to the CDC (Gupta et al., 2005).

Although the number of outbreaks has been relatively constant, the case to fatality ratio has improved markedly. From 1899 to 1950, foodborne botulism was associated with 60% mortality; from 1950 to 1996, the average annual mortality fell to 15.5% (Shapiro et al., 1998), and decreased to 4% during the last decade (Sobel et al., 2004). These advances in survival have come primarily from improvements in critical care (Tacket et al., 1984; Sobel et al., 2004). Further reductions in morbidity and mortality from botulinum intoxication will require better methods for detection and diagnosis of BoNT outbreaks and availability of specific pharmacological treatments (Franz et al., 1997; Dickerson and Janda, 2006).

Perhaps the largest outbreak of foodborne botulism recorded to date occurred in Nan Province, Thailand during 14–18 March 2006 (Ungchusak et al., 2007). The outbreak was traced to consumption of contaminated home-canned bamboo shoots served at a religious festival. The successful handling of this outbreak by the Thai Ministry of Public Health has implications for the appropriate management of a small-scale bioterrorism attack involving deliberate contamination of the food supply or an aerosol attack. A total of 209 people exhibited signs and symptoms of botulism, with abdominal pain, dry mouth, and nausea being the most frequently reported; 134 villagers required hospitalization and 42 required mechanical ventilation. Botulism was suspected as soon as more specific signs such as bulbar muscle paralysis were observed, especially when coupled with respiratory depression. Due to familiarity with botulism in Thailand, the correct diagnosis was reached, and emergency procedures were implemented promptly, which allowed all patients to survive the outbreak. After initial triage, patients were flown to hospitals that had adequate emergency care facilities, including ventilators. In countries where botulism outbreaks are less frequent, health care personnel are often unfamiliar with its clinical presentation (Ruthman et al., 1985). As a result, botulism may not be diagnosed in a timely fashion, leading to delays in

treatment, and in a much poorer prognosis. The lessons learned from this large outbreak suggest that successful management of a bioterrorist attack involving botulism would require early recognition and accurate diagnosis of signs and symptoms, a realistic plan for allocating resources, adequacy of those resources, and coordination among the health care facilities, responsible government agencies, and international partners (Ungchusak et al., 2007). For this outbreak, antitoxin was obtained through informal government channels, and although responses were expeditious, antitoxin from the United States and the United Kingdom did not reach Thailand until 5–9 days after the outbreak. This delay would have resulted in potential deaths in the more severe group were it not for access to ventilators and emergency care. Formal international arrangement for antitoxin delivery is preferable to informal mechanisms, but local strategic stockpiles of critical medicines and supplies are essential to avoid delays in treatment.

B. Wound Botulism

Wound botulism is relatively rare, accounting for only 5% of all outbreaks. The majority of these are caused by serotype A, and the remainder by serotype B (Shapiro et al., 1998). The neurological symptoms of wound botulism differ little from those of foodborne botulism except for the general absence of GI 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 1990, only 47 incidences of wound botulism were documented (Weber et al., 1993). An examination of these cases indicated that wounds susceptible to *C. botulinum* are generally deep with avascular areas but need not appear obviously infected or necrotic. Additional risk factors include compound fractures and extensive crush injuries (Mershon and Dowell, 1973). Contamination of wounds with *C. botulinum* spores leads to germination and colonization at the site of infection. Localized weakness results from production of toxin at the wound, and systemic botulism can occur from toxin transmitted via the bloodstream to distant targets (Weber et al., 1993).

From 1980 to the present time, wound botulism has been observed predominantly in illicit drug users following repeated subcutaneous administration of black tar heroin, or in individuals with nasal or sinus lesions from chronic cocaine abuse (Anderson et al., 1997). During the last decade alone, wound botulism from black tar heroin has exceeded the total reported wound botulism cases during the preceding 40 years by a factor of almost three (Sandrock and Murin, 2001).

For reasons that are not completely understood, wounds are much more likely to be contaminated by *Clostridium tetani* than with *C. botulinum*. Although an aggressive vaccination program has nearly eliminated tetanus in developed nations, the absence of universal tetanus vaccination in many developing countries results in substantial mortality (Vandelaer et al., 2003). A large number of TeNT intoxications occur in neonates, often by infection of the umbilical stump. The mortality rates in developing countries were reported to be 85% for neonatal tetanus and 50% for nonneonatal tetanus during the mid-1980s (Schofield, 1986). For the year 2002, deaths from TeNT were estimated by the World Heath Organization (WHO) at 213,000 worldwide, of which 198,000 occurred in children under 5 years of age (Vandelaer et al., 2003).

C. Intestinal Botulism

1. Infant Botulism

Infant botulism is a consequence of intoxication by BoNT following ingestion or inhalation of clostridial spores that colonize the large intestine; young infants, especially those between 2 and 4 months of age, are susceptible to this form of botulism (Pickett et al., 1976; Arnon, 1995). Germination of spores and growth of vegetative cells lead to production of BoNT; the neurotoxin thus elaborated crosses the intestinal wall and reaches susceptible targets such as skeletal muscle

via the bloodstream (Arnon, 1995). The characteristic symptoms are poor sucking, constipation, generalized weakness, and respiratory insufficiency. The risk factors are not completely understood, but the incidence drops off 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 *C. botulinum* spores in mice (Sugiyama and Mills, 1978). Of all food products that may be contaminated with *C. botulinum* spores, honey has been the one most often implicated in infant botulism; it is therefore recommended that honey not be given to young infants (Arnon et al., 1979).

Although infant botulism was not recognized until a large outbreak occurred in California in 1976 (Pickett et al., 1976), it is currently the most prevalent form of botulism in the United States, accounting for approximately 70% of all cases (Shapiro et al., 1998). Because infant botulism results from a continual production of BoNT, it appears to be more effectively treated by antitoxin than is foodborne botulism. In a recently concluded 5 year randomized clinical trial carried out with a human botulinum immune globulin (BIG-IV), it was found that administration of BIG-IV within 3 days of hospitalization resulted in a 3 week reduction in the mean hospital stay, as well as substantial reductions in the time needed for intensive care and mechanical ventilation (Arnon et al., 2006). In a nationwide open label study, BIG-IV was found to be effective even when administered 4–7 days after hospital admission, although to a somewhat lesser extent than when infusion was initiated at 3 days (Arnon et al., 2006).

2. Adult Intestinal Botulism

Under rare conditions, adults may manifest a syndrome similar to that of 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 (Fenicia et al., 1999).

D. Inhalation Botulism

Inhalation botulism is so rare that only one human outbreak has ever been reported (Holzer, 1962). Three laboratory investigators became intoxicated by BoNT while performing necropsies on animals exposed earlier to an aerosol of BoNT/A that was stabilized by addition of colloids. It is assumed that the BoNT/A was reaerosolized from the animals' fur during the course of performing the necropsies. Signs and symptoms of intoxication, consisting of dysphagia, dysphonia, dizziness, headache, and blurred vision, were observed 3 days later. The patients were hospitalized for approximately 1 week but did not require artificial ventilation. Although BoNT gained entry via the lungs, no alteration of pulmonary function was reported in any of the individuals. In animal experiments (guinea pigs and nonhuman primates), it was demonstrated that no specific pulmonary histopathology resulted 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 damage (Duchen, 1971). However, in a more recent study, histopathological alterations were reported in mice that survived intranasally administered BoNT/A when examined 14 days after toxin challenge. Moreover, immunization with the pentavalent toxoid protected mice from lethality but not from lung damage (Taysse et al., 2005). The discrepancy with earlier results may stem from differences in toxin administration.

Since aerosol dispersal of BoNT can create a toxic cloud over large areas, it is considered to be a likely route for use by terrorists. Consequently, a critical question for effective medical management of potential bioterrorist attacks is whether the conventional vaccine and antitoxin would be effective in treating patients following an inhalation exposure of BoNT. In experiments where guinea pigs were immunized with the pentavalent toxoid, the vaccine was found to be as protective against an

inhalation challenge of BoNT as against challenge by other routes of administration (Cardella et al., 1963). More recently, a vaccine derived from recombinant C fragment of BoNT/B (binding domain of the HC) was shown to protect rhesus monkeys from an inhalation challenge of BoNT/B (Boles et al., 2006).

Both human- and equine-derived antitoxins have been tested for protection against an inhalation challenge of BoNT/A, and both were found to be highly effective. Rhesus monkeys were injected with human hyperimmune globulin or equine $F(ab')_2$ antitoxin, yielding plasma titers of <0.02 to 0.6 IU, and challenged with an ~6 LD_{50} dose of aerosolized liquid BoNT/A 48 h later (Franz et al., 1993). Animals not only survived this challenge but were also protected from any signs of BoNT intoxication. Rhesus monkeys 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 h before death. These signs were similar to those observed with other forms of botulism in nonhuman primates and consisted of, in order of onset, muscle weakness, intermittent ptosis, poor head control, dysphasia, and lateral recumbency (Franz et al., 1993).

Since inhalation botulism does not occur in nature, all outbreaks must be considered as suspicious. Prudence would dictate that each should be treated as a criminal or terrorist attack, unless other causes are found (Arnon et al., 2001). From the limited human and animal data currently available, inhalation botulism does not have a unique presentation; rather, the signs and symptoms resemble those of other forms of botulism. The latent period is comparable with that of foodborne botulism without the early GI signs (Adler, 2006).

Detection of inhalation botulism presents a unique challenge for confirming the outbreak and identifying the source of toxin. There would be no obvious wounds, contaminated food, or history of clinical or cosmetic use, where overdoses, although rare, are possible (Klein, 2004; Chertow et al., 2006). Confirmation of inhalation botulism would have to be based on finding toxin in the blood and perhaps in the nasal mucosa of intoxicated patients, on detecting toxin residues in the environment, or on observing an unusual clustering or distribution of cases. Unfortunately, BoNT cannot always be identified in laboratory samples (Shapiro et al., 1998), and the threshold for detecting toxin or residues in the nasal mucosa or in the environment is currently unknown.

III. PROPHYLAXIS AND TREATMENT OPTIONS

BoNTs are the most potent toxins known to mankind, and exposure to as little as 1 ng/kg by injection or 3 ng/kg by inhalation can result in human fatality (Gill, 1982; Arnon et al., 2001; Adler, 2006). Recovery, especially from type A intoxication, is slow (Keller et al., 1999), and residual physical and psychological signs and symptoms may persist for years after exposure (Mann, 1983; Cohen et al., 1988). Some of the residual problems may be a consequence of the prolonged period of inactivity during intoxication (Wilcox et al., 1990). Treatment consists of intensive care, ventilatory support, if required, and infusion of trivalent equine antitoxin (Tacket et al., 1984). The role of antibiotics in the treatment of botulism is controversial: they may be of benefit in eradicating *C. botulinum* in wound botulism (Sandrock and Murin, 2001), but they are considered to be ineffective in the treatment of intestinal or foodborne botulism (Santos et al., 1981). In addition, aminoglycoside antibiotics and tetracycline may exacerbate the BoNT-induced inhibition of neuromuscular transmission (Santos et al., 1981). There is also concern that if antibiotics are used to treat secondary bacterial infections in intestinal botulism, the subsequent lysis of *C. botulinum* vegetative cells may result in increased toxin absorption. This is considered to be less of a problem currently due to the availability of human-derived botulism antitoxin (BIG-IV), with a long residence time ($t_{1/2} \sim 28$ days), which can neutralize the additional toxin load (Arnon et al., 2006).

A. Antitoxin

1. Equine Botulinum Antitoxin

Trivalent equine antitoxin is administered by intravenous infusion and contains antibodies to BoNT/A (7500 IU), BoNT/B (5500 IU), and BoNT/E (8500 IU) (Shapiro et al., 1998). These potencies are 100-fold greater than those required to neutralize the highest toxin levels detected by the CDC in BoNT-intoxicated patients (Hatheway et al., 1984). The trivalent equine antitoxin is currently the only FDA-approved product for botulism, and is effective in limiting the severity of the intoxication if administered early during the course of illness (Shapiro et al., 1998). The temporal limitation of antitoxin treatment has long been appreciated (Hewlett, 1929), and is related to the fact that clostridial neurotoxins exert their actions inside the nerve terminal, where they are not susceptible to antitoxin action (Simpson, 1981). Accordingly, at the time when signs and symptoms of botulism become apparent, a substantial quantity of toxin has already become internalized, and only the fraction that is still in the circulation is available to be neutralized. In a retrospective study of 134 patients, those who received antitoxin within 24 h after onset of signs and symptoms had a lower fatality rate (10%) than those who received antitoxin after 24 h of onset (15%), or those who did not receive antitoxin (46% fatality). In addition, patients who received antitoxin within 24 h had shorter hospital stays and spent fewer days on a ventilator than those who received antitoxin after 24 h (Tacket et al., 1984).

Although it is commonly believed that circulating BoNT is rapidly cleared from the bloodstream this is not always the case. In the recent Oakland Park, Florida outbreak, detectable levels of toxin were observed in one patient 8 days after receiving a massive overdose of nonapproved BoNT/A during a cosmetic procedure (Chertow et al., 2006). For such severely intoxicated patients, antitoxin administration, even if delayed, may still be effective in limiting the duration of illness, since it would neutralize circulating BoNT and prevent further toxin internalization.

2. Recombinant Monoclonal Antibodies

Although the equine antitoxin is effective in reducing the progression and severity of BoNT intoxication, there are significant limitations to its use. First, it is difficult to generate adequate supplies for the potential population at risk in a mass casualty event. In addition, since it is an equine product, the antitoxin has a brief plasma half-life in humans (5–8 days), and there is substantial risk of adverse reactions ranging from mild hypersensitivity to serum sickness (Black and Gunn, 1980; Hatheway et al., 1984). To overcome these limitations, new formulations of antitoxin are currently under development, including production of recombinant single-chain human BoNT antibodies (scFvs) by phage display technology (Marks, 2004). Initial surface plasmon resonance measurements identified four nonoverlapping epitopes on the BoNT/A C-terminal half of HC (C fragment, binding domain) for a set of scFvs (Amersdorfer et al., 1997).

The measured equilibrium-binding constants were encouraging ($K_d = 7.3 \times 10^{-8}$ to 1.1×10^{-9} M), which indicate a potential for neutralizing capability. To elicit protection against BoNT/A, a combination of three human-compatible monoclonal antibodies proved to be necessary (Marks, 2004). Interestingly, no single monoclonal antibody was found to be protective. These findings suggest that large-scale production of high-potency botulinum antitoxins for human use is technically feasible.

As a further refinement, a yeast display system was used as a technology platform for performing molecular evolution by increasing the affinity of those scFv antibodies that bind to BoNT/A. Yeast-displayed scFv libraries have been constructed by selecting scFvs that bind with increased association rates. A single cycle of error-prone mutagenesis increased the affinity for BoNT/A by 45-fold (from $K_d = 9.43 \times 10^{-10}$ to 2.1×10^{-11} M) (Razai et al., 2005). Thus, it is possible that these and other biotechnology approaches could be harnessed to develop neutralizing monoclonal antibodies directed at all seven serotypes of BoNT. Such technological advances would be expected to result in antitoxin

with relatively long plasma circulation times, a reduced incidence of hypersensitive reactions, and the availability of a sufficient quantity to protect the population at potential risk.

B. Prophylaxis

Prophylaxis generally involves vaccination with a pentavalent (A–E) toxoid. Vaccination provides a high degree of protection and is commonly administered to laboratory investigators who are at risk of exposure. The current vaccine has been available from the CDC as an Investigational New Drug (IND) for the past 45 years (Fiock et al., 1963). The vaccine is administered intramuscularly at 0, 2, and 12 weeks and requires a booster at 1 year to generate long-term protection. A heptavalent vaccine (A–G) originally developed by the U.S. Army (Franz, 1997; Middlebrook and Franz, 1997), will be produced by DynPort Vaccine Co. LLC under the aegis of the National Institute of Allergy and Infectious Diseases (NIAID), and is expected to be available by the end of this decade.

Recently, a vaccine made from the recombinant BoNT C fragment has been reported to protect nonhuman primates from an aerosol exposure of BoNT, and neutralizing antibody titers were detected for up to 2 years following vaccination (Boles et al., 2006). In addition to needle delivery, there is a considerable interest in developing a mucosal vaccine for BoNT. This is based on the premise that the mucosal immune system would be the first line of defense for inhaled BoNT, and administration of mucosal vaccines may be easier to carry out in the general population (Park and Simpson, 2004; Fujihashi et al., 2007).

Although these vaccines are effective, they require multiple inoculations and require up to a year from onset to generate adequate protection. In addition, since the BoNT antibodies remain elevated for a prolonged period, vaccinated individuals may be precluded from use of BoNT for treatment of spasticity or movement disorders that might develop during their lifetime (Jankovic and Brin, 1997). These limitations argue strongly in favor of a supplementary pharmacological approach for the management of botulism.

C. Pharmacological Intervention

From the time that inhibition of ACh release was established as the mechanism of BoNT action, attempts were made to antagonize the neurotoxin by measures that enhance ACh release. Until recently, however, development of a treatment for BoNT intoxication had low priority, in part because early efforts were generally unsuccessful, and in part because effective vaccines and antitoxins were already available. Currently, there is an increased impetus to develop pharmacological treatments following recognition of the potential for overdose with the expanding clinical use of BoNT (Klein, 2004; Chertow et al., 2006; Souayah et al., 2006). In addition, the experience gained in preparation for a potential BoNT threat during the Persian Gulf War made it clear that delays in generating adequate protection by the BoNT vaccine were not consistent with the requirement for rapid deployment of military personnel (Atlas, 1998). In addition, marked sequence variability has been found within BoNT serotypes, where subtypes exhibit differences in susceptibility to antibody neutralization (Smith et al., 2005). Such variability will need to be incorporated in the development of future vaccines and antitoxins to ensure adequate protection.

Some of the earliest putative BoNT antagonists were cholinesterase inhibitors, selected for their ability to prolong the actions of ACh. Carbamate anticholinesterase agents such as neostigmine and physostigmine were investigated in animals (Edmunds and Keiper, 1924), and in nerve-muscle preparations (Guyton and MacDonald, 1947), but they were unable to antagonize the effect of BoNT. More recent studies have tended to confirm earlier findings (Adler et al., 1995), although there have been occasional reports of human botulism responding to the short-acting cholinesterase inhibitor, edrophonium (Cherington, 1998).

Other potential antagonists of BoNT action such as elevated calcium, calcium ionophores, lanthanum, black widow spider venom, 2,4-dinitrophenol, and agents that raise cyclic AMP levels

were examined for their ability to reverse BoNT toxicity. Addition of the above compounds to BoNT-intoxicated nerve-muscle preparations led to increases in the frequency of spontaneous miniature endplate potentials (MEPPs) but resulted in little or no enhancement in the amplitude of evoked endplate potentials (EPPs) (Simpson, 1988; Thesleff, 1989). Since these compounds generally increased spontaneous but not evoked ACh release, they were not considered to be of practical value for treatment of BoNT intoxication.

1. Potassium Channel Blockers

Potassium channel blockers were found to be more effective in antagonizing the paralytic action of BoNT than were the former group of compounds. Their higher efficacy comes from their ability to prolong the duration of the presynaptic action potential (Penner and Dreyer, 1986), leading to a greater influx of calcium during nerve stimulation. Coupling of increased calcium influx to nerve impulses enables the potassium blockers to produce striking increases in the amplitude of EPPs and of nerve-evoked twitch tensions (Adler et al., 1979).

A number of potassium channel blockers have been evaluated for their ability to antagonize the actions of BoNT, including guanidine, 4-aminopyridine, 3,4-diaminopyridine (3,4-DAP), and tetraethylammonium (Lundh et al., 1977; Molgo et al., 1980; Simpson, 1986). Of these, the most promising candidate was 3,4-DAP; 4-aminopyridine exhibited undesirable CNS side effects, and tetraethylammonium caused a marked postsynaptic depression of endplate potentials and muscle contractions that actually exacerbated BoNT-mediated inhibition (Adler et al., 1979, 1995; Simpson, 1988; Thesleff, 1989; Cherington, 1998). When added to nerve-muscle preparations before BoNT, 3,4-DAP produced a marked delay in the time-to-block of nerve-evoked muscle contractions (Simpson, 1986). When applied after BoNT paralysis, 3,4-DAP was able to restore tensions to near-control values (Lundh et al., 1977; Molgo et al., 1980; Simpson, 1986; Adler et al., 1995). Unlike many candidate antagonists, 3,4-DAP could restore tension even several days after total paralysis was established (Adler et al., 1996). In spite of these successes with 3,4-DAP, two fundamental limitations were noted: its efficacy was largely limited to serotype A (Simpson, 1986), and it had a brief in vivo lifetime relative to that of BoNT (Adler et al., 1996). Of the two, the latter is less critical since the short lifetime can be compensated by use of an infusion delivery as shown by Adler et al. (2000).

The basis for the lack of response to 3,4-DAP by the other serotypes is not well understood. At a functional level, serotype A-intoxicated neuromuscular junctions undergo an attenuated but synchronous release of ACh following stimulation; preparations intoxicated by serotypes B, D, and F produce asynchronous release where the ACh quanta are dispersed and cannot summate to produce suprathreshold EPPs (Lundh et al., 1977; Molgo et al., 1980; Thesleff, 1989). It is readily apparent that the lack of synchrony would prevent 3,4-DAP from restoring transmitter release; however, the factors that lead to asynchronous release are not currently understood.

An additional concern with potassium blockers comes from human case reports. These indicate that although the potassium blockers guanidine and 3,4-DAP produced a moderate increase in muscle strength, their use did not lead to the return of spontaneous ventilation in BoNT-intoxicated individuals (Cherington and Schultz, 1977; Davis et al., 1992). It is not clear if human diaphragm or intercostal muscles are less responsive to potassium blockers than are limb muscles, or whether the doses used clinically were insufficient to reverse muscle paralysis (Davis et al., 1992). The latter may be the case, since BoNT/A-paralyzed rat or mouse diaphragm muscles respond vigorously to the actions of 3,4-DAP (Simpson, 1986; Adler et al., 1995). Higher doses of 3,4-DAP were not attempted in these patients to avoid the risk of seizures and other potential side effects. At the present time, the potassium blockers hold promise as potential therapeutic agents, but development of more selective compounds or targeting of the inhibitors to neuromuscular and neuroeffector synapses will be required to exploit their full potential.

2. Inhibitors for Specific Stages of Intoxication

The examples given earlier of treatment strategies are based on antagonizing the actions of BoNT after the neurotoxin has undergone internalization and subsequent cleavage of its target protein. In addition, since these approaches were developed before the intracellular targets were identified, they do not specifically antagonize the action of toxin at the molecular level. Rather, these compounds act by elevating intracellular calcium levels in an attempt to compensate for the toxin-mediated inhibition of ACh release. The discrete stages of clostridial neurotoxin action of binding, internalization, translocation, and catalysis suggest that there are multiple sites for direct pharmacological intervention. These stages are mediated by different domains of BoNT, and in principle, each can be specifically inhibited (Simpson, 1988, 2004). Three areas where significant progress has been made will be discussed in the following sections.

a. Inhibitors of Binding

A reasonable starting point for developing pharmacological countermeasures for BoNT intoxication is the use of receptor antagonists to reduce or prevent the binding of toxin to the nerve terminal. Complications with this approach are that many BoNTs bind to dual polysialoganglioside–protein receptors on the surface of nerve terminals, and that different BoNT serotypes recognize different protein–ganglioside combinations (Dolly et al., 1984; Montecucco, 1986; Yowler et al., 2002; Kohda et al., 2007). This implies that multiple receptor antagonists would need to be developed to protect against the BoNT serotypes responsible for human intoxications.

1. Ganglioside Component of BoNT Receptor. Evidence for involvement of gangliosides in botulinum intoxication is extensive (Van Heyningen and Miller, 1961; Montecucco, 1986; Shapiro et al., 1997; Kitamura et al., 1999). The most direct demonstration, however, comes from the study of Bullens et al. (2002). These authors reported that in diaphragm muscles obtained from a line of knockout mice that lack polysialogangliosides (GalNac-$T^{-/-}$), a paralytic concentration of BoNT/A (~2 ng/mL BOTOX) had no effect on spontaneous or evoked release of ACh. In agreement with this finding, Kitamura et al. (1999) showed that the LD50 of BoNT/A is 40-fold greater in GalNac-$T^{-/-}$ mice than in wild-type littermates. The relative resistance to BoNT/A, but absence of complete protection, suggested that GD3 or GM3 gangliosides, which were upregulated in the knockout mice, may serve as potential receptors for BoNT in these mice. Alternatively, it is possible that the protein receptor may be sufficient for binding of toxin at these higher concentrations.

Pronounced antagonism of neurotoxin binding has been achieved with lectins from *Triticum vulgaris* (TVL) and *Limax flavus* (Bakry et al., 1991). Pretreatment by these lectins led to a concentration-dependent inhibition in the binding of BoNT/B and TeNT to preparations of rat brain membranes, approaching total inhibition at the highest concentration. The most effective lectins were those that had an affinity for *N*-acetyl-α-sialic acid; six lectins with specificities for other carbohydrates were ineffective (Bakry et al., 1991). In complementary experiments on mouse phrenic nerve-hemidiaphragm preparations, TVL delayed the time-to-block of nerve-elicited muscle contractions with all BoNT serotypes examined (A–F). If one defines the time-to-block in the presence and absence of BoNT antagonist as a protective index, the values for the different serotypes ranged from 1.3 to 1.9. Although the physiological actions of TVL appear less striking than its antagonism of binding, it must be borne in mind that a 10-fold decrease in bound neurotoxin can only be expected to produce a 2-fold slowing in the time-to-block (Bakry et al., 1991).

Since the isolated diaphragm muscle has a limited viability in vitro ($\leq$8 h; Adler et al., 1995), it is generally tested with high concentrations of BoNT where protection may be difficult to demonstrate. It was therefore of interest to determine the efficacy of TVL in a physiological preparation where lower toxin doses can be used, and where protection can be assessed over a longer time interval. In a recent study from our laboratory, TVL was injected locally in the rat extensor digitorum longus (EDL) muscle 30 min before a local injection of 0.6 units (U) of BoNT/A. Muscle tension was recorded in situ 7 days after BoNT administration. As shown in Table 16.1, pretreatment by TVL led to

TABLE 16.1
Pretreatment by *Triticum vulgaris* Lectin (TVL) Protects Rat EDL Muscle from Paralysis Following Local Injection of BoNT/A

BoNT/A Dose	Pretreatment	TVL Dose (mg)	Number of Muscles	Twitch Tension (g) Mean ± SEM
None	None		5	69.4 ± 3.4
0.6 U	None		4	2.0 ± 0.9[a]
None	TVL	0.375	3	61.2 ± 10.0
0.6 U	TVL	0.375	3	9.7 ± 3.1[a]
0.6 U	TVL	0.75	6	23.8 ± 6.4[a,b]

Note: The dose of BoNT/A was selected to produce ≥95% inhibition of muscle tension 48 h after injection. TVL was injected 30 min before BoNT/A. Tensions were tested 7 days after BoNT/A administration; doses are expressed as mouse i.p. LD_{50} units (U).

[a] Tensions differ significantly from pre-BoNT/A values ($p < 0.05$).

[b] Tensions differ significantly from muscles injected with BoNT/A alone ($p < 0.05$).

protection, albeit incomplete, of muscle tension following injection of a paralytic dose of BoNT/A. Moreover, protection was sustained for at least 1 week, at which time muscles that were not pretreated with TVL still showed nearly complete paralysis (2.9% of control tension).

The major advantage of the lectins is that they are effective against all clostridial toxin serotypes (Bakry et al., 1991). The disadvantage of using lectins to protect against BoNT is that they must be administered as pretreatments. This limitation is inherent in the basic mechanism of BoNT action; thus no antagonist of surface receptor binding would be expected to be protective once BoNT is internalized and symptoms are manifested.

2. Protein Component of BoNT Receptor. Based on the high-affinity, cholinergic selectivity, and sensitivity to proteolytic enzymes (Black and Dolly, 1986), the binding of BoNT to the nerve terminal was suggested to involve a protein component in addition to polysialogangliosides (Montecucco, 1986). Nishiki et al. (1994, 1996a, 1996b) were the first to identify a protein receptor for BoNT. Using rat brain synaptosomal membranes, these authors demonstrated that BoNT/B bound to synaptotagmin (Syt), a synaptic vesicle protein that also serves as the calcium sensor for evoked transmitter release (Nagy et al., 2006). Binding to Syt was found to be saturable and to be enhanced by the inclusion of the ganglioside G_{D1a} or G_{T1b} (Nishiki et al., 1994). However, since the work was performed in tissue that is not the physiological target for the clostridial neurotoxins, and the authors did not demonstrate that the binding of BoNT to Syt led to toxin internalization, the results were not universally accepted (Middlebrook and Franz, 1997).

Compelling evidence that Syt was indeed the protein receptor for BoNT/B was provided by the seminal work of Dong et al. (2003). These authors demonstrated that Syt isoforms I and II were responsible for the productive binding of BoNT/B to PC12 cells and to motor nerve terminals of diaphragm muscle; Syt-I had lower affinity for BoNT/B and required gangliosides for binding, whereas Syt-II produced higher-affinity binding that did not require gangliosides (Dong et al., 2003). The authors also demonstrated that fragments of Syt-II that contained the toxin-binding domain (luminal region) delayed the time to death in mice challenged by intravenous BoNT/B. Syt was subsequently demonstrated to be the protein receptor for BoNT/G but not for any of the remaining five BoNT serotypes (Rummel et al., 2004).

In addition to serotypes B and G, the protein receptor for serotype A has now been identified. Dong et al. (2006) demonstrated that the synaptic vesicle protein SV2 (isoforms A, B, and C) is the protein receptor for BoNT/A. SV2 is an integral membrane protein with 12 putative transmembrane domains and is normally associated with secretory vesicle membranes (Janz and Sudhof, 1999).

Several lines of evidence implicated SV2 as the protein receptor for BoNT/A: (1) binding of BoNT/A but not BoNT/B was reduced in hippocampal neurons cultured from SV2 knockout mice, (2) fragments of SV2 that contain the toxin interaction domain (luminal loop 4) inhibited binding of BoNT/A to neurons, and (3) SV2B knockout mice were found to be less sensitive to an intravenous challenge of BoNT/A and, in addition, diaphragm muscles from these mice showed a significantly reduced binding of BoNT/A.

SV2 and Syt are thought to become incorporated in the plasma membrane of the nerve terminal during the process of transmitter release, where the secretory vesicle membrane is transiently fused with the plasma membrane (Bonanomi et al., 2006). This explains the presence of synaptic vesicle proteins on the surface of nerve terminal and accounts for the finding that the rate of BoNT intoxication increases with synaptic activity (Hughes and Whaler, 1962).

Recent insight into the binding of BoNT/B has come from X-ray crystallographic studies in which the luminal domain of Syt-II was crystallized with BoNT/B (Chai et al., 2006) or with the C fragment of BoNT/B (Jin et al., 2006). These studies revealed that a helix is induced in the luminal domain of Syt-II, which binds to a hydrophobic groove or saddle-shaped crevice near the C terminus of the BoNT/B HC. Gangliosides bind to nonoverlapping adjacent sites that are separated from the Syt-binding site by 15Å (Chai et al., 2006; Jin et al., 2006). This binding orients the translocation domain of the HC for optimal interaction with the plasma membrane. Mutations in the luminal domain of Syt-II as well as fragments of this peptide were found to inhibit the binding of BoNT/B (Chai et al., 2006). In addition, single-site mutations in recombinant BoNT/B neurotoxin in the region of the Syt-II-binding domain resulted in marked loss of potency when tested in the mouse phrenic nerve-hemidiaphragm preparation, especially mutations A1196K and K1192E (Jin et al., 2006). These findings suggest that peptide fragments of the protein-binding domains and inactive mutant BoNT HC variants have the potential to be developed into inhibitors. More extensive work will be required to determine if these inhibitors have practical application and if they provide benefit over immunological approaches.

Since the details of toxin binding have been reported only for a limited number of serotypes, it is not clear whether the remaining serotypes will also be found to bind to adjacently located protein–ganglioside receptors, or whether the proteins will be derived from transient synaptic vesicle fusions with the plasma membrane. Assuming the former, it is tempting to speculate on the cholinergic selectivity of these neurotoxins. Possibilities include: (1) a high density of synaptic vesicle proteins on cholinergic nerve terminals from the generally rapid transmitter release rates of cholinergic motor neurons (Brooks, 1956; Thesleff, 1989), (2) a high density of the appropriate polysialogangliosides on cholinergic terminals, and (3) favorable localization (geometric arrangement) of the appropriate protein- and ganglioside-binding components.

It must be pointed out, however, that the cholinergic selectivity of the BoNTs is not absolute, and a variety of cell types can be intoxicated in vitro with sufficiently high toxin concentrations or prolonged incubation times (MacKenzie et al., 1982; Schiavo et al., 2000). In addition, direct injection of BoNT into the CNS can give rise to central actions that are not observed during exposure by conventional routes (Bozzi et al., 2006). The finding that noncholinergic cell types and central neurons are not ordinarily affected by BoNT suggests that they may have a limited access to toxin, a lower density of productive receptors, or unfavorable protein–ganglioside geometry.

*b. Inhibitors of Internalization**

Following binding of the clostridial neurotoxins to receptors on cholinergic nerve terminals, the toxins undergo internalization before reaching their ultimate intracellular targets (Schiavo et al., 2000; Simpson, 2004). Internalization is thought to involve endocytosis of the BoNT–receptor complex, acidification of the resulting endocytotic vesicle, dissociation of the LC and HC, and

* Internalization, in this section, is used broadly to describe the entry of toxin or LC into the nerve terminal cytosol and includes both trafficing from the cell surface to the endosoma and translocation across the endosomal membrane.

release of the LC into the cytosol (Koriazova and Montal, 2003; Simpson, 2004). The most direct evidence for internalization comes from experiments in which colloidal gold-BoNT conjugates have been visualized inside cholinergic motor axon terminals (Dolly et al., 1984) and torpedo electric organ synaptosomes (Blasi et al., 1992).

Internalization affords the next opportunity to ameliorate the toxic actions of BoNT. A number of pharmacological agents have been examined for inhibition of this process with various degrees of success. Simpson (1983) demonstrated that pretreatment of phrenic nerve-hemidiaphragm preparations with the lysosomotropic agents ammonium chloride or methylamine hydrochloride delayed the time-to-block of nerve-evoked muscle contractions after exposure to BoNT serotypes A, B, C1, and TeNT. Incubation of nerve-muscle preparations with ammonium chloride and methylamine hydrochloride was effective if applied before, concurrently, or up to 20 min after toxin exposure. The efficacy of the lysosomotropic agents was reduced rapidly with further delays, such that no effect was observed if they were administered 30–35 min after toxin exposure. At optimal concentrations, these compounds produced a twofold delay in the time-to-block (Simpson, 1983).

Other candidates examined for inhibiting BoNT-mediated internalization were the antimalarial agents chloroquine and hydroxychloroquine (Simpson, 1982). These drugs were selected on the basis of interfering with the actions of a large group of peptide hormones and protein toxins that exert their actions following internalization (Goldstein et al., 1979). The maximal efficacies of the above 4-aminoquinolines were similar to those of ammonium chloride and methylamine hydrochloride, and both groups exhibited a comparable therapeutic window. They differed in that effective concentrations of the 4-aminoquinolines also produced a reversible depression of neuromuscular transmission.

Work on antimalarial agents was extended by Deshpande et al. (1997) to identify candidates that did not block neuromuscular transmission, had a longer therapeutic window, and could delay the time-to-block to a greater degree. These investigators examined a large group of 4- and 8-aminoquinoline compounds as well as analogous acridines for their efficacy against BoNT in mouse diaphragm preparations. The most effective compounds were quinacrine, amodiaquine, and chloroquine; 8-aminoquinolines such as primaquine were ineffective. The highest protective index, 3.9, was obtained with 20 μM amodiaquine. This was achieved with no deleterious effects on neuromuscular transmission, and thus defines the present limit for inhibitors of internalization. Unfortunately, the therapeutic window could not be extended; no protection was observed if the antimalarial agents were added $\geq$40 min after exposure to BoNT/A or BoNT/B.

A somewhat different approach for attempting to prevent or reduce the internalization of BoNT was to treat nerve-muscle preparations with the proton ionophores monensin and nigericin (Adler et al., 1994; Sheridan, 1996). These ionophores act by depleting vesicular pH gradients, thereby interfering with several stages in the delivery of active LC in the cytosol. These ionophores were found to be approximately as effective as the other inhibitors of internalization. They were more toxic, however, and high concentrations led to a depression of neuromuscular transmission (Adler et al., 1994; Sheridan, 1996). Toxicity is difficult to avoid with this group of agents since proton gradients are required for a number of cellular reactions such as the synthesis of ATP and filling of synaptic vesicles.

c. *Metalloprotease Inhibitors*

The third area for therapeutic intervention is inhibition of the metalloprotease activity of the BoNT LCs. This field is potentially the most promising, especially since the crystal structures of the LCs for all serotypes have been solved (Agarwal et al., 2005; Arndt et al., 2005, 2006). The presence of a zinc-binding motif in the LC of clostridial neurotoxins, and the finding that zinc is required for neurotoxin-mediated proteolysis of SNARE proteins (Schiavo et al., 1992a; Montecucco and Schiavo, 1993), suggest that three classes of potential inhibitors may be effective in antagonizing the toxic actions of BoNT LC: metal chelators, zinc metalloprotease inhibitors, and exosite inhibitors. Simpson et al. (1993) demonstrated that the zinc chelator *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN) caused a marked slowing in the time-to-block of nerve-evoked muscle

contractions when administered before BoNT in phrenic nerve-hemidiaphragm preparations. The maximum efficacy was equivalent to that achieved with TVL, ammonium chloride, methylamine hydrochloride, or the most potent antimalarial drugs.

In common with the above inhibitors, TPEN was effective against all BoNT serotypes examined. In addition, when coapplied with TVL or the lysosomotropic agents, the protection observed with TPEN was approximately additive with that of the former compounds. These results are encouraging because they demonstrate that, in principle, concerted inhibition of the different stages in the production of toxicity is a viable strategy for managing BoNT intoxication. Sheridan and Deshpande (1995) examined a number of additional chelators on nerve-evoked twitch tensions and concluded that both a high affinity for zinc and membrane permeability are required for antagonism of BoNT.

The results with TPEN in the isolated nerve-muscle preparations were sufficiently encouraging to test this chelator for protection against challenge by BoNT; unfortunately, these results were less encouraging. First, TPEN was found to be highly toxic in vivo, producing rapid lethality at doses above 20 mg/kg in mice (Adler et al., 1997). Second, at the highest tolerated dose, TPEN only increased survival by 2–3 h following a 20 LD_{50} challenge of BoNT/A or BoNT/B. Toxicity of TPEN was also observed with primary and clonal cells. TPEN concentrations $\geq$10 μM produced morphological damage with characteristics of apoptosis (Adler et al., 1999b).

Studies with ion replacement indicated that chelation of zinc was the proximal cause of cytotoxicity, and examination of a variety of chelators suggested that those with high membrane permeability were especially apt to produce cell death (Sheridan and Deshpande, 1998). Based on these findings, metal chelators may have a limited use in the therapy of botulinum intoxication since the requirements for efficacy against BoNT are the same ones that promote cellular toxicity.

A more promising approach is the development of metalloprotease inhibitors to target the catalytic activity of BoNT LC. This endeavor was made possible by the discovery of the zinc metalloprotease activity of the clostridial toxins that began almost 20 years ago when the HEXXH signature sequence of zinc-binding proteins was noted in the TeNT LC by Jongeneel et al. (1989). Their finding suggested that clostridial neurotoxins possessed zinc-dependent protease activity. During the next 4 years, the SNARE protein substrates and serotype-specific cleavage sites were identified and correlated with intoxication in a systematic series of studies (Montecucco et al., 1994; Rossetto et al., 1995). It is noteworthy that an enzymatic activity for BoNT/A was suspected as far back as 1947 by Guyton and Marshall in their pioneering study on botulinum intoxication where it is stated that:

> . . . this minute quantity of toxin necessary to produce poisoning, the duration of poisoning and the physical properties of the toxin all tend to characterize the toxin as a destructive enzyme . . .

Metalloprotease inhibitors have the advantage of being potentially effective after onset of BoNT intoxication. Of the pharmacological agents discussed thus far, metalloprotease inhibitors (and metal chelators) are the only ones not constrained by the brief time window that limits antitoxin efficacy, as well as the efficacy of inhibitors of BoNT binding, internalization, and translocation. To be viable candidates, metalloprotease inhibitors would need to be highly potent, be able to gain access to the internalized BoNT LC in the cytosol, and have unusually high stability to match that of the BoNT LC (Keller et al., 1999; Adler et al., 2001; Keller and Neale, 2001; Foran et al., 2003; Meunier et al., 2003).

Early work with zinc metalloprotease inhibitors focused on the well-characterized agents captopril ((2S)-1-[(2S)-2-methyl-3-sulfanyl-propanoyl] pyrrolidine-2-carboxylic acid) and phosphoramidon (*N*-alpha-L-rhamnopyranosyloxy[hydroxyphosphinyl]-L-leucyl-L-tryptophan). These compounds, however, were found to have little inhibitory activity against BoNT (Adler et al., 1994, 1999a). The poor efficacy of captopril was suggested to stem from unfavorable steric constraints in the binding of proline at the active site of BoNT (Schmidt and Stafford, 2002).

Phosphoramidon analogs in which Leu–Trp was replaced by Phe–Glu to resemble the cleavage site of synaptobrevin exhibited little increase in inhibitory activity; one analog was marginally more potent and two were significantly less potent than the parent compound (Adler et al., 1999a).

Several classes of organic compounds were examined for their ability to inhibit the catalytic activity of BoNT/B, chiefly isocoumarins and phosphonates. Adler et al. (1998) tested a series of isocoumarin compounds that were originally designed as elastase inhibitors. Molecular modeling studies suggested that these compounds may interact favorably with the BoNT/B-active site, and several candidates were able to inhibit BoNT/B LC activity. The most effective compound in this series was 7-*N*-phenylcarbamoylamino-4-chloro-3-propyloxyisocoumarin, which had an IC_{50} of 28 μM when tested in a cleavage assay using a 50-mer synaptobrevin peptide.

Since existing metalloprotease inhibitors showed little promise for development, the focus for drug discovery shifted to peptide inhibitors by systematically modifying key residues in the substrate and examining the consequences of these alterations on enzymatic activity. Schmidt et al. (1998) made single residue changes near the cleavage site of a 17-mer SNAP-25 peptide that was a minimal substrate for BoNT/A. Substitution of Cys in the P1 or P2 position transformed the peptide from a substrate to a competitive inhibitor. The best inhibitors in this series had K_i values of ~2 μM in cell-free assays. Efficacy was attributed to the favorable location of the sulfhydryl group of Cys, which was postulated to interact with the catalytically important zinc in the BoNT-active site. With further elaboration of this approach, more potent inhibitors were developed, as exemplified by the pseudo peptide, 2-mercapto-3-phenylpropionyl-RATKML ($K_i = 0.33$ μM; Schmidt and Stafford, 2002). The most potent inhibitor of BoNT/F ($K_i = 1$–2 nM) was a peptidomimetic in which d-Cys was substituted for Gln-58 in the minimum synaptobrevin fragment (Schmidt and Stafford, 2005).

Sukonpan et al. (2004) also developed substrate-based inhibitors for BoNT/A using a similar 17-mer SNAP-25 peptide (aa 187–203). The authors incorporated α-thiol amide in place of Gln-197 (P1 residue) in the 17-mer SNAP-25 peptide to generate submicromolar inhibitors of BoNT/A catalytic activity. A similar approach with a 35-mer synaptobrevin peptide resulted in inhibitors of BoNT/B LC protease with K_i values in the low micromolar range (Oost et al., 2003). A combinatorial approach using a hinged peptide for BoNT/A and BoNT/B has also been reported (Moore et al., 2006). In spite of their generally high potencies, peptide inhibitors are considered to be unsuitable as therapeutic agents since they are unstable in vivo and would have difficulty gaining access to the nerve-terminal cytosol to inhibit internalized BoNT LC. They have, however, served as templates for synthesis of organic drug candidates and have been used successfully by Burnett et al. (2003, 2007) to select effective compounds from natural products libraries.

Anne et al. (2005) demonstrate that a pseudotripeptide inhibitor and its disulfide-coupled dimer could prevent BoNT/B-mediated inhibition of norepinephrine release from cortical synaptosomes. This action was observed in the presence of inhibitor concentrations >15 μM. The relatively high concentrations required for inhibition of BoNT/B on norepinephrine release contrasts with the 2 nM IC_{50} observed for inhibition of the BoNT/B LC-mediated cleavage of a synaptobrevin peptide in a cell-free enzymatic assay (Anne et al., 2005). The lower potency in cell-based assays appears to reflect the difficulty of these charged peptidomimetic inhibitors to cross plasma membranes, which represents a major limitation of this class of compounds.

Small organic inhibitors with moderately high potency against BoNT protease activity are beginning to emerge. In general, the organic inhibitors described to date are less potent than the most effective peptide-based inhibitors; however, continued work on structure optimization should lead to improvements in their potency, selectivity, and cellular efficacy. Park et al. (2006) performed in silico screens of 2.5 million compounds that were capable of coordinating with the active site zinc of BoNT/A LC and determined a structure commonality for inhibition. Using multiple molecular dynamics simulations, the authors examined the interaction of the initial set of effective compounds with the BoNT/A LC-active site. Modeling studies suggested that inclusion of a hydroxamate would improve interactions with the active site zinc, whereas an additional aromatic ring would

increase interactions with the serotype-specific Phe-193 in the active site. From these insights, the authors were able to synthesize an organic inhibitor with a K_i of 12 μM, which is among the most potent and serotype-selective small molecule inhibitor of BoNT/A thus far reported in a cell-free enzymatic assay.

Boldt et al. (2006b) also screened organic inhibitors of BoNT/A metalloprotease activity on the basis of their interactions with the active site zinc. Initial efforts with arginine hydroxamate led to an inhibitor with a K_i of 60 μM. Subsequently, a hydroxamate library with carboxylic acids was tested. The best agent in this screen was 4-chlorocinnamic hydroxamate (with an IC_{50} of 15 μM). Further chloro substitution led to 2,4 dichlorocinnamic hydroxamic acid, a competitive inhibitor with a K_i of 0.30 μM. The potency of this compound is comparable with that of the best peptide inhibitors for BoNT/A (Schmidt and Stafford, 2002). However, Burnett et al. (2007) reported that this compound was less potent, and had an IC_{50} of 29 μM under their assay conditions. A novel compound, Fmoc-d-Cys(Trt)-OH, was recently tested by Boldt et al. (2006a). This compound was found to be a moderately potent inhibitor of BoNT/A LC protease activity ($K_i = 18$ μM) and was nearly equipotent in its ability to antagonize BoNT/A in neuro-2 cells. The latter is encouraging since most LC protease inhibitors are much less effective in cellular assays than in cell-free assays (Anne et al., 2005).

In addition to the BoNT-active site, other regions of the LC have received attention as potential areas for inhibition. Lebeda and Olson (1994) first suggested that the high substrate selectivity of the family of BoNT proteins depended on ''the critical positioning of the substrate by . . . non-perfectly conserved residues'' and by ''the residues flanking the active-site region.'' This suggestion is consistent with the observation that two exosites are crucial for SNAP-25 binding to the LC of BoNT/A (Breidenbach and Brunger, 2004). The α- and β-exosites were recognized as being potentially important in the development of specific inhibitory drugs, and targeting the peripheral sites of substrate binding has focused on a family of bis-imidazole inhibitors for BoNT/A (Merino et al., 2006).

A recent study by Eubanks et al. (2007) has highlighted limitations of conventional drug development paradigms. The authors found that high-throughput screens did not consistently predict the candidates that would be most effective in their cell-based assays. Some obvious reasons include cytotoxicity and poor membrane permeability of potential inhibitors. However, other reasons are that current drug screens generally use small peptide fragments of substrates, which may have different secondary structures and often lack exosite regions (Chen and Barbieri, 2006). In addition, in cells, the LCs of at least some serotypes (e.g., BoNT/A) are localized to the cytoplasmic surface of the membrane, and are not in free solution (Fernandez-Salas et al., 2004). Eubanks et al. (2007) also observed that efficacy in cell culture did not predict protection in vivo. Complicating factors include absorption, distribution, metabolism, clearance, and systemic toxicity of inhibitor candidates. A prudent approach, therefore, is to combine high-throughput screening with cellular, isolated tissue, and in vivo assays as an iterative strategy, rather than to rely primarily on cell-free assays.

Although metalloprotease inhibitors are not constrained by a brief therapeutic window, recovery from BoNT intoxication may still be delayed because of the time required to replace cleaved SNARE proteins with intact ones. Estimates from pulse-chase experiments suggest that a half-time of ~1 day would be required to replace SNAP-25, 4–5 days to replace synaptobrevin, and ~6 days to replace syntaxin, even if no further cleavage were to occur (Foran et al., 2003). Examination of the fraction of total SNARE proteins cleaved by BoNT reveals that a relatively small fraction is cleaved at the neuromuscular junction at the time of total muscle paralysis. From local injections of BoNT/A in vivo, Jurasinski et al. (2001) estimated that paralysis requires cleavage of less than 35% of the total SNAP-25. Meunier et al. (2003) reported that isolated diaphragm muscles exposed to 2 nM BoNT/A have only 6.5% of their SNAP-25 in the cleaved form, and suggested that the SNAP-25 relevant to transmitter release must exist in a small specialized pool. It is likely that the critical pool of SNAP-25 is even lower than that found by Meunier et al. (2003), since the

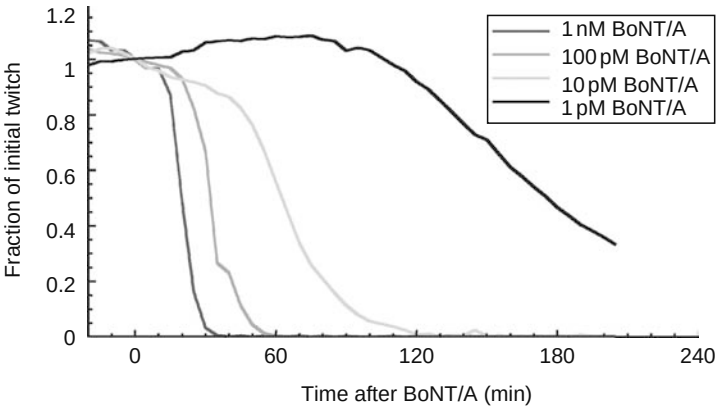

FIGURE 16.1 The effect of BoNT/A on muscle tension in isolated mouse phrenic nerve-hemidiaphragm preparations. Muscles were exposed to BoNT/A at concentrations ranging from 1 pM to 1 nM and the time to paralysis was monitored. Toxins were added to the muscle bath at 0 time from concentrated stock solutions; each concentration was tested on a separate hemidiaphragm muscle. Twitch tensions were elicited by supramaximal stimulation of the phrenic nerve at 30 s intervals. Temperature, 37°C.

concentration of BoNT/A required to paralyze diaphragm muscle is <1 pM (Figure 16.1), considerably lower than the concentration used in the above study. In fact, data from our laboratory on rat EDL muscle indicate that the SNAP-25 cleavage following local injection of a paralytic dose of BoNT/A (1.25 U) is often undetectable (Figure 16.2). The above suggests that after complete inhibition of LC protease activity, recovery of synaptic function will be determined by the turnover rates of the cleaved SNARE proteins, which are in the same range as the turnover rates of the native SNAREs (Foran et al., 2003).

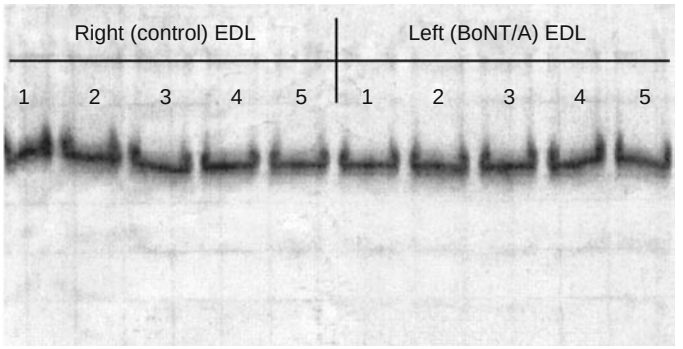

FIGURE 16.2 Western blots of SNAP-25 from rat EDL muscle following local injection of 1.25 U of BoNT/A (15 μL) (left EDL). The right EDL muscles served as uninjected controls. The expected 24 kDa truncated fragment resulting from BoNT/A cleavage of SNAP-25 was not detectable with a paralytic dose of BoNT/A. SMI-81 (Sternberger Monoclonals, Inc) was used for precipitation, whereas rabbit polyclonal antiserum 2777 was used as the primary antibody (BioSynthesis, Inc.). The numbers 1, 2, 3, 4, and 5 correspond to 2, 4, 7, 14, and 30 days after BoNT/A injection, respectively. Neurally elicited twitch tensions were recorded in situ just before excision. Tensions were 2%, 5%, 3%, 32%, and 39% of control, at the above respective time points. Note that the truncated SNAP-25 fragment could not be detected even at day 2, when tensions were 2% of control.

d. *BoNT Inhibitors Derived from Natural Products*

Toosendanin is a triterpenoid compound obtained from the bark of *Melia toosendan* and has been used in traditional Chinese medicine as an antiparasitic agent and agriculture insecticide. Toosendanin was reported to inhibit BoNT intoxication in animal models including nonhuman primates, and to alter the action of a number of ion channels, including the BoNT/A translocation channel (Shi and Wang, 2004; Li and Shi, 2006). Based on reports of its efficacy against BoNT in the Chinese literature, considerable interest has arisen in toosendanin as a potential BoNT antagonist (Sheridan and Parris, 2003; Dickerson and Janda, 2006). In our laboratory, we have found that toosendanin was able to antagonize BoNT/A intoxication in isolated mouse phrenic nerve-hemidiaphragm preparations when administered at the same time or 30 min before BoNT/A, but not when given 30 min after BoNT (Figure 16.3). We have also found that toosendanin is equally effective against BoNT serotypes BoNT/A, BoNT/B, or BoNT/E. The absence of serotype selectivity, coupled with its reported action on the BoNT translocation channel, suggests that toosendanin may be acting on a common step such as a slowing of internalization of the LC into the nerve-terminal cytosol. Current efforts are focused on examining analogs of toosendanin to shed more light on its mechanism of action.

In addition to studies on toosendanin, there are a number of other efforts to develop natural products as BoNT inhibitors, most notably from the laboratory of Dr Bavari. Using the peptide-based inhibitor 2-mercapto-3-phenylpropionyl-RATKML developed by Schmidt and Stafford (2002), Burnett et al. (2007) examined the interaction of this molecule with the active site of BoNT/A LC. The investigators then screened small nonpeptide molecules from the National Cancer Institute's Open Repository and tested these for inhibitory activity using an HPLC assay and in chick spinal motor neurons. Several compounds were effective in the HPLC assay ($K_i = 3$–10 μM), and one, NSC 240898, was found to be effective in producing a concentration-dependent protection of SNAP-25 in cultured spinal cord cells with no evidence of toxicity up to 40 μM.

e. *Removal of BoNT from the Nerve Terminal*

The persistence of BoNT-mediated paralysis is one of the most striking features of intoxication by clostridial neurotoxins. Although persistence is desirable for providing sustained relief of muscle hyperactivity (Jankovic and Brin, 1997), it is a major problem for management of BoNT intoxication (Souayah et al., 2006). In addition to the drug discovery efforts described earlier, a therapy that

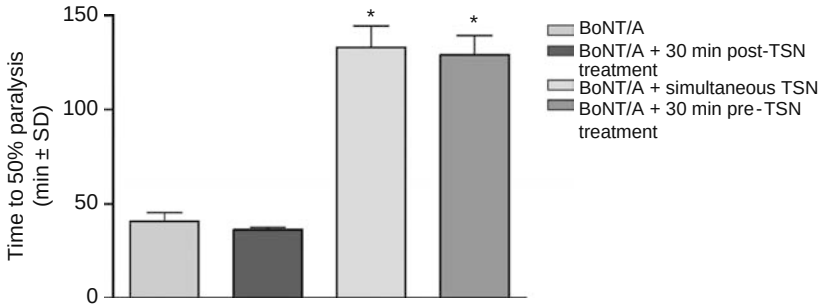

FIGURE 16.3 Toosendanin (TSN) delays the time to 50% paralysis in isolated mouse hemidiaphragm muscle exposed to BoNT/A. TSN (3 μM) was added to tissue baths 30 min before (pre), simultaneously with (simult.), or 30 min following addition of 20 pM BoNT/A (post). Paralysis times were significantly prolonged in muscles when TSN was added before or at the same time as BoNT/A ($p < 0.01$, ANOVA followed by Bonferroni post tests) as indicated by asterisks. Muscles tensions were elicited by supramaximal stimulation of the phrenic nerve at 30 s intervals. Temperature, 37°C.

shortens the period of intoxication would be a major contribution to the development of BoNT treatments.

There are two principal pathways for the elimination of proteins from cells: the ubiquitin proteasome system (UPS) and the lysosomal/autophagy system (Rubinsztein, 2006). These systems are both highly regulated and can be specific for targeting degradation; however, the UPS is generally considered to be the more critical pathway for the specific regulation of protein stability. The essential elements of the UPS consist of ubiquitin (a highly conserved 76 amino acid peptide), a cascade of enzymes to attach ubiquitin to a specific target protein, and the proteasome, which is a large macromolecular complex (Hochstrasser, 1996).

The signature for designating a protein for degradation is attachment of a ubiquitin chain. Ubiquitin serves a number of cellular functions but the most widely investigated is its role in targeting to the proteasome. Ubiquitin is attached to a specific protein through the action of a cascade of enzymes designated E1, E2, and E3, which can sequentially relay ubiquitin to culminate in the covalent ligation of the ubiquitin carboxyl terminus to a lysine side chain of the target protein. The covalent attachment of ubiquitin is exquisitely regulated, and the selection of the protein target for ubiquitination resides primarily in the function of E3 ubiquitin ligases. A specific E3 may have a single or very small number of protein targets that it will recognize to bring about ubiquitination of that target (Figure 16.4). Examples of such specific relationships are the recognition and ubiquitination of the antitumor protein p53 by the E3 ubiquitin ligase MDM2 as well as the regulation of IkK ubiquitination by an SCF ubiquitin ligase complex (Brooks and Gu, 2006).

In cells, the brief duration of BoNT/E LC appears to be related to its more extensive ubiquitination relative to that of BoNT/A LC (Figure 16.4b). The E3 ubiquitin ligases are generally modular, with one domain of the E3 specifically binding and recognizing the target protein and another domain, E3 ligase catalytic domain, functioning to facilitate the attachment of ubiquitin to the target protein lysine residue (Figure 16.5a). Such modular design allows for the generation of chimeric recombinant proteins, which contain a combination of a recognition domain for novel targets together with an E3 ligase catalytic domain to attach ubiquitin to this novel target (Figure 16.5b). This approach of generating chimeric recombinant or ''designer'' ubiquitin ligases is applied to a number of fields, including cancer therapy (Zhou et al., 2000; Oyake et al., 2002; Hatakeyama et al., 2005). The possibility of designating a target protein for degradation may be applicable to the development of therapeutics for BoNT intoxication and is being actively pursued.

In addition to the development of designer ubiquitin ligases for accelerating the elimination of BoNT LC from the presynaptic terminal, studies to determine the role of the endogenous neuronal ubiquitin system in regulating differences in persistence between BoNT/A and BoNT/E have been undertaken. Recent efforts have focused on the identification of cellular protein-binding partners for BoNT/A and BoNT/E LCs. The goal is to identify elements of the UPS that may preferentially recognize one of the two serotypes of BoNT LC and account for the greater rate of UPS degradation of BoNT/E. Two possibilities are emerging. In the first, BoNT/A interacts more extensively with enzymes in the deubiquitinating pathway (systems that actively reverse the ubiquitination process); in the second, cellular E3 enzymes interact more extensively with BoNT/E LC to accelerate its UPS degradation (Amerik and Hochstrasser, 2004). Both alternatives have therapeutic implications. Thus, development of a designer E3 ubiquitin ligase directed to the BoNT/A LC and the delivery of this designer E3 to affected neurons may shorten the duration of intoxication. Alternatively, it may be possible to identify deubiquitination enzymes that are responsible for the persistence of BoNT/A LC, and to inhibit these by small molecule pharmaceutical agents. The era of therapeutics directed toward regulation of protein stability by the UPS is emerging and holds promise in the evolution of novel therapeutic agents to address the persistence of BoNT intoxication.

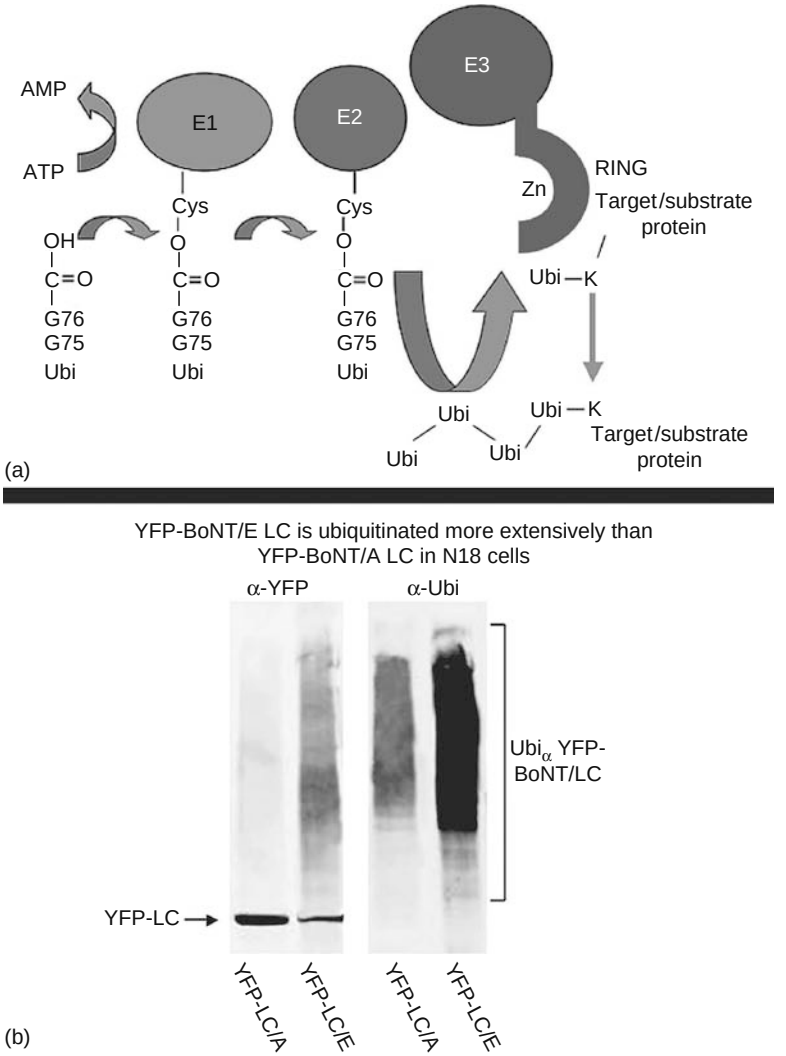

FIGURE 16.4 Cascade of ubiquitination enzymes and the extensive ubiquitination of BoNT/E LC in cells. (a) Ubiquitin activated and attached to lysine (K) residues in target substrate proteins by a cascade of enzymes, designated E1, E2, and E3. The specificity for selecting the target substrate protein for ubiquitin modification resides in the E3 enzyme. (b) Ubiquitination of BoNT/A and BoNT/E LC in N18 neuroblastoma cells. YFP-BoNT/A LC and YFP-BoNT/E LC were transfected into N18 cells, the cells were lysed, and YFP-BoNT LC proteins were immunoprecipitated with anti-YFP antibody. The immunoprecipitated YFP-BoNT LCs were separated and detected by western blotting with antiubiquitin antibody. The YFP-BoNT/E LC is more extensively ubiquitinated than the YFP-BoNT/A LC, as demonstrated by the immunoreactive smearing of the multi-ubiquitinated YFP-BoNT/E LC.

IV. CONCLUSIONS AND FUTURE RESEARCH

Efforts to develop pharmacological inhibitors of BoNT have increased substantially during the last decade. The major focus of the current research is the design and synthesis of specific metalloprotease inhibitors. Early drug discovery efforts were hampered by the lack of information on targets and the absence of the crystal structure for BoNT. Current efforts will be aided enormously by the

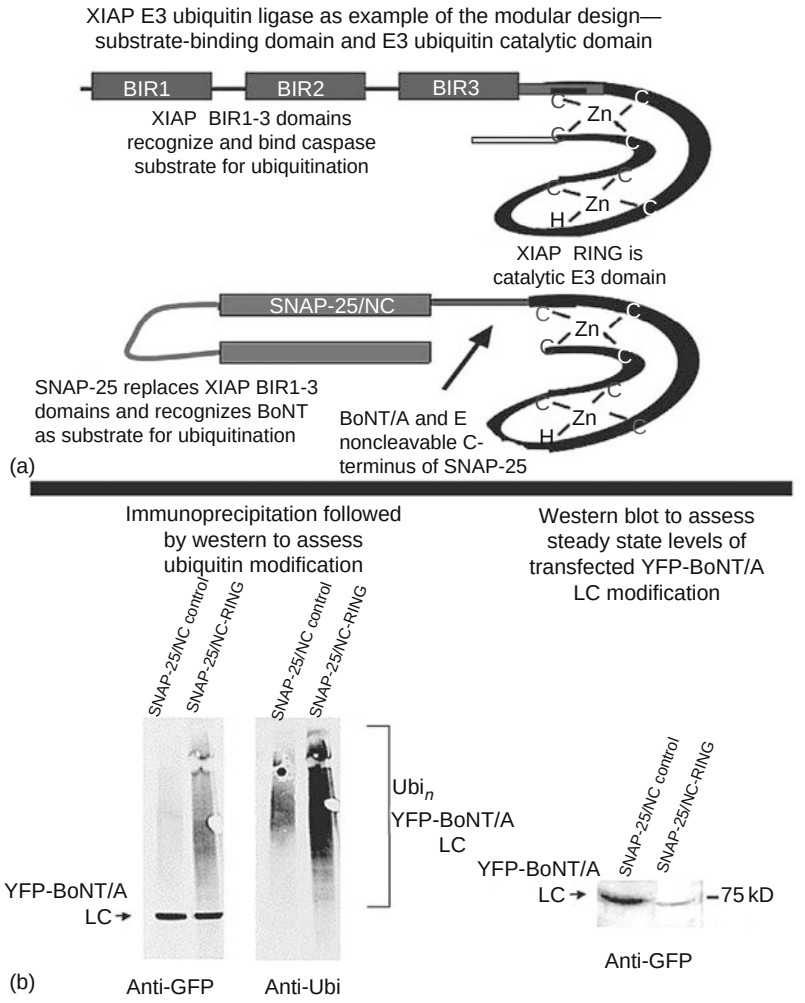

FIGURE 16.5 Modular composition of E3 ubiquitin ligases and increased ubiquitination of BoNT/A LC by a ''designer'' ubiquitin ligase. (a) The ubiquitin ligase XIAP is an example of the modular composition of E3 ubiquitin ligases generally where the amino-terminal BIR domains bind the target substrate caspases, and the carboxyl-terminal RING domain facilitates the attachment of ubiquitin to the target substrate. A designer E3 ubiquitin ligase for BoNT/A LC was constructed by substituting BoNT noncleavable (NC) SNAP-25 for the BIR domains of XIAP to allow targeting of the RING domain toward BoNT/A LC to induce ubiquitination. (b) Demonstration of the activity of the SNAP-25/NC-RING designer ubiquitin ligase. Cells were transfected with plasmid-expressing YFP-BoNT/A LC along with either SNAP-25/NC control plasmid or SNAP-25/NC-RING-expressing plasmid. The cells were lysed and the YFP-BoNT/A LC was assessed by immunoprecipitation. The cells transfected with SNAP-25/NC-RING displayed increased ubiquitination of the YFP-BoNT/A LC and a lower steady-state level of the YFP-BoNT/A LC.

availability of precise structural information and by knowledge of the mechanism of LC-mediated proteolysis of SNARE proteins (Chen and Barbieri, 2006).

Results to date indicate that a number of low molecular weight inhibitors and small peptides are effective against BoNT in cell-free in vitro systems (Sukonpan et al., 2004; Boldt et al., 2006a, 2006b; Park et al., 2006; Burnett et al., 2007; Eubanks et al., 2007).

Development of safe and effective metalloprotease inhibitors with in vivo efficacy will no doubt be difficult. Some of the challenges involve targeting of drugs to the nerve terminal, ensuring their access to the intracellular compartment and increasing the bioavailability of the drugs to match the duration of the toxin (Goodnough et al., 2002; Eubanks et al., 2007). In addition, different inhibitors may be needed for each serotype, requiring multiple parallel efforts. A more complete characterization of BoNT receptors and a better understanding of the internalization process have recently become available and will aid in accomplishing these objectives by refining the drug delivery methodologies (Chai et al., 2006; Jin et al., 2006).

It may also be necessary to accelerate the removal of cleavage products from the nerve terminal and to introduce noncleavable SNARE analogs for a more rapid recovery (O'Sullivan et al., 1999). The latter is especially relevant for treatment of persistent serotypes such as BoNT/A (Keller et al., 1999; Adler et al., 2001; Keller and Neale, 2001; Foran et al., 2003). The progress in understanding the mechanism of action of the BoNTs and detailed structural information gained during the last decade suggest that pharmacological treatments for BoNT intoxication will soon be a reality.

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17 Ricin and Related Toxins: Review and Perspective

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CONTENTS

The opinions or assertions contained herein are the private views of the authors and are not necessarily the official views of the U.S. Army or the U.S. Department of Defense.

I. INTRODUCTION

The attractive and deadly *Ricinus communis* (castor bean plant) has been cultivated for its commercial products, primarily castor seed oil, for at least 4000 years (Olsnes, 2004). Growth of the plant for its oil may have originated in Asia or North Africa, and was likely introduced to the Ancient Greeks and Western civilization through trade with Egypt (Scarpa and Guerci, 1982). Presently, over 1.2 million tons of castor seeds are harvested annually worldwide to produce approximately 220,000 tons of castor oil; the primary producers are India and China (Oil World, 2006). Castor seed contains up to 60% vegetable oil by weight after removal of the spiny seed husk; the oil is highly viscous and very uniform, yielding almost 90% pure, technical grade 12-hydroxy-oleate (ricinoleate) (McKeon et al., 1999). Ricinoleate is a reactive fixed oil that provides the basis for a range of modern products, including dehydrated alkyd resins (used in varnishes, enamels, and paints), biodegradable lubricants (lithium grease), hydraulic fluids, engineering plastics (Polyamide 11), antifungal compounds, cosmetics (lipsticks), soaps, printing inks, plasticizers, wetting agents, and leather dye reagents (Caupin, 1997; McKeon et al., 1999). In addition to castor oil production, the plant serves as a popular ornamental garden bush (temperate regions) or rapidly growing tree (tropical regions).

The seeds of *R. communis* contain large amounts of ricin, a highly toxic protein (Dixson, 1887; Stillmark, 1888), as well as several potent hyperallergens and alkaloids (Thorpe et al., 1988). Toxins are present throughout the plant, but as much as 20 mg of ricin is contained in each gram of castor seed (McKeon et al., 2000). Ricin is water soluble and partitions away from the ricinoleate during oil extraction. However, the presence of toxins limits the safety and value of the castor seed crop, complicates industrial processing, poses a risk to workers, and necessitates additional inactivation steps before the residual seed meal or pomice can be used safely for livestock feed or other biological purposes. It has been estimated that processing of sufficient castor oil to meet U.S. requirements alone can generate up to 30,000 tons of biohazardous waste (McKeon et al., 1999;

Marsden et al., 2005). Efforts are underway to overcome this problem by developing transgenic plants that produce the valuable ricinoleate without making ricin or the hyperallergens (Chen et al., 2005).

A. Introduction to Human Health Effects

The first phytomedical uses of the castor plant are unknown, but likely predate civilization. African tribes use the castor plant leaf as remedy for stomachache, the roots in a paste for toothache, and the seeds as a purgative; the reported curative dose for adults is one unbroken castor seed (Watt and Breyer-Brandwijk, 1962). Purified castor oil has also been ingested as a human nutritional supplement, emetic, or purgative worldwide for many years (Scarpa and Guerci, 1982; Caupin, 1997; Olsnes, 2004). More recently, the potency of purified ricin and related toxins has been combined with the specificity of antibodies, hormones, growth factors, or other ligands to produce experimental therapeutics (e.g., immunotoxins, IgTs) for the treatment of human diseases, including lymphoma or other tumors, graft-versus-host disease, and auto-immune disorders (Vitetta et al., 1983, 1993; Vitetta, 1990).

Along with their beneficial applications for human health, however, the plants that harbor ricin or related toxins are also closely associated with death. Folklore and myths worldwide reflect long-standing recognition of the human toxicity of castor seeds (ricin), jequirity seeds (abrin), or the mistletoe plant (viscumin). In comparison with the most potent bacterial toxins (e.g., clostridial neurotoxins), the toxins found in these plants are much less poisonous on a molar basis, but are more easily obtained, produced, and manipulated. The unbroken seeds themselves provide convenient ''delivery devices'' that can be handled or stored with minimal risk, and even primitive technologies can produce partially enriched toxin from the seeds. Ricin and abrin seeds have been used in Asia for centuries to kill animals and, less frequently, for murder (Stirling, 1924; Olsnes, 2004). Over 750 cases of human poisoning with castor seeds or ricin, including homicide and suicide attempts, have been reported during the past century (Balint, 1974; Rauber and Heard, 1985).

B. Biological Warfare and Terrorism

Attempts to develop ricin as a toxin weapon for modern warfare grew partly from its wide availability in large quantities as a by-product of the castor oil industry, as well as the emergence of industrial-scale processes for its facile production. The thermal instability of the toxin initially limited its use in exploding shells during World War I (WWI), whereas ethical and treaty constraints limited its first use as a poison or blinding agent (e.g., ricin-coated shrapnel or bullets) (Hunt et al., 1918). After considerable efforts, ricin was fully developed for use in special munitions during World War II (WWII), but it was never used on the modern battlefield (Kirby, 2004). The military utility of ricin over conventional weaponry remains questionable; Franz has estimated, for example, that it would require over 8 metric tons of optimally disseminated ricin to create lethal toxin concentrations over a single battlefield of 100 km^2 (Franz and Jaax, 1997).

Although the plant toxins have no documented use as effective military weapons or as agents of mass murder, they retain a mystique as assassination tools or potential bioterrorism agents that fuels ongoing interest from extremist organizations. The potency and delayed toxicity of ricin may have led to its use in a high-profile case of state-sponsored assassination during the Cold War, although this remains unproven (Crompton and Gall, 1980). Incidents involving the possession or planned misuse of ricin by bioterrorists have been investigated or prosecuted by law enforcement agencies worldwide during the past two decades (Franz and Jaax, 1997; Marsden et al., 2005).

Several aspects of ricin, including its significant toxicity, past association with chemical warfare, and wide availability in ton quantities from castor seed meal, have contributed to the international regulation of the toxin as a potential ''weapon of mass destruction.'' Ricin is currently monitored as a Schedule 1 toxic chemical under the *Convention on the Prohibition of the*

TABLE 17.1
Selected Review Articles for Ricin and Related Plant Toxins

Title or Topic Area	Number of References
Ricin (Hunt et al., 1918)	181
Castor bean (ricin) poisoning (Miller, 1939)	10
Ricin (Balint, 1974)	423
Abrin, ricin, and their associated agglutinins (Olsnes and Pihl, 1977)	185
Uses of the castor oil plant (Scarpa and Guerci, 1982)	107
Castor bean toxicity (Rauber and Heard, 1985)	19
Ricin (Parker et al., 1996)	76
Ricin (Franz and Jaax, 1997)	55
Toxin entry: retrograde transport through the secretory pathway (Lord and Roberts, 1998)	50
Ribosome-inactivating proteins: a plant perspective (Nielsen and Boston, 2001)	209
Abrin poisoning (Dickers et al., 2003)	39
Ricin poisoning (Bradberry et al., 2003)	54
History of ricin, abrin, and related toxins (Olsnes, 2004)	100
Ricin: mechanism of toxicity, clinical manifestations, and vaccine development (Doan, 2004)	56
Ricin poisoning (Audi et al., 2005)	113
Prospects for a ricin vaccine (Marsden et al., 2005)	90
Ribosome-inactivating proteins (Stirpe and Battelli, 2006)	216

Development, Production, Stockpiling and Use of Chemical Weapons and on Their Destruction (CWC), and the intentional use of ricin or related toxins as weapons is prohibited under the 1972 *Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction* (BTWC). The possession or transfer of ricin, abrin, or genes encoding functional forms of these toxins is also regulated in the United States by the Centers for Disease Control and Prevention (CDC) Select Agents and Toxins Program (U.S. Department of Health and Human Services, 2005, 2007).

Numerous review articles covering ricin and related toxins are available (Table 17.1). Our goal is to provide an overview for scientists who are working toward practical medical solutions to prevent or mitigate the consequences of chemical warfare or bioterrorism. We summarize the biochemistry and pathophysiology of ricin, and briefly review studies with experimental animal models to aid in preventing, diagnosing, and treating the poorly characterized human response to ricin exposure. Throughout the chapter, we compare ricin with several closely related proteins of comparable potency to clarify the gaps in our current understanding for this important class of plant toxins.

II. OVERVIEW OF RIBOSOME-INACTIVATING PROTEINS

Ricin evolved within a large, diverse family of "ribosome-inactivating proteins" (RIPs) that include numerous other toxins from higher plants, as well as potent bacterial Shiga and Shiga-like toxins from *Shigella dysenteriae* and certain enteropathogenic strains of *Escherichia coli* (Endo et al., 1988b). RIPs are defined by *N*-glycosidase activity that selectively depurinates a highly conserved adenine located within the specific context of a 14 nucleotide region (called the α-sarcin/ricin loop) of the 28S ribosomal RNA (rRNA) subunit of the large (60S) ribosome (Endo and Tsurugi, 1987; Endo et al., 1987; Chen et al., 2006).

The RIP active-site structure and enzymatic function are similar across diverse phylogeny, including those of bacteria, plants, and fungi. Little is known concerning the adaptive radiation or the evolutionary convergence of this family of enzymes. There are no proven selective advantages

to the production of plant RIPs, although it has been proposed that the toxins may function as antiviral, antifungal, or insecticidal agents (Nielsen and Boston, 2001; Stirpe and Battelli, 2006).

A. Type 1 RIPs

Plant RIPs have been classified into three general types based on structure. Type 1 RIPs are monomeric *N*-glycosidase enzymes of approximately 30 kDa molecular weight and a basic isoelectric point; they share a common secondary structure around the rRNA-binding cleft, as well as several specific active-site residues (Barbieri et al., 1993). Type 1 RIPs are frequently found in higher plants, but also have been isolated from at least one mushroom (Yao et al., 1998). The best-studied type 1 RIPs are pokeweed antiviral protein, saporin, and barley translation inhibitor (Nielsen and Boston, 2001). Type 1 RIPs are generally considered nontoxic because they lack an effective cell-binding or internalization apparatus.

B. Type 2 RIPs

Type 2 RIPs are heterodimers composed of an *N*-glycosidase enzyme (A-chain) linked through a disulfide bond to a lectin (B-chain); the B-chain binds target cell surface glycans and facilitates endocytosis (Stirpe and Battelli, 2006). Based on available X-ray crystal structures, the active-site regions of the type 2 RIP A-chains share several key amino acid residues and a common structural architecture with the type 1 RIPs (Rutenber et al., 1991; Monzingo et al., 1993; Gao et al., 1994; Ren et al., 1994; Weston et al., 1994; Hosur et al., 1995; Tahirov et al., 1995; Krauspenhaar et al., 1999).

Ricin and the other deadly plant toxins discussed throughout this chapter, including abrin, pulchellin, modeccin, volkensin, and viscumin, are homologous type 2 RIPs with lectin affinity for terminal galactose or *N*-acetylgalactosamine. Abrin and pulchellin are produced in the seeds of *Abrus precatorius* (jequirity seeds) and *Abrus pulchellus*, respectively (Olsnes et al., 1974b; Olsnes and Pihl, 1977; Griffiths et al., 1987; Hughes et al., 1996; Ramos et al., 1998; Dickers et al., 2003). The primary amino acid sequences of the A-chains of abrin-c and pulchellin are 86% identical (Silva et al., 2005). Abrin and ricin share extensive identity and structural similarity across both the A- and B-chains (Rutenber et al., 1991; Weston et al., 1994; Tahirov et al., 1995).

Modeccin and volkensin are closely related toxins produced by the plant family Passifloraceae; modeccin is from the fruits and large roots of *Adenia digitata* (Green and Andrews, 1923a, 1923b; Watt and Breyer-Brandwijk, 1962), and volkensin is from the roots of *Adenia volkensii* (Stirpe et al., 1985; Chambery et al., 2004, 2006). Viscumin (mistletoe lectin, ML-I), ML-II, and ML-III are distinct type 2 RIPs produced by the mistletoe, *Viscum album* (Olsnes et al., 1982; Franz, 1986; Holtskog et al., 1988; Tonevitsky et al., 2004).

High structural similarity notwithstanding, the biological potencies of type 2 RIPs vary significantly (Endo et al., 1988a; Stirpe et al., 1988; Stirpe and Battelli, 1990; Barbieri et al., 1993). Not all type 2 RIPs are considered toxins; examples of relatively nontoxic heterodimeric RIPs include nigrin b from *Sambucus nigra* (elderberry) (Van Damme et al., 1996), ebulin lectins from *Sambucus ebulus* (dwarf elder) (Girbes et al., 1993b), and lectins from *Eranthis hyemalis* (winter aconite) (Kumar et al., 1993). For comparison, ricin and abrin are toxic to mice by injection in the range of 1–20 μg/kg body weight, whereas nigrin b is not toxic even at the dose of 1.6 mg/kg (Stirpe and Battelli, 1990; Girbes et al., 1993a). For some type 2 RIPs, the lack of toxicity is due to a truncated or otherwise nonfunctional carbohydrate-binding B-chain (e.g., basic nigrin b). The source of variation in toxicity among others can be more subtle and may reflect differences in target cell receptors, processing or intracellular routing of the toxins (Moisenovich et al., 2002; Barbieri et al., 2004).

C. Type 3 RIPs

Type 3, the least common structural class of plant RIP, appears to reflect an unusual adaptation in enzyme production or storage. Type 3 RIPs are synthesized as inactive precursor molecules with

a polypeptide insert in the active-site region of the *N*-glycosidase domain (Walsh et al., 1991; Bass et al., 1992; Chaudhry et al., 1994). Proteolytic processing of the pro-RIP is required to remove the insert and thereby convert these molecules into active *N*-glycosidases; once processed, the type 3 RIPs resemble the nontoxic type 1 plant RIPs in overall net charge and catalytic activity (Chen et al., 2001; Nielsen and Boston, 2001).

III. BIOCHEMISTRY AND PATHOPHYSIOLOGY OF RICIN

Ricin, also referred to as ricin-D (Ishiguro et al., 1964a), *R. communis* agglutinin II, or RCA_{60} (Nicolson and Blaustein, 1972), is the most completely studied member of the type 2 RIP toxins. It is a heterodimeric glycoprotein composed of an A-chain (RTA) and a B-chain (RTB) held together by a single interchain disulfide bond (Ishiguro et al., 1964a, 1964b; Funatsu et al., 1970; Monzingo and Robertus, 1992). Upon reduction of the covalent disulfide, there remains a relatively weak, noncovalent association between RTA and RTB (estimated dissociation constant of 10^{-6} M) (Lewis and Youle, 1986).

The total quantity of ricin produced or stored within castor seeds depends on the genetic composition of the plant and the growth conditions. As a general guide, published purification methods may yield 1–2 mg of ricin/g of castor seed meal starting material (Ishiguro et al., 1964a; Olsnes and Pihl, 1977; Parker et al., 1996).

The solution molecular weight of ricin holotoxin, determined by sedimentation and diffusion measurements, is 77 kDa with an apparent molecular weight ranging from 65 to 70 kDa by gel electrophoresis (Funatsu et al., 1970; Balint, 1974). The isoelectric point reported for ricin varies widely from 5.4 to 8.8, depending on the source of protein and extent of glycosylation or other cotranslational or posttranslational modifications (Funatsu et al., 1970; Balint, 1974; Hegde and Podder, 1992). Several isoforms (isotoxins) can be produced within a single castor seed, and different plant variants or environmental conditions can result in production or storage of different relative amounts of isotoxins (Olsnes and Pihl, 1982).

As an additional source of complexity, the ricin gene is part of a multigene family within the castor bean plant that encodes for the production of pro-ricin (the natural ricin precursor), several apparently inactive polypeptides, and a well-studied, structurally related lectin called *R. communis* agglutinin I (RCA_{120}) (Tregear and Roberts, 1992; Pinkerton et al., 1999; Helmy and Pieroni, 2000).

RCA_{120} is a 120 kDa glycoprotein tetramer that is composed of two ricin-like dimers, each of which can be further dissociated by disulfide reduction into subunits of approximately 27 and 33 kDa; one subunit shares high identity with RTB, whereas the other is unique to RCA_{120} (Hegde and Podder, 1992, 1998). RCA_{120} has only about 3% of the potency of ricin in cytotoxicity assays with human cells, but it is a powerful red blood cell agglutinin (Corwin, 1961; Hegde and Podder, 1992). RCA_{120} is poorly absorbed through the digestive tract in animals, and is virtually nontoxic in mice compared with ricin (Olsnes et al., 1974c).

A. Cell Binding and Internalization

The RTB subunit of ricin is a 262-amino acid (approximately 34 kDa) glycoprotein containing two functional lectin (carbohydrate-binding) domains, each of which is capable of binding a galactose or *N*-acetyl galactosamine residue, primarily through specific hydrogen bonds (Lamb et al., 1985; Rutenber et al., 1991; Sphyris et al., 1995). RTB binds reversibly to exposed galactose moieties of glycoconjugates on the target cell surface with an apparent association constant in the range of 10^7–10^8 M^{-1} at 37°C, resulting in as many as 3×10^7 molecules of ricin bound/target cell under saturating conditions (human HeLa cells) (Sandvig et al., 1976; Olsnes and Pihl, 1977).

The importance of carbohydrate binding for ricin toxicity is supported by the observation that cell lines lacking galactosyl transferase are resistant to ricin (Foddy and Hughes, 1986). The potency of ricin or related toxins does not correlate directly, however, with the number of receptors present

on the target cell surface; for example, some human cell lines *in vitro* may express 100-fold higher amounts of ricin receptors than modeccin receptors, yet modeccin is more toxic to the cells (Sandvig et al., 1976; Olsnes et al., 1978a, 1978b).

There is little discernible cell-type specificity for the RTB–carbohydrate interaction, and presently no compelling evidence for involvement of more selective ricin coreceptors. If there are undiscovered protein coreceptors, or biologically important subsets of galactosylated receptors, then these are likely shared among the many different cell types sensitive to ricin.

B. Intracellular Trafficking

The ricin–receptor complex enters target cells by endocytosis through both clathrin-dependent and -independent uptake pathways, and can be detected in endosomes and the trans-Golgi network (TGN) (van Deurs et al., 1986, 1988; Sandvig and van Deurs, 1996; Iversen et al., 2001). Because ricin binds to heterogeneous cell surface glycoconjugates, the toxin may utilize more than one mode of internalization and subcellular trafficking. Perhaps 5% of endocytosed toxin reaches the TGN; most of the toxin appears to be extruded from cells after cycling back to the cell surface, or it is rendered harmless by routing to lysosomal degradation (van Deurs et al., 1986, 1989; Sandvig et al., 2002, 2004).

It has been proposed that some internalized ricin escapes directly from endosomes to the cytosol (Beaumelle et al., 1993). However, several other experimental studies indicate that the toxin first passes through the TGN and subsequently undergoes retrograde transport to the endoplasmic reticulum (ER) (Lewis and Youle, 1986; Hudson and Grillo, 1991; Iversen et al., 2001).

1. Retrotranslocation to the Cytosol

A critical fraction of RTA that reaches the ER from the TGN will travel further within the target cell from the lumen of the ER into the cytosol through ''retrotranslocation,'' the reverse process of normal protein import to the ER (Bonifacino and Rojas, 2006). This peculiar ''upstream'' movement of a fraction of internalized toxin, essentially traversing the entire pathway of normal host cell glycoprotein production in reverse, probably makes an important contribution to the potency of ricin (Wesche et al., 1999). The relatively nontoxic type 2 RIP, nigrin b, in comparison, does not appear to follow the same unusual intercellular routing pattern as does ricin; in one study, 94% of internalized nigrin b was degraded and extruded by HeLa cells, whereas only 79% of ricin was removed in the same system (Battelli et al., 1997).

RTA retrotranslocation likely requires the reduction of the ricin interchain disulfide and dissociation of RTB from RTA (Di Cola et al., 2001). Expression of individual ricin subunits in the ER lumen of plant cells shows that the toxicity observed from the retrotranslocation of RTA is mitigated when RTA and RTB are coexpressed; the disulfide-bonded holotoxins formed in the ER lumen appear unable to reach the cytosol and are secreted instead (Frigerio et al., 1998).

2. Role of Target Cell ERAD Pathway

By analogy with other A-B toxins, translocation of RTA has been proposed to exploit the ER-associated protein degradation (ERAD) pathway that normally functions to recognize misfolded host proteins and shunt them back to the cytosol for degradation (Zhang et al., 1995; Rapak et al., 1997; Wesche et al., 1999; Schmitz et al., 2000; Roberts and Smith, 2004). According to this hypothesis, hydrophobic surfaces are exposed to solvent on separation of the toxin A- and B-chains in the ER, resulting in reversibly unfolded states that mimic misfolded target cell proteins; unfolded A-chains are subsequently misrouted to the ERAD pathway.

The mechanism of RTA unfolding in the ER is unknown, but could involve target cell proteins, lipid membranes, or both. Interaction with negatively charged lipids was shown *in vitro* to induce significant structural changes in RTA, leading to speculation that the ER membrane may play a role

in the unfolding of RTA and subsequent export to the cytosol via ERAD (Day et al., 2002; Roberts and Smith, 2004).

Several lines of evidence support the involvement of specific ERAD translocons in the movement of RTA from ER to cytosol. A fraction of internalized RTA (less than 1%) was coimmunoprecipitated with Sec61α, a known component of the highly conserved sec61p core ER translocon machinery (Wesche et al., 1999). Additionally, studies with mutant cell lines defective in ERAD components suggest that the extent of ricin translocation correlates with the total amount of ERAD activity occurring in yeast or CHO cells (Teter and Holmes, 2002; Teter et al., 2003).

If RTA unfolds to exploit the target cell ERAD pathway, then it must refold or otherwise escape the normal fate of misfolded cytosolic proteins, which typically includes degradation by ATP-dependent proteases (proteasome). One protective mechanism for avoiding degradation appears to be an inherently low number of exposed lysine residues on RTA (2 per monomer); exposed lysine residues are a primary target for ubiquitination, and the presence of ubiquitin routes misfolded proteins to the proteasome (Deeks et al., 2002).

Another structural feature that may facilitate the retrotranslocation of RTA is its inherent ability to adopt metastable conformations in solution (McHugh et al., 2004). In this model, a significant fraction of RTA would transiently adopt a partly unfolded state in which the hydrophobic surfaces of the RTA C-terminal domain are exposed to the ERAD mechanism on dissociation of RTB. The N-terminal domain of RTA, which is more stable in solution than is RTA (Olson et al., 2004), would provide a structural ''anchor'' for refolding RTA to the functional *N*-glycosidase conformation, perhaps with the aid of appropriate chaperones. Potential chaperones for refolding in the cytosol include host cell proteins or the rRNA substrate itself (Argent et al., 2000).

C. Catalytic Inactivation of Ribosomes

1. RTA Structure and Function

The RTA subunit of ricin is a 267 amino acid (approximately 32 kDa), highly efficient enzyme that binds the large ribosomal subunit of eukaryotic cells and catalyzes hydrolysis of the *N*-glycosidic bond between the adenine base and ribose at position A4324 (rat liver ribosome sequence number); the scissile bond is located within a conserved GAGA motif of the α-sarcin/ricin loop of the 28S rRNA already mentioned (Montanaro et al., 1973; Sperti et al., 1973; Endo and Tsurugi, 1987). The *N*-glycosidase activity of RTA does not directly break the rRNA phosphodiester chain, but depurinated RNA becomes more susceptible to acid-, base-, or enzyme-catalyzed hydrolysis (Ogasawara et al., 1999).

In order for RTA to achieve its optimal catalytic rate, the rRNA substrate must meet specific structural requirements, including an RNA stem, bulged nucleotide, and loop structure (Endo et al., 1991; Chen et al., 1998; Amukele et al., 2005). Using artificial polynucleotide RNA substrates containing the required GAGA loop *in vitro*, purified RTA has a k_{cat}/K_m of up to $4.5 \times 10^5\ M^{-1}\ s^{-1}$ (Endo et al., 1991; Chen et al., 1998). The catalytic efficiency of RTA *in vivo* for the ribosome rRNA is much greater, approaching $10^8\ M^{-1}\ s^{-1}$ (Endo et al., 1991; Lord and Roberts, 1996). It has been estimated that, under physiological conditions, a single molecule of RTA in the cytosol may inactivate over 1500 mammalian cell ribosomes/min (Olsnes and Pihl, 1982; Sandvig and van Deurs, 1996).

When the three-dimensional structure of RTA was solved by X-ray crystallography over 20 years ago, a long cavity on the enzyme surface was identified and postulated to correspond with the RNA substrate-binding site (Montfort et al., 1987). Many of the amino acids found within this cavity are invariant residues shared among the RIPs, including Tyr21, Phe24, Arg29, Tyr80, Tyr123, Gly140, Ala165, Glu177, Ala178, Arg180, Glu208, Asn209, and Trp211 (RTA numbering) (Funatsu et al., 1991; Katzin et al., 1991). Based on subsequent site-specific mutagenesis and systematic amino acid deletions of RTA, several of these residues were shown to participate in the

catalytic mechanism of RTA (Schlossman et al., 1989; Frankel et al., 1990; Ready et al., 1991; Kim and Robertus, 1992; Morris and Wool, 1992; Munishkin and Wool, 1995).

The reaction mechanism of RTA has been compared with the nucleosidase-catalyzed cleavage of adenosine from ribose in AMP, and is believed to proceed through formation of an unstable ribo-oxacarbenium ion transition state (Mentch et al., 1987; Chen et al., 1998). The crystal structures of RTA bound with substrate analogs suggest that the ribose ring is captured by the enzyme between the side chains of Tyr80 and Tyr123; a conserved Arg180 is positioned to hydrogen bond and protonate the adenine ring during catalysis, while the carboxylic acid of Glu177 stabilizes the developing positive charge on the ribose ring in the ribo-oxacarbenium transition state (Montfort et al., 1987; Katzin et al., 1991; Rutenber et al., 1991; Monzingo and Robertus, 1992; Day et al., 1996). Interestingly, RTA retains catalytic activity when Glu177 is replaced by site-specific mutagenesis, perhaps because of an active-site rearrangement that repositions Glu208 to act in place of Glu177 (Schlossman et al., 1989; Frankel et al., 1990).

2. Inhibition of Target Cell Translation

The ribosome conformations required for optimal RTA binding and catalysis are still under study (Endo et al., 1991; Macbeth and Wool, 1999; Chan et al., 2004; Mansouri et al., 2006). In addition to the rRNA, RTA may interact with specific protein components of the ribosome. Rat ribosomal proteins L9 and P0, both located in the acidic stalk of the ribosome, can be chemically cross-linked with RTA (Vater et al., 1995). Likewise, yeast ribosomal protein L3 interacts with pokeweed antiviral protein, and the binding is dependent on the presence of two specific amino acid residues that are highly conserved among eukaryotes (Hudak et al., 1999).

Enzymatic depurination of the α-sarcin/ricin loop by RTA selectively alters the structure of the GTPase center of eukaryotic ribosomes, resulting in a reduced affinity of elongation factor EF2 in the presence of guanine nucleotides (Montanaro et al., 1973; Olsnes et al., 1975; Fernandez-Puentes et al., 1976; Brigotti et al., 1989; Endo et al., 1991). The failure of EF2 and aminoacyl-tRNA to bind productively with the eukaryotic ribosome essentially stops the translocation step of the elongation cycle required for protein synthesis, leading ultimately to target cell death.

RTA and related RIPs are toxic to most eukaryotes, but not to prokaryotes. As a result, recombinant RTA is difficult to express in yeast cells, but it can be readily overexpressed and properly folded by *E. coli*. The inability of RTA to disrupt protein synthesis effectively in prokaryotic cells may be due to significant divergence in the sequences of ribosomal proteins or the precise three-dimensional structure of rRNA near the RTA cleavage site (Gale et al., 1981; Endo et al., 1991; Vater et al., 1995).

D. Secondary Catalytic Activities

1. Polynucleotide Substrates

Although rRNA is clearly the preferred substrate, ricin and related RIPs possess weaker *N*-glycosidase activity for other polynucleotide substrates *in vitro*. Depending on the reaction conditions, RTA retains activity for RNA substrate analogs with smaller stem-loop structures that express the GAGA loop sequence (Gluck et al., 1992; Allerson and Verdine, 1995; Chen et al., 1998). Cellular DNA apparently is not a biological target of RTA, but unstructured DNA sequences of d [GAGA] without stem-loop structures are slow substrates for RTA *in vitro* (Amukele et al., 2005). Comparison of the polynucleotide adenosine glycosidase activities of 52 different RIPs showed that each was able to catalyze the removal of adenine from isolated DNA *in vitro* (Barbieri et al., 1997).

2. Lipid Substrates

Early studies with purified ricin demonstrated calcium-independent phospholipase type A_1 and A_2 activity, as well as nonspecific acyl hydrolase lysophospholipase type A_1 and A_2 activity

(Helmy et al., 1999; Lombard et al., 2001). The lipase activity was tentatively ascribed to RTB, owing partly to the presence of a serinyl residue with a suggestive primary sequence context (RTB Ser70), and to the sequence homology of RTB with plant lipases (Moulin et al., 1994; Lombard et al., 2001).

More recently, the ricin lipase activity has been localized to a specific Ser–His–Asp triad composed of amino acid residues from both RTA and RTB; the putative catalytic serinyl residue is RTA Ser221. The hydroxyl group of Ser221 is within potential hydrogen bonding distance of RTA His40 which, in turn, may hydrogen bond across the subunit interface with RTB Asp94 (Morlon-Guyot et al., 2003). One caveat for this proposal is the lack of the expected sharp turn, or serine hydrolase ''nucleophilic elbow,'' in the RTA backbone at Ser221. Nevertheless, mutation of Ser221 to alanine abolished ricin lipase activity, as did treatment of ricin with the serine-hydrolase inhibitor diethyl *p*-nitrophenylphosphate (Morlon-Guyot et al., 2003). The presumed catalytic triad was found to be conserved in the primary sequence of abrin, but was absent from MLI and the nontoxic type 2 RIP, nebulin 1 (Morlon-Guyot et al., 2003).

The biological role, if any, for the lipase activity of ricin remains unknown. Experimental disruption of the lipase activity by site-specific mutagenesis of RTA Ser221 slowed but did not stop the RTA translocation rate in A431 cells or monocytes; the cytotoxicity of ricin mutants lacking a serinyl residue at RTA 221 was reduced by 35% compared with ricin (Morlon-Guyot et al., 2003). The effects of this mutation on RTA stability within the target cell, however, also may have contributed to the observed reduction in cytotoxicity. It has been difficult to isolate the biological effects of ricin lipase activity in experimental model systems; RTA alone, which lacks the putative interchain lipase site, is sufficient to destabilize lipid bilayers (Day et al., 2002; Sun et al., 2004). Moreover, the phospholipase turnover numbers observed for ricin *in vitro* have been estimated to be too slow to destabilize the lipid bilayer on a timescale that could account for cytotoxicity (Sun et al., 2004).

IV. CYTOTOXICITY OF RICIN

A. Importance of Protein Synthesis Inhibition

Complete elucidation of the mechanisms by which ricin kills target cells remains an area of active study, but it is clear that the *N*-glycosidase activity of RTA is the essential triggering event. Inhibition of protein synthesis precedes other detectable alterations in target cell biochemistry. Ricin blocks amino acid incorporation in crude microsome preparations before changes occur in energy metabolism or oxidative phosphorylation; the toxin has essentially no effect on mitochondrial respiration in isolated mitochondria or tumor cells (Waller et al., 1966; Dirheimer et al., 1968; Lin et al., 1971). Likewise, the first observable cytotoxic effect of ricin in cell culture is typically the inhibition of protein synthesis, followed by a reduction in DNA synthesis (Lin et al., 1970; Lin et al., 1971; Onozaki et al., 1972; Refsnes et al., 1974; Nicolson et al., 1975; Olsnes et al., 1976; Refsnes et al., 1977).

After addition of ricin, abrin, or modeccin to cell culture medium *in vitro*, there is a characteristic time delay before the inhibition of protein synthesis becomes apparent; the lag time decreases with increasing toxin concentration, but even at high concentrations it is as long as 20–30 min (Refsnes et al., 1974; Olsnes et al., 1976). The cause of this time delay is unknown, but it may partly reflect the complex intracellular trafficking of the toxins.

Different cell types exhibit different sensitivities to ricin *in vitro*. For example, the concentration of ricin in tissue culture medium that leads to an approximately 50% inhibition of protein synthesis is 13–22 pM for human lymphoma (Daudi cells), AKR/A, lipopolysaccharide blasts, or mice spleen cells, but it is 0.4 pM for nonparenchymal rat liver cells (Fulton et al., 1986). Ricin causes cytotoxicity in cultured human HeLa cells at concentrations as low as 15 pM (Eiklid et al., 1980; Rao et al., 2005), with an approximately 50% loss of viable cells occurring after a 12 h exposure to

15 nM ricin (Rao et al., 2005). Exposure of HeLa cells to 1 nM ricin for 24 h resulted in >30% loss of viability compared with control cells (Gan et al., 2000).

Ricin may inhibit the synthesis of specific proteins to different extents; in a myeloma cell line, for example, the synthesis of IgA is inhibited more rapidly than is bulk protein synthesis (Ko and Kaji, 1975). There is currently insufficient experimental evidence, however, to conclude that preferential inhibition of the synthesis of specific proteins significantly influences ricin cytotoxicity *in vivo*.

B. Comparison with Related Plant Toxins

The relative amounts of ricin or related toxins required to kill target cells *in vitro* vary depending on the cell type studied and the experimental methodology, but several trends are evident. Modeccin is significantly more toxic to cultured cells than is ricin, abrin, viscumin, or volkensin (Olsnes et al., 1978b; Olsnes and Pihl, 1982; Stirpe and Battelli, 1990). Volkensin, the least cytotoxic of the toxins discussed herein, is about 10- or 40-fold less toxic to human HeLa cells than is ricin or modeccin, respectively (Stirpe and Battelli, 1990).

Differences in cytotoxicity result from more than just the relative catalytic efficiencies of the toxin A-chains. For example, ricin is about 10-fold more cytotoxic than viscumin, despite the fact that the *N*-glycosidase activities of the two toxins are almost the same (Barbieri et al., 1993; Eck et al., 1999). Barbieri et al. (2004) systematically compared ricin and over a dozen other type 2 RIPs and confirmed that, despite 100- to 1000-fold differences in *N*-glycosidase activity, there was no apparent correlation between enzyme activity and overall cytotoxicity.

Some differences in potency among type 2 RIP toxins can be explained by their binding to different subpopulations of target cell receptors. Cell lines exhibiting a reduced number of ricin receptors are resistant to both ricin and abrin *in vitro*, but are not resistant to modeccin or diphtheria toxin (Olsnes and Refsnes, 1978; Olsnes et al., 1978b; Li et al., 1980). Ricin and viscumin bind to different sites of the plasma membrane of mouse 3T3 fibroblasts, despite sharing 64% primary sequence identity and similar carbohydrate-binding sites within their respective B-chains (Moisenovich et al., 2002).

C. Role of Apoptosis

Ricin and related RIP toxins ultimately kill target cells, whether in isolated human cell culture systems or whole animal models, by complex pathways that involve both necrosis and apoptosis (Griffiths et al., 1987, 1988; Chang et al., 1989; Morimoto and Bonavida, 1992; Hughes et al., 1996; Komatsu et al., 1998, 2000; Oda et al., 1999; Baluna et al., 2000; Brigotti et al., 2002; Tamura et al., 2002a). As with other cellular toxins, the pathway of cell death may depend on the specific target cell type, the extracellular milieu, or the time-weighted concentration of toxin exposure.

It is generally accepted that ricin-induced apoptosis requires RTA, and is a downstream consequence of the inhibition of protein synthesis (Williams et al., 1997; Langer et al., 1999; Oda et al., 1999; Vervecken et al., 2000). There is recent evidence, however, to suggest that apoptosis in yeast cells exposed to ricin may require more than RTA catalytic activity, perhaps involving the subcellullar trafficking of the toxin (Tamura et al., 2002a; Li et al., 2006).

Cultures of bovine pulmonary endothelial cells undergo apoptotic cell death when exposed to 15.5 pM ricin or 4.5 pM abrin over a period of up to 40 h (Hughes et al., 1996). Transmission electron microscopy demonstrates characteristic changes associated with apoptotic cell death, including heterochromatin condensation at the nuclear periphery, progressive degeneration of residual nuclear and cytoplasmic structures, and crenulation of the nuclear membrane (Hughes et al., 1996).

Ricin, modeccin, diphtheria toxin, or *Pseudomonas* toxin induces apoptosis in human lymphoma U937 cells through an increase in caspase-3 and caspase-6 activities, but not caspase-1 activity

(Komatsu et al., 1998). Treatment with a serine protease inhibitor *in vitro* abolishes the apoptotic cell death and DNA fragmentation induced by toxins without effecting caspase-3 and caspase-6 activities, whereas an inhibitor of caspase blocks all features of apoptosis (Komatsu et al., 1998). Redistribution of intracellular Zn^{2+} occurs as an early apoptotic event following ricin exposure in U937 cells, and exogenously added Zn^{2+} mitigates apoptosis by completely preventing the activation of caspase enzymes (Tamura et al., 2002b).

Ricin induces apoptosis in human HeLa cells in a concentration- and time-dependent manner *in vitro* (Gan et al., 2000; Rao et al., 2005). Exposure to 15 nM ricin leads to increased cell permeability, activation of abnormal caspase-3 activity, increased levels of reactive oxygen species, DNA fragmentation, and depletion in the levels of intracellular glutathione (Rao et al., 2005). Caspase-3 activation begins 1–4 h after ricin exposure, leading to proteolysis of the nuclear repair enzyme, poly(ADP-ribose) polymerase, and the DNA fragmentation factor-45 (DFF45/ICAD), as well as caspase-activated DNase activity (Rao et al., 2005). Apoptotic cells and degradation of DNA into oligonucleosomal-sized fragments are clearly evident by 4 h after exposure (Rao et al., 2005).

Viscumin, ML-II, and ML-III each cause apoptotic changes in exposed human cells *ex vivo*; ML-III is the most potent of the MLs at inducing apoptosis in human lymphocytes, followed by ML-II and viscumin (Bussing et al., 1996; Pevzner et al., 2005). Site-specific mutagenesis of the active site of the viscumin A-chain has provided the most direct evidence that apoptosis induced by the type 2 RIPs correlates with their primary ability to disrupt protein synthesis (Langer et al., 1999).

V. EFFECTS OF RICIN ON LABORATORY ANIMALS

The toxicity of ricin for laboratory animals was first reported over a century ago (Muller, 1899), and dozens of subsequent studies have been conducted using intravenous (i.v.), intraperitoneal (i.p.), subcutaneous (s.c.), intramuscular (i.m.), inhalation, gastrointestinal (GI), cutaneous, or ocular instillation exposure methods (Hunt et al., 1918; Balint, 1974; Parker et al., 1996; Greenbaum and Anderson, 2006). Table 17.2 compares the lethality of ricin by different exposure routes in several animal species. Acute toxicity is usually expressed as the amount of toxin required per kilogram of body weight to kill 50% of challenged animals under defined experimental conditions (LD_{50}). For inhalation studies, toxicity is expressed as the time weighted concentration lethal for 50% of test animals (LCt_{50}), or as an estimated LD_{50} based on the calculated absorbed dose of toxin. Published LD_{50} values for ricin vary considerably, which likely reflects interlaboratory differences in the quality of toxin preparations, the mode of exposure, the postexposure observation interval, and other differences in methodology (Hunt et al., 1918).

A. Injected Ricin

Injected ricin kills laboratory animals in a concentration- and time-dependent manner with steep lethality curves (Fodstad et al., 1976, 1979; Olsnes and Pihl, 1977). After administration of ricin to experimental animals by injection, there is a characteristic time delay before signs of intoxication appear. The delay time decreases with increasing amounts of toxin, but it is always several hours, perhaps reflecting the time required for sufficient toxin to reach the target ribosome and disrupt protein synthesis (Olsnes and Pihl, 1977; Fodstad et al., 1979). In laboratory rats, for example, liver protein biosynthesis is unchanged compared with control levels for the first 3 h after injection (i.p.) with 500 μg/kg ricin, but steadily declines to approximately 15% of that of control groups by 10 h (Lin et al., 1971).

The first signs of ricin intoxication in laboratory animals are typically lethargy and refusal to eat, followed later by a rapid drop in body temperature with characteristic shivering, and, ultimately, hypotension; death is usually delayed until 10 h to 5 days after exposure, depending on the amount of toxin injected and the species of animal (Flexner, 1897; Waller et al., 1966; Balint, 1974; Fodstad et al., 1979). Laboratory animals surviving the first 6–7 days after injection of sublethal doses of

TABLE 17.2
Comparison of the Lethal Effects of Ricin in Different Animals

Animal	Lethal Dose of Ingested Castor Seed (g/kg)[a]	Lethal Dose of Injected (i.m.) Ricin (μg/kg)[b]	Lethal Dose of Injected (i.p. or i.v.) Ricin (μg/kg)[c]	Relative Resistance to Injected (s.c.) Ricin[d]
Hen	14.0	—	—	—
Goat	5.50	3	—	—
Cow	2.00	—	—	—
Pig	1.30	—	—	—
Sheep	1.25	—	—	—
Rhesus monkey	—	—	1–10 (i.v.)	—
Dog	—	0.6	1.2 (i.v.) (Fodstad et al., 1979)	200
Cat	—	0.2	—	200
Mouse	—	—	0.8–10 (i.p. or i.v.) (Fodstad et al., 1976, 1979; Fulton et al., 1986; Stirpe and Battelli, 1990)	100
Guinea pig	—	0.8	<1.1 (i.v.) (Ehrlich, 1891a; Hunt et al., 1918; Olsnes and Pihl, 1977)	60
Rat	—	—	0.5–2 (i.v.)	12
Rabbit	0.90	0.1	0.5 (i.v.)	12
Goose	0.40	—	—	—
Horse	0.10	—	—	—

Note: Dashes indicate data not available.

[a] Average lethal dose of castor seed p.o. from a single study of Mieszner and colleagues (Mieszner and Rewald, 1909; Hunt et al., 1918; Balint, 1974).

[b] Lethal dose determined from a single study using a highly purified preparation of ricin; the postexposure observation interval was 3 days for goat and unspecified for other animals (Field, 1910; Hunt et al., 1918).

[c] Selected literature values for the parenteral toxicity of ricin in laboratory animals. The postexposure observation interval was generally 2–7 days; see individual references for details of methodology.

[d] Relative resistance to injected ricin (s.c.) estimated by comparing the minimum fatal and maximum nonfatal doses and considering the time to death (Hunt et al., 1918). A larger number indicates relatively greater resistance to ricin; tabulated values are normalized by assigning 100 arbitrarily to the laboratory mouse.

ricin, however, appear to recover completely after 1–3 weeks, based on subjective observation (Fodstad et al., 1979).

Early studies of animals poisoned with lethal amounts of purified ricin or castor seed homogenates reported the appearance of severe, multiorgan pathology at necropsy, including hemorrhages in the intestines, mesenterium, and omentum, with gross pathological changes in the liver, spleen, kidney, and lymphoid tissues (Stillmark, 1888; Flexner, 1897; Hunt et al., 1918; Waller et al., 1966; Derenzini et al., 1976; Olsnes and Pihl, 1977). Administration of lethal amounts of ricin to rats, cows, or goats by injection leads to pathological changes in the liver and lymphoid tissue, including enlargement of the mesenteric lymph nodes with perinodal hemorrhages, bleeding into intestinal mucosa, and gross changes in the liver and spleen (Waller et al., 1966).

Employing highly pure toxin preparations and histological evaluation of necropsy tissue, however, Fodstad et al. (1979) were unable to confirm several of the severe pathological changes reported earlier for liver, kidneys, or mesenteric lymph nodes of laboratory mice or dogs following injection (i.v.) of ricin or abrin. Instead, they observed a transient decrease in serum hemoglobin and more subtle evidence of ultrastructural pathology in the liver of animals exposed to lethal or sublethal amounts of toxin (Fodstad et al., 1979). Differences among studies may be caused partly by variations in the amounts or potency of ricin used.

The effects of injected ricin are not limited to local tissue damage, and this has suggested the possibility of preferential target cells or organ systems. For example, rats injected i.m. with ricin show significant intestinal pathology, including intestinal hemorrhages, macrophage infiltration of the villi, and apoptosis of cells lining the small intestine, particularly within the ileum and villous lamina propria (Leek et al., 1989). The severity of changes in the GI tract following an i.m. injection is similar to findings with the related Shiga toxin RIP, and suggests that the toxins may trigger systemic immune system effects on lymphoid cells remote from the portal of toxin entry into the animal (Leek et al., 1989).

The B-lymphocytes may represent one of the preferential targets of ricin toxicity *in vivo*. Lymphatic tissues from animals exposed to ricin show extensive hyperplasia and cellular necrosis with edema, hyperemia, and hemorrhage (Waller et al., 1966). Rats injected (i.m.) with ricin or abrin develop numerous apoptotic-like bodies in ileal crypts, para-aortic lymph nodes, and Peyer's patches (Griffiths et al., 1987). The finding of apoptosis in whole animals may be due to a direct effect of ricin on cells of the lymphatic tissue, as is observed with isolated cells *in vitro*, or it may partly reflect the numerous pathological sequelae of toxin exposure, including severe shock (Griffiths et al., 1988; Howat, 1988).

B. Biodistribution

Whole-body autoradiography of mice after injection (i.v.) of ^{125}I-labeled ricin showed that the toxin accumulates in the spleen, liver, adrenal cortex, and bone marrow; approximately 50% of the injected ricin was found in the liver (Fodstad et al., 1976). Several strains of mice, including DBA, C3H, and B6D2 mice, were given a comparable dose of ricin, and the concentrations found in liver, spleen, and kidneys were found to be highest in the DBA strain (Godal et al., 1984). Some of the ricin in the liver was present in the form of dissociated RTA that appeared to be chemically modified, but retained the ability to inhibit protein synthesis *in vitro* (Godal et al., 1984). Ricin is cleared or degraded from the circulation with a half-life of about 5 h in mice (Olsnes and Pihl, 1977).

Injection (i.v.) of laboratory rats with ^{125}I-ricin resulted in an accumulation of about 70% of the toxin in the spleen, liver, and muscle at 30 m, with detectable levels decreasing to about 11% of injected toxin by 24 h after exposure (Ramsden et al., 1989). Injection (i.m.) of rats with ricin showed concentration of the toxin at the injection site and the ipsilateral para-aortic lymph nodes beginning as early as 4–8 h after exposure (Griffiths et al., 1986).

In both rats and mice, injected ricin is excreted mostly in the urine during the first 24 h after exposure, with less than 2% of injected toxin appearing in the feces (Griffiths et al., 1986; Blakey et al., 1988; Ramsden et al., 1989).

After injection (i.v.) of mice with radioactively labeled abrin, the toxin concentrates in the liver and spleen, and is cleared or degraded from the animals with a half-life of 17 h (Olsnes and Pihl, 1977).

C. Inhalation Toxicity

Exposure of animals to lethal ricin aerosols or dust clouds under controlled, experimental conditions causes irreversible damage to the respiratory tract resulting in epithelial necrosis, pulmonary edema, and ultimately death (Griffiths et al., 1995a, 1995b; Wilhelmsen and Pitt, 1996; Wilhelmsen, 2000). The inhalational toxicity of ricin depends on the aerosol particle size and the mode of aerosol exposure (e.g., nose-only, head-only, or whole-body exposure). Most particles larger than about 10 μm do not reach the critical level of the bronchiolaralveoli and, therefore, pose less of a pulmonary threat than do ricin particles in the size range 0.5–3 μm (Griffiths et al., 1995a, 1995b; Parker et al., 1996; Wilhelmsen and Pitt, 1996; Brown and White, 1997; Kende et al., 2002).

Roy et al. (2003) compared the lethality of different particle size distributions of ricin aerosols in laboratory mice; aerosols composed of particles in a size distribution >3 μm were significantly less

toxic than were ricin aerosols composed of an optimal 1 μm particle size. Additionally, the trachea and nasopharyngeal regions of mice exposed in a whole-body aerosol chamber contained significantly more ricin than did those animals exposed in a nose-only exposure chamber (Roy et al., 2003).

Inhalation toxicology of ricin has been studied most often in rodents (Griffiths et al., 1995a, 1995b; Brown and White, 1997; Kende et al., 2002; DaSilva et al., 2003; Roy et al., 2003). In laboratory mice, depending on the strain and aerosol exposure method, the LCt_{50} for ricin aerosol exposure is about 9 mg/min/m^3. Based on a comparative study of several strains of mice, BALB/C mice were the most resistant to ricin with whole-body aerosol LCt_{50} levels that correspond to an LD_{50} of approximately 11–15 μg/kg (Wilhelmsen, 2000; DaSilva et al., 2003). Other strains of laboratory mice showed apparent LD_{50} values ranging from approximately 3 to 9 μg/kg (Wilhelmsen, 2000). In the laboratory rat, depending on the strain and exposure method, the reported LCt_{50} values for ricin aerosol range from 4 to 13 mg/min/m^3 (Griffiths et al., 1995a; Brown and White, 1997).

Aerobiologists at the U.S. Army Medical Research Institute of Infectious Diseases (Fort Detrick, MD) have developed a reproducible, head-only ricin aerosol exposure model for laboratory nonhuman primates (NHP) that yielded acute LCt_{50} values for African green monkeys or rhesus monkeys corresponding to approximately 6–10 or 15 μg/kg, respectively (Wilhelmsen and Pitt, 1996). Exposure of NHP to aerosolized ricin (particle size 1–2 μm) caused a dose-dependent toxicity that is delayed from 8 to 24 h; early anorexia and lethargy were frequently observed, followed by gastric distress, hypothermia, hypotension, acute respiratory distress, and death. Rhesus monkeys exposed to the equivalent of approximately 20–40 μg/kg ricin by aerosol died from acute respiratory distress about 36–48 h after exposure; necropsy revealed fibrinopurulent pneumonia, acute inflammation of the trachea and airways, and massive pulmonary alveolar flooding (Wilhelmsen and Pitt, 1996).

Unlike all other routes of exposure, the gross pathological changes caused by ricin inhalation are observed almost exclusively in the respiratory tract, and are generally characterized by a diffuse pulmonary edema with multifocal areas of necrosis and inflammation (Griffiths et al., 1995a; Doebler et al., 1996; Poli et al., 1996; Vogel et al., 1996; Wilhelmsen and Pitt, 1996; Brown and White, 1997; Wilhelmsen, 2000).

The differential effect of ricin on specific cell types within the respiratory tract represents an important data gap in our understanding of ricin toxicity. Inhaled ricin causes high-permeability pulmonary edema and necrosis of the pulmonary epithelium, which is consistent with a direct cytotoxic effect on the epithelial cells (Doebler et al., 1995, 1996; Poli et al., 1996; Vogel et al., 1996; Wilhelmsen and Pitt, 1996). Additionally, pulmonary epithelial necrosis may be initiated by ricin-mediated activation of regulatory cell populations, paracrine inflammatory mediators, and leukocyte infiltrates activated and recruited after respiratory intoxication (Brown and White, 1997). Destruction of lymph nodes by ricin also may involve effects on specific cell types. Ricin binds to type I and II pneumocytes *in vitro*, and these cells appear to be a major target for ricin within the lung after respiratory exposure *in vivo* (Griffiths et al., 1995a; Wilhelmsen and Pitt, 1996; Brown and White, 1997).

DaSilva et al. (2003) examined the expression levels of 1178 mRNA species in pulmonary tissues obtained postmortem from BALB/c mice following inhalation exposure to ricin. This approach identified at least 34 specific genes with statistically significant changes in expression as early as 12 h after ricin exposure, including several known mediators of inflammation.

D. Gastrointestinal Toxicity

Ricin is markedly less toxic to animals by the GI route than it is by parenteral injection or inhalation. In laboratory mice, for example, the acute LD_{50} for ricin exposure intragastrically or orally (p.o.) has been reported to be 1–20 mg/kg (Parker et al., 1996), which is 100- to 1000-fold less toxic than by

injection (i.v.) or inhalation (1–10 μg/kg). Decreased potency via the GI route may arise from a low absorption of ricin holotoxin from the intestinal tract; in some animals, <0.1% of the ingested toxin is absorbed (Olsnes and Pihl, 1977; Ishiguro et al., 1983).

The susceptibility of different animal species to poisoning with castor seed has been shown to vary by as much as 100-fold (Table 17.2) (Hunt et al., 1918; Balint, 1974). It is unclear to what extent this reflects differences in the amount of actual toxin ingested owing to variations in seed content, digestive processes, or protective regurgitation, versus more subtle innate or biochemical mechanisms of resistance to ricin in some animals.

E. Ocular Toxicity

Ricin and abrin are severely irritating to the eyes and adnexa, causing congestion and pseudomembranous conjunctivitis after administration of very dilute solutions containing microgram quantities of toxin (Hunt et al., 1918; Grant and Schuman, 1993). Ocular instillation of 1–100 μg ricin in laboratory animals, including rabbits, guinea pigs, and mice, causes extensive ocular inflammation that may lead to permanent corneal damage and blindness (Hunt et al., 1918; Grant and Schuman, 1993). Ehrlich found that larger amounts of toxin (approximately milligram quantities) applied to mouse eyes caused panophthalmitis and loss of the eye (Hunt et al., 1918). Ricin also may be absorbed systemically after ocular instillation and cause damage to internal organs (Strocchi et al., 2005).

F. Comparisons among Related Plant Toxins

Ricin and related type 2 RIP plant toxins are comparably lethal to laboratory mice under controlled conditions (Table 17.3). Variations in the LD_{50} values for a single toxin reported by different laboratories are comparable with variations among different toxins. Few controlled animal studies are available for several of these toxins, and comparisons among laboratories are limited by differences in toxin preparations, animal strains, and methodologies employed. The postexposure observation period is a particularly important variable; for example, the literature values for the acute toxicity of abrin vary by 80-fold depending on whether intoxicated animals are observed for 24 or 48 h after exposure (Dickers et al., 2003).

There are likely differences among species in susceptibility to related plant toxins but, as with ricin, these are often obscured by interlaboratory variability. In one comparative study of abrin (i.v.) lethality in different animals, the MLD for mice was 10-fold greater (0.7 μg/kg) than that for rabbits (0.03–0.06 μg/kg), with rats and guinea pigs showing intermediate sensitivity (Fodstad et al., 1979); these findings parallel those for ricin (Table 17.2).

Stirpe and colleagues compared the postmortem pathology of laboratory rats exposed to supralethal amounts of ricin, abrin, volkensin, modeccin, or viscumin, and found subtle but consistent differences (Stirpe and Battelli, 1990). Each of the five toxins has a delayed effect and causes ascites and early damage to the lymph nodes, the spleen, and the Kupfer sinusoidal cells of the liver. The liver is the primary site of necrosis for ricin, volkensin, or modeccin, whereas the primary lesion detected in parenchymal organs following abrin exposure is necrosis of the pancreatic acinar cells (Stirpe and Battelli, 1990). Differences in pathology may reflect the relative uptake of target cell populations or differences in toxin biodistribution caused by variations in the carbohydrate specificity of the toxin B-chains. For example, volkensin and modeccin lead to greater interneuron toxicity and destruction of neurons in the ipsilateral substantia nigra and intralaminar thalamus of the rat than do ricin or abrin (Wiley and Stirpe, 1987, 1988).

G. Sublethal Exposure

Outside of limited descriptions or subjective commentary describing surviving animals of acute lethality studies, little is published describing the sublethal or chronic effects of ricin on specific

TABLE 17.3
Comparison of the Parenteral Lethality of Ricin and Related Toxins in Laboratory Mice

Toxin	LD_{50} (μg/kg)[a]	Exposure Route
Ricin[b]	0.8–10 (Olsnes and Pihl, 1973a; Fodstad et al., 1976, 1979; Olsnes and Pihl, 1982; Fulton et al., 1986; Stirpe and Battelli, 1990)	i.p. or i.v.
Abrin[c]	0.6–20 (Lin et al., 1969; Olsnes and Pihl, 1973b, 1982; Stirpe and Battelli, 1990; Dickers et al., 2003)	i.p. or i.v.
Pulchellin	30 (Silva et al., 2005)	i.p.
Modeccin[d]	2–5.3 (Gasperi-Campani et al., 1978; Stirpe et al., 1978; Stirpe and Battelli, 1990)	i.p.
Volkensin[e]	1.7 (Stirpe et al., 1985; Stirpe and Battelli, 1990)	i.p.
Viscumin	2.4 (Olsnes et al., 1982)	i.v.

[a] Postexposure observation interval for determining acute toxicity was 2–7 days unless noted otherwise.

[b] The acute parenteral LD_{50} of ricin in mice is approximately 0.8–10 μg/kg with a minimum lethal dose of 0.7–2 μg/kg (Fodstad et al., 1976, 1979; Stirpe and Battelli, 1990). The LD_{50} value of 0.8 μg/kg was measured 7 days after exposure in 25–27 g BALB/c mice (Fulton et al., 1986).

[c] MLD of abrin by injection (i.v.) is 0.7 μg/kg in mice (Fodstad et al., 1979). Over 40-fold variation in the MLD for abrin was observed among laboratory animal species, ranging from 0.03 to 0.06 μg/kg in rabbits to as high as 1.2–1.4 μg/kg in dogs (Fodstad et al., 1979).

[d] Modeccin has a delayed LD_{50} of 2.3 μg/kg if lethality is measured 10 days after exposure (Stirpe et al., 1978). The postmortem pathology observed after modeccin injection is similar but not identical to that observed following ricin or abrin poisoning (Flexner, 1897; Green and Andrews, 1923b; Waller et al., 1966; Derenzini et al., 1976; Olsnes and Pihl, 1977; Stirpe et al., 1978).

[e] Volkensin has an acute LD_{50} of 0.3 μg/kg in rats by parenteral injection (measured at 48 h) (Stirpe et al., 1985). The delayed toxicity of volkensin measured 14 day after injection is even greater, with the observed LD_{50} in rats dropping to 60 ng/kg in rats (Stirpe et al., 1985).

tissues or organs. Fodstad et al. (1979) found that repeated injections (i.v.) of sublethal amounts of ricin or abrin biweekly in laboratory mice or dogs caused neither observable signs of acute toxicity nor accumulated toxicity; the histological and ultrastructural (electron microscopy) changes observed in sublethal exposures were comparable with those found in animals exposed to lethal concentrations of toxin. Moreover, continued laboratory observations, pathology, and biochemical studies for 4 months after exposure found that acute or chronic exposure to sublethal amounts of ricin or abrin does not cause delayed damage in dogs (Fodstad et al., 1979).

H. Toxicity of Isolated A-Chain

Purified A-chains from the type 2 RIPs generally are much less toxic than are the holotoxins, provided that no contaminating B-chain is present. For example, highly purified RTA injected i.p. in BALB/c mice yielded an LD_{50} of approximately 40 mg/kg, making it over 1500-fold less toxic than is ricin under the same conditions (Fulton et al., 1986).

I. Synthetic and Chimeric Toxins

Synthetically produced toxins and genetically engineered toxin chimeras are areas of emerging interest because of their possible application as new medical modalities (e.g., IgTs) and powerful research tools, as well as their potential misuse as toxin weapons to confuse traditional medical countermeasures (Olsnes and Pihl, 1986; Millard, 2005). With the advent of facile production systems for recombinant proteins, the large-scale production of type 2 plant RIP toxins or toxin chimeras is increasingly practicable.

It is possible to produce type 2 RIPs synthetically by recombinant production of the individual A- and B-chains, followed by incubation in the presence of an appropriate reduction/oxidation system to form the interchain disulfide bond. The efficiency and yield of the *in vitro* production method vary greatly, but it can produce toxic heterodimers. For example, recombinant pulchellin heterodimers produced by this approach yielded a parenteral LD_{50} of 45 μg/kg in mice, compared with 30 μg/kg for the natural toxin (Silva et al., 2005).

Production of toxin chimeras is possible by applying similar technology to recombine isolated A- and B-chains from closely related type 2 RIPs (Olsnes and Pihl, 1986). For example, hybrid toxins composed of the modeccin A-chain and RTB have been produced and found to be even more toxic to Vero cells than are native ricin or modeccin (Sundan et al., 1983). Isolated abrin A-chain can be combined with RTB, or abrin B-chain with RTA, to produce chimeras with almost the same toxicity as the native toxins (Olsnes et al., 1974a). Similar experiments have been conducted combining viscumin A-chain and RTB to produce a chimera with cytotoxicity intermediate between that of viscumin and ricin (Tonevitskii et al., 1994).

Moreover, it is possible to use genetic engineering to expand upon the natural set of toxins further by producing chimeras from the components of the nontoxic type 2 RIPs. If the B-chain from cinnamomin, a relatively nontoxic type 2 RIP, is replaced with RTB, then the resultant chimera acquires cytotoxicity comparable with that of ricin holotoxin (Wang et al., 2006).

VI. EFFECTS OF RICIN ON HUMANS

Judging from laboratory studies of other mammals, ricin is likely to be highly toxic and potentially fatal to humans by any means of exposure, but the signs, symptoms, and severity of illness are expected to vary significantly with the amount and quality of toxin introduced as well as the exposure route. Only limited reports are available describing the toxicity of ricin or any of the related plant toxins in humans, and most of this literature describes the consequences of relatively benign oral ingestion of unknown amounts of toxin (Balint, 1974; Knight, 1979; Crompton and Gall, 1980; Kinamore et al., 1980; Fine et al., 1992; Audi et al., 2005).

A. Castor Seed Ingestion

Ricin poisoning by the accidental or intentional ingestion of castor seeds is rarely fatal if modern medical care is available (Balint, 1974; Rauber and Heard, 1985). Stillmark and Beauvisage reviewed 150 cases of human ricin poisoning before 1894 and identified 9 fatalities (Hunt et al., 1918). A review of castor seed poisoning reports from the early 1900s until 1984 revealed only three deaths, two verified by autopsy, for a mortality rate of approximately 1.9% (Rauber and Heard, 1985). According to a review of the American Association of Poison Control Center's Toxic Exposure Surveillance System data from 1983 through 2002, 47% of the 4387 exposures reported had no effects, 20% had minor effects, 4% had moderate effects, and 0.1% had major effects (Watson et al., 2003; Doan, 2004).

Ingestion of castor seeds results in a relatively nonspecific syndrome, resembling infectious, food-borne, or chemical gastroenteritis that typically manifests within a few hours after exposure (Rauber and Heard, 1985). Table 17.4 summarizes the wide range of signs and symptoms associated with human intoxication. The effects vary considerably among individuals, are apparently dose dependent, and can range from mild GI disturbances with subjective weakness or prostration, to severe hypotension, profuse and possibly bloody diarrhea, dehydration, and multisystem organ failure (Hunt et al., 1918; Rauber and Heard, 1985). The signs and symptoms of ricin p.o. in humans generally parallel the effects observed in experimental animals, although the onset of illness (or perhaps its recognition) occurs more rapidly in humans (Hunt et al., 1918; Rauber and Heard, 1985).

It is widely cited that approximately 1 mg/kg ricin p.o. is fatal for humans; this is significantly lower than the measured intragastric LD_{50} of approximately 20 mg/kg for laboratory mice

TABLE 17.4
Signs and Symptoms Reported to Result from Castor Seed Ingestion

Abdominal pain (Meldrum, 1900; Möschl, 1938; Kászas and Papp, 1960; Ingle et al., 1966; Malizia et al., 1977; Kopferschmitt et al., 1983; Wedin et al., 1986; Challoner and McCarron, 1990; Aplin and Eliseo, 1997)
Anuria (Meldrum, 1900; Bispham, 1903; Kopferschmitt et al., 1983)
Blurred vision (Kopferschmitt et al., 1983)
Bradycardia (Challoner and McCarron, 1990)
Confusion (Koch and Caplan, 1942; Wedin et al., 1986)
Convulsions (Möschl, 1938)
Dehydration (Ingle et al., 1966; Malizia et al., 1977; Kopferschmitt et al., 1983; Challoner and McCarron, 1990)
Diarrhea, bloody diarrhea (Hutchinson, 1900; Möschl, 1938; Ingle et al., 1966; Malizia et al., 1977; Spyker et al., 1982; Kopferschmitt et al., 1983; Wedin et al., 1986; Challoner and McCarron, 1990)
Disorientation (Möschl, 1938; Kászas and Papp, 1960; Malizia et al., 1977; Levin et al., 2000)
Drowsiness (Koch and Caplan, 1942)
Fluid and electrolyte loss (Rauber and Heard, 1985)
Hematemesis (Meldrum, 1900)
Hematuria (Malizia et al., 1977; Spyker et al., 1982; Challoner and McCarron, 1990)
Hyaline casts on urine microscopy (Malizia et al., 1977)
Hyperbilirubinemia (Palatnick and Tenenbein, 2000)
Hyperglycemia or hypoglycemia (Challoner and McCarron, 1990; Levin et al., 2000)
Hypertonia (Möschl, 1938; Rauber and Heard, 1985)
Hypophosphatemia (Challoner and McCarron, 1990)
Hypotension (Rauber and Heard, 1985)
Hypovolemic shock (Meldrum, 1900; Bispham, 1903; Kopferschmitt et al., 1983)
Increased creatine kinase activity (Challoner and McCarron, 1990)
Increased hepatic transaminase and lactate dehydrogenase activities (Challoner and McCarron, 1990; Levin et al., 2000)
Intravascular hemolysis (Malizia et al., 1977)
Leucocytosis (Malizia et al., 1977; Kopferschmitt et al., 1983; Challoner and McCarron, 1990)
Light-headedness (Challoner and McCarron, 1990)
Melena (Rauber and Heard, 1985)
Metabolic acidosis (Challoner and McCarron, 1990; Levin et al., 2000)
Miosis (Malizia et al., 1977)
Muscle cramps (Bispham, 1903; Kopferschmitt et al., 1983; Rauber and Heard, 1985)
Mydriasis (Hutchinson, 1900; Balint, 1974)
Oliguria (Meldrum, 1900; Bispham, 1903; Kopferschmitt et al., 1983)
Oropharyngeal irritation (Möschl, 1938; Challoner and McCarron, 1990)
Peripheral cyanosis (Koch and Caplan, 1942; Kopferschmitt et al., 1983; Rauber and Heard, 1985)
Prerenal impairment, secondary to hypovolemia (Kopferschmitt et al., 1983)
Proteinuria (Malizia et al., 1977; Spyker et al., 1982; Challoner and McCarron, 1990)
Renal failure (Rauber and Heard, 1985)
Sweating (Meldrum, 1900)
Tachycardia (Ingle et al., 1966; Kopferschmitt et al., 1983; Wedin et al., 1986; Challoner and McCarron, 1990)
Tachypnea (Bispham, 1903; Wedin et al., 1986)
Transient increased hepatic enzyme levels (Challoner and McCarron, 1990; Levin et al., 2000)
Vomiting (Hutchinson, 1900; Bispham, 1903; Möschl, 1938; Kászas and Papp, 1960; Kopferschmitt et al., 1983; Wedin et al., 1986; Challoner and McCarron, 1990; Palatnick and Tenenbein, 2000)

(Hunt et al., 1918; Kopferschmitt et al., 1983; Wedin et al., 1986; Franz and Jaax, 1997; Palatnick and Tenenbein, 2000). Although humans may be particularly susceptible to ricin poisoning, there is little basis for an informed estimate because of the great variation in the amount of toxin contained in castor seeds, as well as variations in the circumstances of poisoning (Hunt et al., 1918; Balint, 1974). There are also wide differences expected in the amount of toxin released

from chewing or swallowing seeds, unknown degradation and absorption kinetics from the GI tract, and natural variations in potency and composition among castor seed strains. For rare cases when castor seed ingestion is fatal, death typically occurs later than the third day after exposure from multiorgan failure; autopsy findings include ulceration of the mucosa of the stomach and small intestine, nephritis, and necrosis of the liver and mesenteric lymph nodes (Rauber and Heard, 1985).

Whole castor seeds contain oil, phytochemicals, and other potentially purging components that may induce protective vomiting or otherwise reduce GI absorption of ricin in humans. One must recognize, therefore, that the highly successful clinical outcome of treating accidental or intentional cases of oral poisoning with castor seeds may not accurately predict the human morbidity or mortality associated with consumption of pure or stabilized ricin.

B. Castor Seed Allergy

Some workers may develop a well-characterized allergic syndrome from inhalation exposure to castor seed dust, but this is not believed to result from ricin. Patients with castor seed allergic syndrome typically present with acute onset conjunctivitis, rhinitis, sneezing, urticaria, and wheezing, and they generally improve by reducing exposure to castor seed products and conventional allergy treatments (e.g., epinephrine) (Figley and Elrod, 1928; Topping et al., 1982). Both Type I and IV allergic responses have been documented following dermal exposure to castor seeds or its by-products; in some cases, the immune reaction may cause debilitating hives and asthma (Lockey and Dunkelberger, 1968; Kanerva et al., 1990; Metz et al., 2001).

Castor seed allergic syndrome is believed to result primarily from the CB-1A fraction of the seed extract (Ratner and Gruehl, 1929; Grabar and Koutseff, 1934; Brown and White, 1997). The CB-1A extract contains hyperallergenic 2S albumin proteins (McKeon et al., 2000; Breiteneder and Radauer, 2004). In one study, up to 96% of individuals with allergy to castor seed dust possessed IgE specific for 2S albumin (Bashir et al., 1998). Additional low molecular weight allergens are also likely present in the castor seeds; for example, contact dermatitis has been attributed to zinc ricinoleate oil found in some cosmetic products (Magerl et al., 2001).

Although it is less common than is the castor seed allergic syndrome, workers chronically exposed to subacute ricin or other RIPs may develop Type I allergies directly against the toxins; allergies to purified RIPs can be demonstrated by immune sensitization and IgE induction (Hunt et al., 1918; Forster-Waldl et al., 2003; Szalai et al., 2005).

C. Inhalation Toxicity

As with other protein toxin weapons, we expect that the generation of primary or secondary ricin aerosols, especially within an enclosed space, poses a potential biological warfare or bioterrorism risk (LeClaire and Pitt, 2005; Millard, 2005). Due to the technical challenges of generating highly toxic and persistent protein aerosols, we expect the risk of lethality to be less than the risk of operational disruption, prolonged incapacitation from ocular or respiratory tract inflammation, and increased burden on medical and logistical assets.

Documented cases of human poisoning by ricin aerosol exposure are unknown. Cautious inferences may be drawn from observations of NHP exposed to ricin under controlled laboratory settings. Human toxicity from ricin inhalation would be expected to occur after a latency period of 24–72 h that may be characterized by loss of appetite and listlessness. Based on extrapolation from NHP studies, other signs and symptoms expected in humans after ricin inhalation may include listlessness, high fever, dyspnea, and coughing or bloody sputum that is delayed for 4–8 h after exposure, as well as bilateral abnormalities on chest radiographs, arterial hypoxemia, neutrophilic leukocytosis, and elevated protein levels in bronchial aspirates (Balint, 1974; Wilhelmsen and Pitt, 1996; Franz and Jaax, 1997).

D. Injected Ricin

The lethal dose of ricin by injection for humans is unknown, but it has been estimated to be in the range of 1–10 μg/kg bodyweight based on analogy with NHP studies and from extrapolation of human clinical trials (see below).

In one well-publicized assassination case attributed to injection (i.m.) of an unknown amount of ricin (estimated to be as much as 500 μg), the first signs and symptoms appeared within 24 h after exposure and included fatigue, nausea, vomiting, and fever; over the next 48 h, the patient developed necrotic lymphadenopathy at the site of injection, GI hemorrhage, hypovolemic shock, and renal failure (Crompton and Gall, 1980). Death occurred by 72 h after exposure, and autopsy revealed pulmonary edema and hemorrhagic necrosis of the small bowel, and hemorrhages in lymph nodes near the injection site, myocardium, testicles, and pancreas (Crompton and Gall, 1980).

A 20 year old man allegedly attempted suicide by injection (s.c.) of an unknown amount of ricin from crude castor seed extract (Targosz et al., 2002). The victim entered the hospital about 36 h after injecting the toxin with symptoms of nausea, dizziness, weakness, chest and abdominal pain, and myalgia with paraesthesia of the extremities. Signs included hypotension, anuria, metabolic acidosis, and a bleeding diathesis. Hepatorenal and cardiorespiratory failure preceded death from an asystolic arrest approximately 54 h after injection.

E. Sublethal Effects

Sublethal effects of injected ricin have been documented in isolated case reports, and are difficult to distinguish from those of many other toxic or infectious agents. A 36 year old chemist who allegedly injected himself (i.m.) with an unknown amount of ricin prepared from homogenized castor seed, for example, complained of headache and rigors approximately 10 h later, then developed anorexia and nausea, a sinus tachycardia, erythematous areas around the puncture wounds, and local lymphadenopathy at the injection sites (Fine et al., 1992).

F. Human Clinical Trials

Clinical trials with ricin or IgTs provide another important source of information for predicting the effects of ricin in humans. In addition to Phase 1 studies with ricin holotoxin (Fodstad et al., 1984), various glycoforms of RTA, chemically deglycosylated (dg) RTA, or recombinant RTA have been covalently conjugated with tumor-specific antibodies and tested for the ability to safely and selectively target and kill tumors (Vitetta et al., 1993; Ghetie and Vitetta, 1994). IgTs based on dgRTA were found to possess a longer biological half-life than does native RTA (Blakey et al., 1988; Wawrzynczak et al., 1991).

Cancer patients treated with i.v. ricin every 2 weeks at doses as low as 14 $\mu g/m^2$ (about 0.5 μg/kg) experience flu-like symptoms of fatigue and myalgia with occasional episodes of nausea or emesis; most patients tolerate doses up to 18–20 $\mu g/m^2$ (Fodstad et al., 1984). Ricin is cleared from circulation within 24 h after a bolus injection, with apparently first-order kinetics and 99% clearance by 18 h (Fodstad et al., 1984).

Consistent with animal studies comparing RTA and ricin, humans tolerate much higher levels of RTA than ricin. In Phase I/II clinical trials of two experimental IgTs containing dgRTA administered (i.v.) to Hodgkins' lymphoma patients, for example, dose-limiting toxicity was observed at levels of approximately 15 mg/m^2 (about 0.5 mg/kg) (Schnell et al., 2003).

1. Lethality Extrapolations

By comparing the maximum sublethal doses for laboratory mice with the maximum tolerated doses (MTDs) obtained from human clinical trials, and assuming a similar LD_{50}/MTD ratio for the two species, an estimate of the LD_{50} of ricin or abrin for humans would be about 0.6–1 μg/kg (i.v.)

(Faguet and Agee, 1997). For comparison, the parenteral LD_{50} of ricin for laboratory mice is in the range of 0.8–10 μg/kg (Table 17.2), and a human lethal dose by injection based on one case report was estimated to be 1–10 μg/kg (Crompton and Gall, 1980; Gill, 1982; Franz and Jaax, 1997). Although there is concordance among these lethality estimates, we caution that there is no convincing scientific basis to conclude that the toxicity of ricin in laboratory mice is predictive of human morbidity or mortality.

2. Vascular Leak Syndrome

A significant source of human morbidity for ricin or RTA administered i.v. apparently is due to endothelial cell toxicity that manifests clinically as a vascular leak syndrome (VLS) (Vitetta, 1990; Baluna and Vitetta, 1997; Engert et al., 1997; Schnell et al., 1998, 2002, 2003; Baluna et al., 2000; Ghetie and Vitetta, 2001). Patients exposed to ricin or RTA IgTs experience symptoms of myalgia and fatigue, followed by hypoalbuminemia, edema with resultant weight gain, and, in some cases, mild cardiopulmonary symptoms (tachycardia and dyspnea) (Vitetta et al., 1993; Baluna and Vitetta, 1997; Baluna et al., 2000; Ghetie and Vitetta, 2001). Signs and symptoms of VLS can be reversed by removal of the IgT or by corticosteroid treatment (Baluna et al., 2000). Interestingly, the RTA-induced VLS that occurs in humans, or human endothelial cell models *in vitro* (Lindstrom et al., 1997), is not observed in normal laboratory mice, rats, guinea pigs, or dogs exposed to comparable levels of ricin or IgT (Soler-Rodriguez et al., 1992, 1993).

Although cytotoxicity from the nonspecific uptake of RTA or IgTs by vascular endothelial cells may contribute to VLS pathogenesis, this simple explanation does not fully explain the human sensitivity relative to other species. Baluna et al. (1999) compared the primary amino acid sequences of several polypeptides that cause human VLS, including RTA conjugates, other IgTs, and interleukin-2, and proposed that the endothelial cell damage underlying VLS is caused partly by a three residue motif containing a central aspartic acid; the proposed sequence motif occurs in the amino acid sequence of RTA at positions L74, D75, and V76.

G. Comparison with Abrin Toxicity

The human fatal dose of abrin via parenteral routes has been estimated to be approximately 0.1–1 μg/kg based on case reports of accidental or intentional ingestion. No serious toxic effects were observed in terminal cancer patients treated with as much as approximately 0.3 μg/kg (i.v.) abrin (Dickers et al., 2003). As with ricin, however, most documented cases of abrin poisoning in humans have involved chewing or swallowing *A. precatorius* (jequirity) seeds, a route of exposure that is much less dangerous and which predominantly causes GI toxicity (Gunsoulus, 1955; Hart, 1963; Davis, 1978; Fernando, 2001).

Symptoms following p.o. ingestion of jequirity seeds generally can be expected to begin within a few hours, although there are reports of postexposure delays lasting 1–5 days (Davis, 1978; Fernando, 2001). Delayed onset of symptoms may result from various factors, including the content of abrin in the seeds and the mastication of the seeds before swallowing. Early signs and symptoms, like those reported for ricin (castor seed), are relatively nonspecific and include nausea (Guggisberg, 1968; Davis, 1978), severe vomiting (Davis, 1978; Fernando, 2001), abdominal pain (Davis, 1978; Fernando, 2001), and diarrhea (Davis, 1978; Fernando, 2001). As intoxication progresses, signs and symptoms can include GI bleeding (Fernando, 2001), bloody diarrhea (Davis, 1978; Frohne et al., 1984), hematemesis (Davis, 1978), tachycardia (Davis, 1978; Fernando, 2001), headaches (Guggisberg, 1968), dilated pupils (Gunsoulus, 1955), irrationality (Gunsoulus, 1955), hallucinations (Gunsoulus, 1955; Frohne et al., 1984), drowsiness (Davis, 1978; Fernando, 2001), weakness (Davis, 1978), tetany (Gunsoulus, 1955), tremors, seizures (Davis, 1978; Frohne et al., 1984), flushed skin (Gunsoulus, 1955), fever (Davis, 1978; Frohne et al., 1984), and unspecified cardiac dysrhythmias (Davis, 1978).

VII. DIAGNOSIS AND DETECTION OF RICIN EXPOSURE

The primary diagnosis of ricin exposure is through clinical signs and symptoms, but these can be expected to vary significantly depending on the amount of toxin ingested and the route of exposure. Attempts to provide a diagnostic picture for humans exposed to supralethal amounts of ricin toxin are almost entirely speculative and based largely on a few unsubstantiated case reports that describe intentional injection of unknown quantities of toxin.

In the absence of an unambiguous history of ricin exposure, the preferred diagnostic method is specific immunoassay of ricin in serum, respiratory secretions, or other clinical samples associated with poisoning. Most of the methods described for ricin detection are experimental or are under development. The CDC and the Federal Laboratory Response Network have the capability to detect ricin in environmental specimens using validated polymerase chain reaction (PCR) tests and time-resolved immunofluorescence assays, with cell-based bioassays to confirm ricin activity. The U.S. Department of Defense has produced experimental field immunoassays, but commercial distribution of field test kits currently is limited.

Ricin can be detected in the blood or other bodly fluids of exposed animals using competitive radioimmunoassays or enzyme-linked immunosorbent assays (ELISA). These methods generally do not distinguish between active ricin molecules versus partially degraded or otherwise inactivated toxin. The postexposure time limit for accurate antibody-based detection of ricin in biological samples varies and depends on the route of exposure and the absorbed dose. In the laboratory, ELISA detects ricin in oro-nasal swabs of NHP exposed to ricin aerosol up to 24 h after exposure (Franz and Jaax, 1997). Likewise, ELISA detects ricin in selected tissues of laboratory rats up to 48 h after an i.m. challenge (Leith et al., 1988).

Radioimmunoassays can detect ricin in blood at concentrations as low as 50–100 pg/mL (Godal et al., 1981). Colorimetric ELISA methods can detect ricin spiked into *ex vivo* human serum or urine samples accurately at levels as low as 1 ng/mL (100 pg/well) with acceptable inter- and intra-assay variation (Poli et al., 1994). Chemical luminescence-based ELISA technology improves the detection limit to 0.1–1 ng/mL ricin, but the coefficients of variation may be as high as 50% (Poli et al., 1994). Combination of a sensitive immunological assay with PCR amplification may achieve a limit of detection for ricin at levels as low as 10 fg/mL (Lubelli et al., 2006).

Aptamers, based on either RNA or DNA sequences, offer a potential replacement or adjunct to antibody-based detection of ricin (Proske et al., 2005). Both DNA- and RNA-based aptamers bind ricin and can approach the specificity of an antibody–antigen interaction (Hesselberth et al., 2000; Tang et al., 2006). Ricin has been detected at levels of approximately 14 ng/mL using fluorescently labeled RNA-based aptamers and capillary electrophoresis under nonequilibrium conditions (Haes et al., 2006).

Rapid differentiation between active and inactive ricin is possible using assays that measure the inhibition of protein synthesis (May et al., 1989). Several rapid screening assays have been published that exploit the ability of ricin at very low levels to disrupt cell-free translation of enzymes such as luciferase (Langer et al., 1996; Hale, 2001; Mei et al., 2006); this approach detects active ricin at levels as low as 0.3 ng/mL (Mei et al., 2006). Confirmatory assays for ricin activity can be developed that directly detect the adenine product using radiolabeled substrate, colorimetric methods, or liquid chromatography–mass spectrometry methods (Brigotti et al., 1998; Heisler et al., 2002; Hines et al., 2004). It may be possible to distinguish among the catalytic activity of ricin and related RIPs in a rapid detection format by the use of carefully selected synthetic substrates and antibody binding in an electrochemiluminescence-based assay (Keener et al., 2006).

Ricin is a highly immunogenic toxin, and paired acute and convalescent sera should be obtained from survivors for measurement of antibody response. Conceptually, the detection of circulating antiricin antibodies formed in response to sublethal toxin exposure is possible for exposed individuals who survive for 2–3 weeks (Hunt et al., 1918). However, humoral immunoglobin M responses would likely be of short duration, and no immunological memory would be anticipated without boosting.

In cases where the toxin is metabolized, excreted, or otherwise not available for immunological or chemical detection, it may be possible to verify an exposure to ricin or castor seeds for forensic purposes by detecting other unique components of the *R. communis* plant. PCR can detect residual castor seed DNA in most ricin preparations. Ricinine, an alkaloid (3-cyano-4-methoxy-*N*-methyl-2-pyridone) produced by castor seeds, has been proposed as a biomarker for ricin exposure; the detection limit for ricinine by electrospray ionization tandem mass spectrometry is as low as 0.083 ng/mL in urine of exposed subjects (Johnson et al., 2005).

VIII. PHYSICAL PROTECTION

A. Field Methods

Avoidance of contamination and physical barriers provides the best protection for noninfectious toxins (U.S. Department of Health and Human Services, 2007). Detection and monitoring of exposure levels requires reliable, field-expedient procedures for detecting active ricin, especially aerosols. A comprehensive description of the available and emerging technologies for ricin detection in nonclinical, environmental settings is beyond the scope of this chapter. Methods for field detection of ricin parallel those described above for medical diagnosis and include mass spectrometry, as well as the use of antibodies combined with physical detection platforms, such as evanescent wave fiber optics (Narang et al., 1997), microfluidics, or fluorescent antibodies coupled with charge-coupled device cameras (Rowe-Taitt et al., 2000; Ligler et al., 2003).

Before entering a potentially contaminated area, first-responders should conduct a risk assessment of the specific situation and deploy suitable physical protection, including personal protective equipment (PPE). The most important hazards to protect against are primary and secondary toxin aerosols. Audi et al. (2005) summarize PPE available for protection against ricin in a field situation, including level-B PPE with self-contained breathing apparatus, disposable, penetration-resistant Tyvek suits with gloves, air-purifying respirators (P-100 filters), and full-face respirators for eye and face protection.

B. Inactivation of Ricin

Ricin is not inactivated significantly by freezing, chilling, or storage at ambient temperature. Ricin stored at 4°C in the liquid state with a suitable preservative (e.g., sodium azide) retains significant potency for years. Isolated RTA is less stable in solution at 4°C than is the holotoxin, but either can be stored below −20°C for long periods in 10% glycerol.

Heating a 1% (w/v) solution of ricin to ≥85°C for 30 min results in complete inactivation as judged by toxicity in laboratory mice (Hunt et al., 1918). Dry heat of ≥100°C for 60 min in an ashing oven or steam autoclave treatment at >121°C for 1 h reduces the activity of pure ricin by >99% (Wannemacher et al., 1989). Heat inactivation of impure toxin preparations (e.g., crude ricin plant extracts) may vary. Heat-denatured ricin can undergo limited refolding (<1%) to yield active toxin. Isolated RTA and RTB are more easily inactivated by heating than is the holotoxin (Olsnes et al., 1975; Taira et al., 1978).

Ricin also is inactivated by a 30 min exposure to concentrations of NaOCl ranging from 0.1% to 2.5%, or by a mixture of 0.25% NaOCl plus 0.25 N NaOH (Wannemacher et al., 1989). In general, solutions of 1.0% NaOCl are effective for decontamination of ricin from laboratory surfaces, equipment, animal cages, or small spills (Wannemacher et al., 1989; NIOSH, 2003; Wannemacher et al., 1993; Burrows and Renner, 1999).

It has been observed that treatment of ricin or RTB with chloramine-T reduces toxic activity, although optimal conditions for complete inactivation are not established (Funatsu et al., 1970; Balint, 1974; Sandvig et al., 1976).

Irradiation causes a dose-dependent loss of activity for aqueous solutions of ricin, but complete inactivation is difficult to achieve; 75 MRad reduced activity 90%, but complete inactivation was

not achieved even at 100 MRad (Haigler et al., 1985). Gamma irradiation from a laboratory ^{60}Co source can be used to partially inactivate aqueous solutions.

Abrin is largely inactivated by incubation >60°C for 1 h (Olsnes and Pihl, 1977). Systematic, comparative studies of the relative stabilities of the type 2 plant RIPs to denaturation by heat or inactivation with conventional laboratory decontamination solutions are unavailable presently; this represents an important practical data gap in the literature.

IX. TREATMENT AND MEDICAL COUNTERMEASURES

A. Emergency Treatment

There is currently no specific treatment available for poisoning with ricin or related plant toxins. Medical management treats signs and symptoms, employing i.v. replacement of fluid and electrolytes to prevent dehydration from vomiting and diarrhea. The major treatment goals for patients who have ingested toxin are to improve perfusion through fluid resuscitation, vasopressor therapy, and the replenishment of electrolytes (Kinamore et al., 1980; Rauber and Heard, 1985; Challoner and McCarron, 1990; Audi et al., 2005). All patients potentially exposed to ricin or related toxins should be monitored closely for signs of hypotension, hepatoxicity, and bone marrow suppression (Darby et al., 2001).

Most documented human cases of ricin poisoning have resulted from intentional or accidental oral ingestion, and this route of exposure is fatal for <1% of cases if modern clinical care is provided (Rauber and Heard, 1985; Challoner and McCarron, 1990). For oral poisoning, intestinal decontamination is unlikely to be beneficial once vomiting has begun; gastric lavage is controversial, but can be considered for nonvomiting patients presenting within an hour from ingestion (Vale, 1997). Early GI lavage with a single dose of activated charcoal p.o. (3 g charcoal/25 mL water) may reduce further systemic absorption of the toxin, although the extent of ricin adsorption to charcoal is unknown (Chyka and Seger, 1997; Vale, 1997).

Early induction of emesis by home administration of syrup of ipecac, followed by aggressive clinical management that includes i.v. fluid administration, has been recommended for treating children aged 2–6 years who have consumed seeds containing ricin or abrin (Kinamore et al., 1980).

For accidental ocular exposure to ricin, extensive rinsing with 10% lactose solution may reduce toxicity if performed immediately after exposure (Strocchi et al., 2005).

For aerosol inhalation, the complete lack of useful clinical experience leaves one to speculate based on animal studies or human poisoning from comparable chemical agents. The first priorities should be immediate evacuation and decontamination to reduce the risks of further exposure or secondary aerosols for the patient and health care provider, combined with providing adequate ventilation and any necessary airway support. Pulmonary edema likely will evolve during the 12–36 h following exposure, and this may be amenable to treatment with anti-inflammatory drugs, bronchodilators, oxygen, endotracheal intubation, and, in extreme cases, continuous or mechanical supplemental positive end-expiratory pressure (Franz and Jaax, 1997; Audi et al., 2005).

B. Experimental Vaccines

Paul Ehrlich used ricin and abrin to lay the foundations of molecular immunology by demonstrating the specificity of the immune response to foreign proteins, as well as the importance of agglutinating serum proteins (antibodies) in mediating protective and passive immunity; he also predicted the use of antibodies as a diagnostic tool for toxin exposure (Ehrlich, 1891a, 1891b, 1960; reviewed in Hunt et al., 1918; Olsnes, 2004). Over one century later, there is still no ricin vaccine approved by the U.S. Food and Drug Administration for human use, although experimental vaccines are under advanced development in laboratory and clinical trials.

1. Toxoid Vaccine

Heat-denatured ricin is highly immunogenic in experimental animals and serves as a suitable animal vaccine to protect against ricin challenges (Hunt et al., 1918). As early as WWII, the U.S. Army developed a toxoid vaccine for human use that was prepared from formalin-inactivated ricin. A second-generation toxoid vaccine composed of denatured ricin adsorbed to aluminum-based adjuvant was developed after the 1991 Persian Gulf War. Toxoid vaccines were effective at preventing death in experimental animals, including NHP, exposed to ricin toxin aerosols, but the respiratory tracts of surviving animals were not protected completely against bronchiolar and interstitial pulmonary inflammation (Griffiths et al., 1996; Poli et al., 1996).

Likewise, a formalin-treated abrin toxoid vaccine was shown to reduce lethality in rats exposed to a supralethal aerosol challenge with toxin; the abrin toxoid delayed the onset of pulmonary toxicity, but could not prevent significant lung damage (Griffiths et al., 1995b).

2. Ricin Subunit Vaccines

Inherent barriers to the production and widespread use of the formalin-inactivated toxoid, including local reactogenicity, the potential for reversion, and expectations of a difficult manufacturing process, have led to an ongoing interest in development of improved ricin vaccines (Lord et al., 1987; Lemley and Wright, 1992; Lemley and Creasia, 1995; Griffiths et al., 1999; Smallshaw et al., 2002; Marsden et al., 2004; Olson et al., 2004).

Wannemacher and colleagues developed a subunit vaccine based on dgRTA that is highly immunogenic and effectively protects mice against supralethal ricin aerosol challenge; dgRTA is effective when administered i.m. with aluminum-based adjuvant, or intranasally with experimental mucosal adjuvant (Kende et al., 2006). Important limitations remain, however, for using dgRTA as a human product, including a complicated manufacturing process that results in heterogeneity of the final product, potential reversion of *N*-glycosidase activity, and a strong tendency of the chemically deglycosylated material to self-aggregate in solution.

Following the development of dgRTA, several recombinant immunogens were proposed based on modified forms of RTA with active-site substitutions that eliminate residual *N*-glycosidase activity without disrupting the antigenic properties of the molecule (Aboud-Pirak et al., 1993; Lemley and Creasia, 1995; Griffiths et al., 1998; Smallshaw et al., 2002). These candidates effectively eliminate several of the potential safety concerns raised for ricin toxoid or dgRTA, but do not address the important manufacturing problems of immunogen stability and aggregation.

The tendency of RTA-based vaccines to self-aggregate under physiological conditions may be related to hydrophobic domains exposed by the absence of the natural RTB chain (Olson, 1997, 2001; Olson and Cuff, 1999). Based on an analysis of the functional architecture of the toxin, it was hypothesized that compacting the RTA subunit to a single domain (RTA N-terminal domain residues 1–198) might reduce the solvent-exposed hydrophobic surface and, thereby, offer an improved platform for presentation of neutralizing epitopes (Olson et al., 2004, 2005). Removal of the C-terminal domain of RTA also eliminates key residues of the rRNA-binding site that are required for toxic *N*-glycosidase activity.

Polypeptides based on the RTA1–198 domain concept can be expressed in large quantities; remain folded as judged by circular dichroism and infrared spectroscopy; are more stable than RTA and exhibit dynamic light scattering indicative of monodisperse monomers under physiological conditions without significant aggregation (McHugh et al., 2004; Olson et al., 2004). Moreover, a representative domain immunogen based on this concept, RTA1–33/44–198, showed no detectable toxin activity, and protected mice or NHP against supra-lethal ricin aerosol exposure (Olson et al., 2004, 2005).

Marsden et al. (2004) have proposed a subunit vaccine based on their finding that ricin can be largely inactivated by the recombinant insertion of a specific 25 residue internal peptide into the active-site region of RTA. This concept is reminiscent of the type 3 RIPs, yielding a pro-processed

form of RTA that is essentially inactive unless the internal peptide is removed by proteolysis. A recombinant construct based on modified ricin holotoxin containing this 25 residue inactivation peptide was not toxic when injected into rats at a concentration that was lethal for native ricin. Moreover, treated animals developed significant antiricin antibody titers, suggesting that the construct holds promise as an experimental vaccine (Marsden et al., 2004).

Vitetta and colleagues have focused their vaccine development efforts on elimination of the tripeptide motif in RTA that has been implicated in the toxic VLS described above. RTA-induced damage to endothelial cells was observed in human clinical trials, as well as in model systems measuring pulmonary vascular leak or vascular leak in human skin xenografts in SCID mice (Baluna et al., 1999). To overcome this safety concern, a recombinant variant of RTA-containing amino acid substitutions to disrupt the *N*-glycosidase RIP site (Y80A), as well as the putative VLS-promoting site (V76M), is under development (RTA Y80A/V76M or RiVax) (Smallshaw et al., 2002; Vitetta et al., 2006; Peek et al., 2007).

RiVax immunization (i.m.) protects laboratory mice against a ricin challenge at least 10-fold greater than that survived by nonimmunized controls (Smallshaw et al., 2002). A pilot clinical trial was conducted on humans under an Investigational New Drug application submitted to the Food and Drug Administration, in which three groups of five normal volunteers were injected three times at monthly intervals with 20, 33, or 100 μg of RiVax. The vaccine was apparently safe, and elicited ricin-neutralizing antibodies in one of five individuals in the low-dose group, four of five in the intermediate-dose group, and five of five in the high-dose group (Vitetta et al., 2006). A stable, optimized vaccine formulation of RiVax with Alhydrogel has been developed for follow-on clinical studies (Peek et al., 2007).

Although there have been inconsistent results reported regarding the ability of RTB to induce protective antibodies, the development of a modified RTB vaccine or inactivated RTA-RTB chimera merits further investigation. Recombinant B subunit-based vaccines are useful against other members of the A–B (*n*) family of toxins, such as Shiga toxins (Boyd et al., 1991; Ryd et al., 1992; Acheson et al., 1996; Byun et al., 2001; Mukherjee et al., 2002), diphtheria (Pappenheimer et al., 1972), cholera (Michalski et al., 1993; Bergquist et al., 1997), and tetanus. Moreover, recombinant RTB can be expressed by mammalian cells grown in culture (Chang et al., 1987), and it may be especially useful as a mucosal immunogen to stimulate antibodies that would prevent ricin attachment to the epithelial surfaces of the intestinal or respiratory tract (Medina-Bolivar et al., 2003).

Finally, we note that conventional, i.m. administration of subunit vaccines in general may not protect completely against the lung pathology caused by exposure to ricin aerosols. Results of animal studies strongly suggest that the presence of secretory antibodies may be critical in completely preventing lung injury (Poli et al., 1996; Yan et al., 1996; Griffiths et al., 1997, 1998, 1999). Efforts are underway to apply alternate vaccine delivery strategies and novel adjuvants that may enhance the total IgG or the protective mucosal immune response to ricin (Kende et al., 2002, 2006; Matyas et al., 2004).

3. Neutralizing Epitopes

Protective immunity to ricin in laboratory animals is mediated by the formation of high-affinity, neutralizing antibodies (Houston, 1982; Godal et al., 1983; Foxwell et al., 1985; Chanh et al., 1993; Hewetson et al., 1993; Lemley et al., 1994; Yan et al., 1995, 1996; Griffiths et al., 1995b; Kende et al., 2002; Maddaloni et al., 2004). Several groups have described monoclonal antibodies (MAbs) that partly or completely protect animals against ricin poisoning when premixed with toxin or administered by passive transfer (Colombatti et al., 1986; Chanh et al., 1993; Lemley et al., 1994; Gao et al., 2002).

Specific structural epitopes have been identified on ricin that can elicit MAbs capable of neutralizing ricin *in vitro* and *in vivo* (Katzin et al., 1991; Aboud-Pirak et al., 1993; Maddaloni

et al., 2004). Lowell and colleagues identified a 10 amino acid region of RTA (residues 97–106) that forms a solvent-exposed loop over the lip of the active-site cleft where the substrate rRNA presumably binds (Aboud-Pirak et al., 1993; Lebeda and Olson, 1999). Linear peptides based on this region were insufficient to neutralize holotoxin, suggesting the importance of a specific three-dimensional conformation for optimal MAb binding (Aboud-Pirak et al., 1993). Later studies by another group also produced a neutralizing MAb that binds an epitope on this loop (RTA residues Y91-F108) (Maddaloni et al., 2004).

A second neutralizing epitope (identified by MAb RAC 18) was shown to be located at RTA residue A178, adjacent to the key catalytic residue E177 (Ready et al., 1991; Maddaloni et al., 2004). Antibodies to this epitope could theoretically interfere with RTA catalysis by blocking access to the active site. Finally, a third neutralizing epitope (identified by MAb RAC17) binds RTA at residues 66–69 (Maddaloni et al., 2004).

Experiments based on the *in vitro* priming of peripheral blood mononuclear cells identified several human T-cell epitopes on RTA, including one located within RTA175–183 that contains the catalytic E177 and apparently requires I175 (Tommasi et al., 2001; Castelletti et al., 2004). An examination of neutralizing antibodies produced by 15 different patients who had been treated with RTA-based IgTs identified a continuous, immunodominant epitope within RTA residues 161–175; this polypeptide sequence comprises the epitope recognized by MAb RAC18 in animal studies, and is adjacent to a human T-cell epitope of RTA175–185 (Castelletti et al., 2004; Maddaloni et al., 2004). Additional and comparative T-cell epitope mapping studies may be possible in the future from human volunteers immunized with RiVax or other RTA-based immunogens in the course of ongoing clinical trials.

C. Experimental Antitoxins

There is currently no antitoxin available that is approved for human use against ricin poisoning. Studies with experimental animals showed that the i.v. administration of ricin antibodies within 1–2 h of toxin exposure may improve survival rates. Mice can be protected by passive transfer of polyclonal antisera or MAbs (Houston, 1982; Foxwell et al., 1985; Colombatti et al., 1987; Chanh et al., 1993; Lemley et al., 1994).

In one study, antibodies raised to either RTA or RTB protected mice equally well against ricin challenge (Foxwell et al., 1985). A more recent study, however, found that only a subset of neutralizing MAbs produced against RTA were protective *in vivo*, and none of the anti-RTB MAbs tested could protect animals against ricin poisoning (Maddaloni et al., 2004).

Passive pretreatment of animals with an aerosolized cocktail of polyclonal antiserum offered some protection against lung lesions in an aerosol challenge model (Poli et al., 1996). Comparable levels of protection could be obtained by stimulating secretory antibodies through mucosal immunization (Yan et al., 1996; Griffiths et al., 1997, 1998, 1999).

Along with antibodies, RNA-based aptamers may serve as a potential structural scaffold for the development of selective therapeutics that bind ricin in the circulation and prevent it from reaching cellular targets (Hesselberth et al., 2000; Lee et al., 2006).

The use of antibody- or aptamer-based approaches to protect against ricin holds potential as a possible pretreatment for armed forces, or as an adjunct to PPE for civilian first-responders and other special populations required to enter contaminated areas. However, extracellular antitoxin would probably be of limited use in a bioterrorist or civilian mass casualty scenario, because the therapeutic window for administration is likely to be short (a few hours), the delay to onset of symptoms is relatively long, and there presently is no method of immediately detecting ricin exposure. It also remains to be determined whether specific combinations of MAbs are required for optimal *in vivo* protection against the toxin. Moreover, in some cases, MAbs that bind toxin with high avidity and block enzymatic activity of RTA *in vitro* actually enhance the toxicity of ricin *in vivo* (Maddaloni et al., 2004).

D. Other Experimental Therapeutics

Several research groups have explored the development of active site-directed inhibitors of RTA or, alternatively of RTB receptor antagonists, as therapeutic adjuncts to vaccination or for use as clinical antidotes against ricin or RTA-IgT toxicity. Discovery of safe and effective ricin therapeutics is an especially challenging problem for several reasons. The toxin binds to a ubiquitous receptor (galactose) that can be expected to be present in relatively high concentration compared with exogenous antagonists. Internalization is rapid compared with the onset of symptoms and, once inside the cell, ricin is essentially inaccessible to exogenous therapies; development of an intracellular therapeutic for such a permissive toxin poses enormous safety risks. Finally, a successful RTA active-site inhibitor must disrupt or prevent the strong charge–charge interactions of the RTA–ribosome complex without itself causing toxic interference with the binding of essential translational machinery.

Nevertheless, several efforts have yielded small molecule inhibitors with promising characteristics *in vitro*. Active-site inhibitors with RTA-binding constants that range from millimolar to nanomolar have been described (Orita et al., 1996; Tanaka et al., 2001; Miller et al., 2002). Nanomolar inhibitors based on the oligonucleotide stem-loop RNA inhibitors of RTA face considerable drug development obstacles with respect to bioavailability and biological stability (Lipinski et al., 2001; Amukele et al., 2005). If an effective and essentially irreversible RTA inhibitor could be devised, however, it might be practically useful as a pretreatment for military forces or civilian first-responders entering potentially contaminated areas with relatively short notice (i.e., scenarios where traditional vaccination is impractical and PPE is unavailable or undesirable).

X. CONCLUSION AND PERSPECTIVE

The impressive biomedical literature describing ricin notwithstanding, we are only just beginning to understand the complexity of the plant RIPs. Ricin has been the most extensively characterized plant RIP largely because of its relative abundance and ease of isolation. Increased awareness of the biological effects of less common, related toxins has grown rapidly during the past decade as methods for detecting, characterizing, producing, and bioengineering plant proteins continue to expand. As a result of this trend, this family of proteins can be expected to play an increasing role in future design of toxins for medical uses, as well as engineered toxin weapons (Millard, 2005).

Basic research is needed to elucidate precisely how ricin and other nonpore-forming toxins may exploit translocation machinery present in the ER membrane to reach the cytosol of target cells. Knowledge of the specific translocons and protein chaperones involved in translocation or refolding RTA within the target cell may reveal new classes of therapeutic targets for blocking cytotoxicity, or permit artificial shunting of the subcellular routing of toxin toward unproductive pathways (e.g., lysosome or ATP-dependent proteases). Current approaches to these questions include isolation of protein–protein complexes essential for RTA retrotranslocation, as well as the use of libraries of defined ‘‘knock-out’’ mice to assess the importance of specific intracellular pathways.

The data gaps in our understanding of how to treat poisoning with type 2 RIPs appear primarily above the cellular level in trying to understand the failure of interrelated cellular and organ systems, on which the organism depends for survival. Despite more than 150 years of laboratory study, including seminal work by Ehrlich in defining passive immunity, the effects of ricin and its homologs on specific cells of the immune system remain enigmatic and incompletely understood. Are there specific immunotoxic effects that influence the protective host response to ricin?

Although ricin and related plant RIPs are widely available and among the most toxic natural substances known, there are fortunately few human fatalities documented. With modern medical treatment, oral poisoning by castor seed homogenates or crude ricin preparations is unlikely to pose a significant threat to public health. In contrast, we know almost nothing about the management or consequences of human poisoning by inhalation exposure, such as might occur if the toxin were

employed as an indoor aerosol. A few animal studies raise the possibility that vaccination may not fully protect at-risk populations from the effects of ricin aerosols, necessitating research to confirm these observations and quantify the acute and chronic effects in vaccinated survivors.

The aerosol toxicity of ricin has been documented in several laboratory animals, including rhesus (Wilhelmsen and Pitt, 1996) and African green monkeys, but it remains unclear which, if any, of these effectively model the human response to ricin. Is it necessary to conduct animal trials with medical countermeasures for ricin in an NHP aerosol model, or will other animal models suffice to predict the human response? The unexpected appearance of VLS in human clinical trials underscores the difficulty in extrapolating from animal models. Data addressing these questions will be critical in devising practical and effective strategies for coping with ricin as a toxin weapon.

Current prioritization for medical research must include improved models for management of the pulmonary damage, as well as effective postexposure treatments aimed at elimination or irreversible inactivation of the toxins. Additional unanswered questions pertaining to the clinical course of ricin poisoning include: What are the target cells and pharmacokinetics that underlie the predictably delayed onset of illness, the early signs of general malaise and reduced reflexes, or the final convulsions, dyspnea, and hypotension? What is the window of opportunity after ricin exposure for the productive administration of antitoxins or other toxin scavengers?

Continued vaccine studies are needed to determine the longevity of the protective immune response that is induced by recombinant RTA-based immunogens like RTA1–33/44–198 or RiVax. Additionally, it may be possible to develop RTA-based vaccines that combine the physicochemical advantages of the RTA1–198 platform for storage and stability with the desirable safety profile of RiVax.

Although the type 2 plant RIPs are similar in structure and function, experimental ricin vaccines have not been able to protect animals against challenges with abrin, or vice versa (Ehrlich, 1891a, 1891b; Hunt et al., 1918; Griffiths et al., 1995b). Consequently, it presently would require multiple vaccines to fully protect against the threat posed by ricin and related plant toxins. This obvious shortcoming underscores the importance of continued basic research to identify shared epitopes, or to apply emerging technologies in the development of cost-effective, multivalent vaccines that may protect against multiple type 2 RIPs with a single vaccination.

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18 Screening Smokes: Applications, Toxicology, Clinical Considerations, and Medical Management

Bryan Ballantyne and Harry Salem

CONTENTS

I. INTRODUCTION AND GENERAL CONSIDERATIONS

Screening smokes have a major military use in concealment operations by the generation of a fog-like atmosphere composed of light-scattering particles that prevent or markedly limit the visibility

The contents of this chapter are not to be construed as an official Department of the Army position, unless so designated by other authorizing documents.

of, and actions of, troops or vehicle movements where occult operational characteristics are of tactical importance. As noted in Section II, they are also used for a variety of other military and civilian purposes during which the user and bystanders may be exposed to the smoke; these include various types of training, rescue operations, and fire simulation. Screening smokes are produced by the deliberate burning or vaporization of a material or formulation to form an opaque cloud, and they are often differentiated from obscurants, which are usually defined as naturally occurring particles suspended in air that block or attenuate the transmission of parts of the electromagnetic spectrum, such as visible or infrared radiation or microwaves (NRC, 1997). Examples of obscurants are fog, mist, and dust.

In many military situations, the user will be protected by appropriate clothing and respirators donned before generation of the smoke. However, in some military uses, and in training sessions and civilian applications, there may be accidental or, in some cases, intended exposure of unprotected individuals. For the latter applications, particularly, it requires to be determined that smoke materials will not cause adverse health effects in the long- or short-term. This, coupled with the marked variations in the chemistry of different smoke materials, requires that the acute and repeated exposure toxicity of the screening smoke and any formulation constituents is determined, and the absence of significant adverse effects may be required to be confirmed by appropriate studies with human volunteer subjects as discussed in Section V.A. In this respect, and with reference to military applications, screening smokes should not be of a biologically reactive nature that would permit them to be classified as chemical warfare agents under the Geneva Convention. However, some severely injurious materials used for screening smokes have on occasion been used for offensive antipersonnel purposes in warfare situations; for example, white phosphorus (AOL, 2005; BBC, 2005a).

II. APPLICATIONS OF SCREENING SMOKES

Screening smokes have a wide spectrum of applications in military and civilian situations. The selection of which smoke is to be used in military situations depends on the tactical needs. For example, smokes that block visible wavelengths and near infrared are used to inhibit the effective use of viewers such as binoculars, weapons sights, night observations sights, and laser range finders. Smokes that also block into the far infrared interfere with battlefield viewers and weapons guidance systems, such as homing systems or antitank and air-defense missiles. Other multispectrum screens can obscure radar systems and high-energy microwave-directed weapons. Some of the principal uses of screening smokes are summarized below.

1. *Military Concealment Operations*. Deliberately generated smokes are used in military situations for local visual concealment of troop operations, such as movement and placement of artillery and other fire capabilities. They may also be employed against opposing forces to cause impairment of coordinated and operational activities. Generally these are white/gray smokes. Additionally, some screens are used for infrared obscuration purposes.
2. *Military Signaling Smokes*. These are usually colored smokes used to mark specific locations. They thus find application for indicating and coordination of certain local operational commands, for identification of friendly units, and for identifying enemy positions and targets. Some colored smokes are used to simulate fire and explosive situations for training purposes. They are generated by volatilizing and subsequently condensing a pyrotechnic mixture that contains an organic dye.
3. *Security Operations for Rescue*. Screening smokes may find a use for distraction and concealment during hostage rescue operations.
4. *Simulation for Firefighting Training*. To limit hazards to firefighters, and others, during training exercises, there is a clear requirement for an obscuring smoke simulant that is devoid of adverse effects. Cinnamic acid, which produces a white moderate obscuring smoke, is a candidate (Ballantyne and Clifford, 1978).

5. *Peacekeeping Operations*. The use of screening smokes to cause distraction, disorientation, and obscure potential targets in civil disturbances, although infrequently used, is possibly a useful adjunct (Ballantyne, 2006a).

The wide range of uses for screening smokes indicates that different and varying populations may be exposed and, as discussed below, this has implications for health-hazard assessments and advising on the protective and precautionary measures that may be needed.

III. SMOKE GENERATION

With smokes used for screening purposes, it is the intention to produce a dispersion of obscuring light-scattering particulate material in the atmosphere. A method frequently used for the production of a screening smoke is by pyrotechnic generation from substances that are thermostable. The pyrotechnic mix is basically usually composed of an oxidizing agent, such as potassium chlorate, and a combustible material such as sulfur or a sugar, the activation (ignition) of which results in an exothermic reaction that volatilizes the screening agent, which then condenses to produce a cloud of solid or supercooled liquid droplets that form an obscuring particulate cloud, the droplets of which may agglomerate into larger particles. A cooling agent may sometimes be added to prevent excessive decomposition of the screening agent; for example, with a dye. The burning time of the screening smoke mix can be regulated by adjusting the proportions of oxidant and combustible material, and by the use of a cooling agent such as sodium bicarbonate. Pyrotechnically generated smokes may be delivered by bomb, shell, mortar, or grenade; for example, phosphorus, zinc-hexachloroethane, and colored smokes. For easily volatile materials or formulations, the material may be introduced into a heated manifold such as the exhaust manifold of a vehicle; for example, diesel-fuel and fog-oil smokes.

The effectiveness of screening smokes depends on obscuration by particles that reflect, refract, and scatter light rays. The effectiveness of a smoke increases with its atmospheric concentration, as shown in Table 18.1.

IV. EXPOSURE PATTERNS AND GENERAL PROTECTIVE MEASURES

The multiple uses of screening smokes indicate that, depending on the application, various and differing individuals may be exposed to screening smokes. These may include protected and unprotected military personnel, civilian instructors, trainees, and bystanders. The latter might include members of the public who become accidentally exposed due to changing meteorological conditions and those who live or work near military training facilities. Also, production workers engaged in the filling and processing of grenades and other dispersion devices can be occupationally exposed to the basic active constituents of the munition. Unprotected individuals may have exposure of skin, eyes, and respiratory tract. This can result in local adverse effects at the sites of contamination or, if exposure dosages are sufficiently great, there may be systemic effects resulting from absorption of material following inhalation or from percutaneous absorption. Physical personal protection against potential local and systemic adverse toxicity can be afforded by protective clothing (including gloves) and by respirators with appropriate absorbent cartridges. Standard military respirators will give both respiratory tract and eye protection (Eaton and Young, 1989).

In view of the differing exposure patterns and targets it is considered necessary (advisable) that the following exposure guidelines be developed for collective protection of potentially exposed individuals:

1. An occupational exposure guideline (OEG), or permissible exposure guidance level (PEGL), for those who may be exposed recurrently and for sustained periods of time;

TABLE 18.1
Relationship between Smoke Concentration and Visibility for Several Typical Smokes

Smoke	Concentration (mg m^{-3})	Visibility (m)[a]
Fog oil	31.0	10
	6.2	50
	1.6	200
Diesel fuel	39.0	10
	7.9	50
	2.0	200
Red phosphorus	62.0	10
	12.0	50
	3.1	200
Zn/HCE	69.0	10
	14.0	50
	3.5	200

Source: Data after Eaton, J.C. and Young, J.Y., In *Medical Criteria for Respiratory Protection in Smoke, Technical Report 8902*, US Army Biomedical and Research Laboratory, Fort Detrick, Frederick, Maryland, 11, 1989; NRC, *Toxicity of Military Smokes and Obscurants, Volume 1*, National Research Council, National Academy Press, Washington DC, 1997.

[a] Visibility = path length for a 10% transmission.

this may be as an 8 h time weighted average (TWA_8) threshold limit value (TLV), which may need qualification by a STEL (short-term exposure limit) or a C (ceiling) value.

2. Emergency exposure guidance levels (EEGLs for 15 min, 1 h, and 6 h) for emergency situations resulting in exposure of personnel for less than 24 h.
3. Short-term public emergency guidance levels (SPEGLs) for an emergency situation potentially resulting in an exposure of the public to training smokes.
4. Permissible public exposure guidance levels (PPEGLs) for repeated accidental exposures of the public located or working near facilities using screening smokes in the open.

In developing SPEGL and PPEGL values, it is assumed that sensitive sectors of the population may be exposed (e.g., elderly, children, pregnant females, chronically ill subjects). The SPEGL values are derived (estimated) by using an uncertainty factor of 10 on the EEGL values, to account for the possibility of sensitive subpopulations in the nearby communities (i.e., SPEGL = 0.1 EEGL). The PPEGL values are derived from the PEGL values also by dividing them by an uncertainty factor of 10.

As a result of injuries from smoke generators, the UK Army Council has drawn attention, in Council Instruction 260, to the dangers of using such generators in confined spaces or remaining immediately downwind of a concentration; the Instruction advised that they should never be used in buildings and recommended the use of respirators when working in high concentrations of smoke. Similarly, the U.S. army recognizes the hazards of using smoke generators in confined spaces and has issued appropriate guidance instructions (NRC, 1997).

Because smoke materials may contaminate equipment, vehicles, and the environment (e.g., soil, foliage, water sources, and wildlife), it may be necessary to conduct certain ecotoxicological studies and to develop and advise on decontamination procedures.

V. TOXICITY ASSESSMENT AND HEALTH-HAZARD EVALUATION OF SCREENING SMOKES

In view of the many different basic materials used for screening smoke production, and because of the numerous methods that may be used for the generation of a smoke cloud, it is fundamental that toxicity studies be carried out specifically for each material and for formulations, and the end smoke toxicologically assessed in the physical and chemical form to which individuals may be exposed. There is a clear need to determine the likelihood of the smoke causing local adverse effects, and if there is an indication for absorption of material by the various routes of exposure, then it will be necessary to investigate the potential for systemic toxicity. Should laboratory toxicological studies indicate a potential for adverse effects by acute or repeated exposure, then it may be considered desirable to conduct carefully controlled, and appropriately designed, studies in human volunteer subjects. These various aspects of assessing the safety-in-use or otherwise of particular screening smokes are discussed in more detail below.

A. Laboratory Toxicological Studies

As noted in Section IV, the principal routes of exposure to screening smokes are by inhalation and by skin and eye contact. Because multiple exposures may occur with military uses and some training purposes, there is a need to conduct both acute (single exposure) and repeated exposure studies by the various routes of exposure. Additionally, investigations for particular special end points may be considered necessary; for example, genotoxicity. The following information should be available on the smoke material before the planning and conduct of studies; physical properties (including solubility, partition coefficients, molecular weight, particle size distribution); chemical and likely biological reactivity; intended use; method of cloud generation; subjects likely to be exposed.

1. Inhalation Toxicology Studies

Screening smokes for laboratory animal studies should be generated in as close a method as possible to simulate that used in real situations, or likely to be employed, in the field. There should be analytical measurement of the exposure concentration and measurement of the mass median aerodynamic diameter (MMAD) of the smoke particulates. The latter allows calculation of the respirable fraction of the smoke and its depth and degree of penetration into the respiratory tract. Methods for the generation and monitoring of particulate clouds and aerosols have been discussed in detail by Hext (1999). Both acute and repeated general inhalation studies are required for a full evaluation of the potential for immediate, delayed, and cumulative toxicity. Also, special studies may be required to determine the potential of the inhaled material to cause respiratory sensitization and thus being asthmagenic (see Section V.A.4).

Acute studies should ideally involve the determination of the effects of exposure to various concentrations of the smoke for different periods of time to obtain information on concentration related pharmacological and toxic effects, including the calculation of a timed LC_{50} if the material is sufficiently toxic. Also, exposures should be conducted for different periods of time at a concentration around that anticipated as being likely in operational use, to define safe and permissible exposure times under working conditions. As a minimum, acute studies with screening smokes should be monitored for signs of toxic and pharmacological effects, local ocular effects, body weights, and gross pathology. It is not usual to conduct detailed biochemistry, hematology, physiological, and urine monitoring or extensive histology in acute studies, but where there is clinical evidence for acute respiratory tract injury, the removal of tissues from the respiratory tract for subsequent histological examination is recommended. Also, and if the material has peripheral chemosensory effects, it may be necessary to quantitatively determine the concentrations at which this appears by both inhalation and by eye contact because of the harassing effects of sensory irritation (Ballantyne, 2006b). This has to be considered as part of the process in establishing

permissible exposure concentrations; inhalation peripheral chemosensory irritation may be estimated in animals by a mouse RD_{50} study (Ballantyne, 1999a, 2006b).

Repeated exposure studies are required principally to study the following general features (Ballantyne, 1989, 1999b). Increased sensitivity to local and systemic toxic and pharmacological effects compared with acute studies to determine the potential for latent and cumulative organ and tissue toxicity, and to give a reliable ''no observed adverse effect level'' (NOAEL) and a threshold for adverse effects (lowest observed adverse effect level, LOAEL). Depending on the reasons for conducting a repeated exposure study by exposure to a screening smoke, the duration of the study may vary from a few days to a lifetime. Short-term repeated studies (by definition varying from a few days to 28 days) usually give valuable information on the potential for adverse effects by repeated exposures. These should ideally be followed by subchronic studies, which last for 15%–20% of the lifetime of species exposed, about 90 days in rodents, and give a better determination of LOAEL and NOAEL. Chronic (lifetime) studies are conducted principally if there is a suspicion of an oncogenic potential. The most frequently employed general monitors for toxicity include signs, body weights, food and water consumption, peripheral blood hematology, urinalysis, necropsy for gross pathology, organs weights, and processing organs and tissues for histology. Other (special) investigations may be required on the basis of the exposure characteristics, potential bioreactivity, and known or suspect toxicology. These may include, with investigations on inhaled dyes and irritant materials, measurement of pulmonary retention of dyes, biochemical estimates of pulmonary injury, assessment of lung connective tissue, and respiratory function tests to assess functional changes in pulmonary physiology.

1. *Dye retention and elimination rates* are usually assessed by sequential removal of lungs from animals at various times during the exposure period, and also sequentially at various times during a recovery period following the final exposure. In this way, it is possible to determine both cumulative processes and elimination rates of dye in the lungs (Sun et al., 1987). After removal of lungs at the specified times, they are homogenized in an appropriate solvent, the homogenate centrifuged, and the supernatant collected. The pellet is then resuspended and repeatedly extracted until no more color is seen in the solvent; the combined extracts are then analyzed for dye content. When a dye mixture is under investigation, the retention and elimination of all components should be evaluated to determine if there is a differential handling between the materials.
2. *Biochemical and cytological estimation of lung injury* and inflammation can be undertaken by examining cell populations and enzyme activities in bronchoalveolar lavage (BAL) fluid (DeNicola et al., 1981; Duniho et al., 2002; Sciuto, 2006). Cytological determinations should include macrophage and neutrophil numbers, and biochemical measurements include lactate dehydrogenase, acid phosphatase, alkaline phosphatase, β-D-glucuronidase, glutathione reductase, acid proteinase, cathepsins B and D, and total protein.
3. *Lung connective tissue biochemistry*, as an indication of developing pulmonary fibrosis and as an adjunct to histology, involves measurement of total lung tissue collagen and of BAL fluid hydroxyproline peptides (Pickrell and Mauderly, 1981; Pickrell et al., 1983).
4. *Respiratory function tests* should be conducted before exposure and, at least, after the final exposure and at the end of a recovery period. Components that may be studied include compliance, total lung capacity, functional residual capacity, forced vital capacity, resistance, and CO diffusing capacity (Harkema et al., 1982; Sun et al., 1987).

Details of the determinants for repeated exposure studies, and the choice of species, dosing procedures, design, monitoring, and interpretation of such studies have been discussed by Ballantyne (1999b).

2. Cutaneous Toxicity Studies

The unprotected user or bystander could have skin contact with screening smokes, and depending on the nature, physical properties, and chemistry of the smoke there may be a potential for local and systemic adverse effects. Thus, it is considered that the following studies should be conducted:

1. Skin irritation (inflammation) from cutaneous exposures that are acute (primary irritation) and repeated (cumulative irritation).
2. Potential for skin sensitization and allergic contact dermatitis.
3. Depending on the chemistry of the material, it may be necessary to study the potential for phototoxicity and photosensitization.
4. It is unlikely that there will be significant percutaneous toxicity under the usual conditions of exposure to screening smokes. However, if primary and cumulative skin irritation tests suggest that there may be systemic effects, then it will be appropriate to conduct more detailed monitored studies for percutaneous toxicity.

3. Ocular Toxicology

In view of the fact that the eyes of unprotected individuals may become contaminated with screening smokes, depending on the physical nature and chemistry of the material, there may be local injury to the eye. There is thus a requirement to determine the eye-irritating potential of the smoke material by acute and possibly repeated exposure. This may be undertaken by using animals that are also exposed to the smoke for inhalation studies. The nature and severity of any eye injury, and its duration, need to be monitored in detail, and thus it may be desirable to have a subgroup of screening smoke-exposed animals specifically examined for ocular toxicity. Details of the design, recording, and interpretation of in vivo eye-irritation tests have been discussed in detail by Ballantyne (1999c). For screening purposes, in vitro tests for prediction of potential eye irritation are available (Ballantyne, 1999c, 2006c). If the screening smoke causes inflammatory changes in the eye, it probably also causes a peripheral chemosensory effect on the eye, which will produce discomfort, excess lacrimation, and blepharospasm to the eye, resulting in harassment and distracting effects. This needs to be quantitated by studies on peripheral chemosensory irritation of the eye; for example, the guinea pig blepharospasm test (Ballantyne, 1999a, 1999c). Also, if a material is injurious to the eye or is capable of penetrating the cornea or sclera, it may be necessary to examine eyes for adverse functional effects, such as measurement of intraocular pressure by tonometry (Ballantyne et al., 1977; Ballantyne, 1999c) and in vivo quantitation of alterations in corneal thickness by pachymetry (Ballantyne, 1986, 1999c; Myers et al., 1998).

4. Special End-Point Studies

Although most individuals are exposed to screening smokes for only a single occasion or a few repeated exposures, in some instances there may be longer term multiple exposures; for example, troops in action, trainees, or public residing at or near facilities using screening smokes, as well as those involved in manufacturing and filling operations. Also, in certain situations there may be exposure of heterogeneous populations; for example, children, women of fertile age, pregnant women, ill and susceptible individuals. For these reasons, and depending on the frequency and magnitude of exposure, site of use, chemical reactivity of the smoke, and known or suspect toxicology, the following are examples of specialized toxicology studies that may require to be conducted.

Genetic Toxicology. This may be used as a guide to the potential of a chemical or formulation to cause certain longer term adverse effects, of which carcinogenicity is a major example. Initially, in vitro studies, such as the *Salmonella typhimurium* reverse mutation assay and Chinese hamster ovary (CHO) forward gene mutation test (HGPRT locus), should be undertaken, followed by in

vivo studies such as the bone marrow cytogenetics study. Details of suitable in vitro and in vivo mutagenicity and clastogenicity studies, their mechanistic basis, and the conduct and interpretation of such investigations have been presented by Anderson (1999) and Tweats and Gatehouse (1999).

Immune-Mediated Hypersensitivity Reactions. In view of the likely inhalation of respirable particulates and skin contact with screening smokes, and depending on the chemistry of the material, the potential for immune reactions should be considered; the need for studies on the potential for allergic contact dermatitis has been discussed in Section V.A.2. Standard whole animal studies monitoring breathing rates by plethysmography are not reliable approaches for determining the potential for respiratory sensitizing potential (Botham et al., 1988; Blackwell, 2006). Changes in breathing pattern can result from respiratory tract primary irritation as well as by an immunologically mediated mechanism and the results require confirmation by the use of chemical–carrier conjugate, which presents preparative and analytically problems (Botham et al., 1988). Possibly more reliable methods are the mouse IgE test (Kimber and Dearman, 1997, 1999), cytokine fingerprinting (Dearman et al., 1996, 2002; Kimber and Dearman, 1999), and the eosinophil peroxidase assay (Bozeman et al., 1990; Tagari et al., 1993; Blackwell, 1998, 2006).

Developmental Toxicity. In certain situations, women who are pregnant or of childbearing age may be in an exposure situation. It thus follows that there is a need for studies on the potential for developmental toxicity; typical protocols for such investigations have been reviewed by Tyl (1999).

Toxicokinetics and Metabolism. If there is an indication that systemic toxicity is occurring from exposure to a smoke, then it may be necessary to conduct studies to quantitatively determine the rate and extent of absorption, biodistribution, metabolism, and elimination (ADME) of the suspect constituent. These measurements will permit conclusions regarding systemic, organ, and tissue doses, and thus allow quantitative risk assessments. Studies on the bioconversion of the material may give information on the relative contributions of parent material and its metabolites to the genesis of toxicity.

B. Human Volunteer Studies

Although preclinical in vivo and in vitro toxicology testing will detect many of the potential adverse effects relevant to humans, with some xenobiotics, no matter how exhaustive the preclinical screening in laboratory animals and test systems, some potentially adverse responses may not be detected or not be a monitoring feature of the test systems. This applies particularly, but not exclusively, to reflex physiological responses, some of which may be significant as triggers in the exacerbation of active or latent disease processes (Ballantyne, 1977). Thus, in several cases it is justifiable to undertake carefully designed and controlled studies with informed volunteer subjects, provided there is a wide margin of safety as assessed by preclinical investigations (Lee et al., 2004). Controlled human volunteer studies may be regarded as necessary for the following reasons (Ballantyne, 2005): (1) to confirm the absence of a particular adverse effect when this is equivocal on laboratory testing, (2) to obtain quantifiable dose–response relationships where a detailed hazard evaluation or risk assessment is necessary for the in-use application to a human population, (3) to investigate the pharmacokinetics and metabolism where species variations in laboratory studies make it difficult to determine which animal model is most appropriate for a comparative risk assessment with respect to humans (Wilks and Weston, 1990), (4) to obtain information on the effectiveness and operational characteristics of specific in-use situations by carefully conducted clinical and field trials, and (5) to meet specified national/international regulations. The prime concern in any human volunteer study should be that the volunteers are not exposed to any unacceptable risk (Van Gelderen et al., 1990). All proposals for studies involving human volunteer subjects must be reviewed and approved by an ''Ethics Committee'' or ''Institutional Review Board'' that is independent of the sponsor of the study. The Review Body should have responsibility for deciding that the proposal is justified, it can be conducted, the

procedures are acceptable, and the study carries a definable minimum of risk for the volunteers. The conditions under which human volunteer studies are permissible include the following (Ballantyne, 2005): (1) the objectives for the investigation must be clearly defined, as must the reasons why the objectives cannot be satisfied by nonhuman studies, (2) the protocol should include justification for the study, rationale for the route and mode of exposure, number of volunteers necessary, and monitoring procedures, (3) a risk–benefit analysis should be carried out in advance of the study (Wilks and Minton, 1999), (4) the investigators should be appropriately qualified (and documented), (5) compensation for participation in a study should not be an inducement. Informed consent should be obtained from the volunteers, who should sign a consent form for participation in the study. Informed means that they will be told of the purpose of the study and its benefits, what procedures are involved, and the risks (if any) to which they may be exposed. It should be made clear that they have the right to refuse to participate and that they may withdraw from the study at any stage.

The need for human volunteer studies and their nature and design will vary with the material to be studied, its use, and the route and degree of exposure. Thus, it is not possible to give a specific itemized list of human studies required on a generic basis. However, the following are some of the more frequently employed investigations:

1. *Skin Irritation and Sensitization*. If there are species variations in the response to laboratory tests to assess local cutaneous toxicity, or if the findings are marginal or equivocal, then it may be considered appropriate to confirm, or otherwise, the cutaneous reaction in humans. This may be conveniently conducted using patch tests or repeated insult patch tests (Hermansky, 1999).
2. *Defining Threshold Effect Levels*. If a physiological or biochemical effect is known to be produced by exposure to a material, then studies may be conducted to determine threshold and no-effects concentrations/dosages for that effect. It is then possible to relate these to levels considered to represent potentially hazardous overexposure conditions and permit estimates for safety factors in operational use.
3. *Small-Scale Clinical Trials*. Such trials involve exposure of individuals to the active substance or dissemination formulation with clinical monitoring for the specific organ, tissue, or system of concern with respect to potential adverse effects. For example, quantifiable exposure to a screening smoke with monitoring for respiratory function tests.
4. *Operational Trials*. Such trials are intended to simulate in-use situations for assessment of operational effectiveness and usefulness, to determine potential problems, and for training. Such trials should have independent institutional review board approval, use only fully informed volunteer subjects, ideally begin at threshold levels, and be conducted with appropriate medical cover. Such studies should be designed to combine the major intended objectives for conducting the trial with monitoring to determine the effects of the trial conditions on physiological and biochemical homeostasis and to detect any unsuspected effects from the exposure conditions. Depending on the nature of the trial, the chemicals involved, mode of delivery, and known or suspect adverse health effects, the supportive monitoring may need to include cardiovascular status (notably blood pressure, cardiac rate, ECG), respiratory function tests, chest radiograph, peripheral blood hematology, blood clinical chemistry, urinalysis, and ophthalmic investigations (including visual fields, refraction, tonometry, and pachymetry). These medical monitoring studies are of value not only in assessing safety-in-use for the healthy population and defining any operational restrictions on the active material, its formulation, and its delivery system, but also may draw attention to susceptible subpopulations, including those with established ill health. It is important that written and signed detailed records should be kept of all aspects of trials and of discussions involving the local approval committee and of the independent review body.

C. Environmental and Ecotoxicological Considerations

Screening smokes, by their intended use, are deliberately released into the environment, albeit in localized and very small amounts. Thus, the possibility for environmental problems, including ecotoxicological issues, needs consideration. Particular aspects that may need to be taken into account include local decontamination, phytotoxicity, effects on domestic and wild animals, avian and aquatic toxicity, contamination of food and drinking water, effects on plant sewage organisms.

VI. SPECIFIC SCREENING SMOKE MATERIALS

A. Phosphorus Smokes

1. Red Phosphorus Smoke

Chemistry and Generation. Most red phosphorus smokes used for visual and infrared screening are generated explosively from grenades and mortar shells using a mixture of red phosphorus and butyl rubber (≈95:5), which results in the production of a particulate cloud containing phosphorus oxyacids and phosphine (Davies, 1999). Butyl rubber reduces the cloud pillar effect found with pure red phosphorus. Red phosphorus is less reactive than the white phosphorus allotrope. The composition of the phosphoric acids in red phosphorus/butyl rubber static burn has been determined as follows: orthophosphate 22.8%, pyrophosphate 19.6%, tripolyphosphate 13.3%, tetrapolyphosphate 11.5%, P_5–P_{13} 32.8% (Brazell et al., 1984). The relative proportions of the various phosphoric acids in smoke changes with time following generation, but the predominant component remains orthophosphate (Ballou, 1981; Mitchell and Burrows, 1990). MMAD measurements of aerosol particles generated for toxicity studies have ranged 0.4–1.6 μm and geometric standard deviations of 1.5–1.9 (Ballou, 1981; Aranyi, 1984; Aranyi et al., 1988).

Toxicity. Inhalation of the smoke from ignited red phosphorus causes respiratory tract pathology, which may be lethal. *Ortho*-phosphoric acid is the principal combustion product in the smoke (Burton et al., 1982; Ramsey et al., 1985), which has a pK_a of around 2 for its first proton (Albert, 1979) and causes respiratory tract irritation and inflammatory changes. Ballantyne (1998) studied the acute inhalation toxicity of unformulated red phosphorus ignited in an air stream and using 1 h exposures to rabbits, rats, mice, and guinea pigs. Values for lethal respiratory tract exposure are given in Table 18.2, which shows a wide range of 1 h LC_{50} values with the rabbit and rat being the most resistant species and mouse and guinea pig being the most susceptible; the 1 h LC_{50} value for guinea pig was 0.036 of that for the rabbit and 0.05 of that for the rat. Most animal deaths occurred during exposure, although a small proportion of animals survived a few hours or days postexposure. The exposure concentration–mortality data for rabbits and rats showed a clear graded linear relationship. Rabbits, rats, and mice that died demonstrated similar respiratory tract histopathology. This consisted of laryngotracheal epithelial or mucosal necrosis with acute inflammatory cell infiltration, pulmonary congestion and edema, alveolar hemorrhages, polymorphonuclear cell infiltration of alveolar walls, bronchiolitis, and macrophage aggregations in alveoli and bronchioles. Guinea pigs exposed to high concentrations of red phosphorus smoke showed no lesions in the larynx and trachea, and only alveolar capillary congestion in the lungs, indicating lethal toxicity was probably a consequence of asphyxia secondary to laryngospasm in the guinea pig. This accords with a steeper slope on the concentration–mortality data for guinea pigs compared to rats or rabbits. The greater susceptibility of guinea pigs to the toxicity of phosphorus smokes has also been commented on by Mitchell and Burrows (1990). Also, Burton et al. (1982) described a similar histopathology in rats exposed to red phosphorus smoke aerosols to that seen by Ballantyne (1998). The reduced histopathological findings in survivors from potentially lethal exposure concentrations indicate that reversibility and healing may occur, although the nature of the lesions (particularly necrotic, ulcerative, and epithelial) may predispose to secondary infection. Aranyi et al. (1988) exposed rats acutely for 3.5 h to 1000 mg m^{-3} red phosphorus/butyl rubber smoke and found that pulmonary

TABLE 18.2
Acute Lethal Toxicity of Red Phosphorus Smoke

Species	Inhalation Toxicity Value (with 95% confidence limits) as P[a]	as OPA[b]
1 h LC_{50} (mg m^{-3})		
Rabbit	1,689 (1,200–3,586)	5,337 (3,792–11,332)
Rat	1,217 (970–1,489)	3,846 (3,065–4,705)
Mouse	271 (196–670)	856 (691–2117)
Guinea pig	61 (52–80)	193 (14–253)
$L(CT)_{50}$ (mg min m^{-3})		
Rabbit	101,869 (73,338–215,584)	321,906 (231,748–681,245)
Rat	73,237 (56,483–89,740)	231,429 (178,486–283,578)
Mouse	16,438 (14,523–19,38)	51,944 (45,893–61,108)
Guinea pig	3,641 (3,113–4,783)	11,506 (9,837–15,114)

Source: Data after Ballantyne, B., *Toxic Sub. Mech.*, 17, 251, 1998.

[a] Expressed as phosphorus.

[b] Expressed as *o*-phosphoric acid equivalents.

bactericidal activity to inhaled [35S]-*Klebsiella pneumoniae* was depressed. Also, tracheobronchial lavage showed a decrease in the number of recovered cells, increased alveolar macrophage levels of ATP, and decreased alveolar macrophage 5′-nucleotide activity (Aranyi et al., 1988). Acute exposure of rats to red phosphorus smoke at 1813 mg m^{-3} for 180 min and to dogs at 1882 mg m^{-3} for 240 min produced conjunctivitis, which resolved within 3 days (Weimer et al., 1977).

Repeated exposure studies to red phosphorus smoke for 1 h day^{-1} for 5 days $week^{-1}$ in mice (180 exposures) and rats (200 exposures) produced cumulative respiratory tract toxicity (Marrs et al., 1989a). Aranyi et al. (1988) exposed rats to red phosphorus/butyl rubber smoke at concentrations ranging from 300 to 1200 mg m^{-3} for 2.25 h day^{-1}, 4 days $week^{-1}$, for 4 and 13 weeks. They found pulmonary bactericidal activity to inhaled [^{35}S]-*K. pneumoniae* was depressed after repeated exposures for 13 weeks, but not after 4 weeks; because acute studies had shown decreased bactericidal activity, it was concluded that during the short-term repeated exposures some adaptation had occurred, but the depression recurred under the subchronic repeated exposure conditions. Rats exposed for 4 and 13 weeks had increased tracheobronchial lavage ATP levels in alveolar macrophages, with decreased 5′-nucleotidase activity. Bronchiolar fibrosis was seen in all rats after 4 and 13 weeks exposure to smoke concentration $\geq$750 mg m^{-3}, which did not resolve in a recovery period. In another repeated exposure study, Weimer et al. (1980) studied the effects on rats, mice, guinea pigs, and rabbits of mean concentrations of red phosphorus smoke at 22 mg m^{-3} and 165 mg m^{-3} for 8 min day^{-1}, 5 days $week^{-1}$, for 12 weeks. The low exposure concentration resulted in a cumulative inhalation exposure dosage (CT) of 10,705 mg min m^{-3} and mean daily CT of 178 mg min m^{-3}. The corresponding values for the high-concentration group were 81,691 mg min m^{-3} and 1,319 mg min m^{-3}. During the eighth exposure week there was a concentration-related reddening and swelling of the eyelids in rats. During the first 3 exposure days rats had an increasing breathing rate. However, there was no evidence of histopathological changes in the respiratory tract of any of the species sacrificed after the final exposure. Pulmonary function tests conducted on guinea pigs demonstrated that 3 weeks into the exposure regime, pulmonary resistance decreased at both concentrations, but only in males, and was not present after 6, 9, or 12 weeks of exposure. The authors concluded that, under the conditions of the study, exposure to smoke from red phosphorus/ butyl rubber mix did not cause any short-term or cumulative toxicity and that 165 mg m^{-3} could be regarded as a NOAEL for short-term repeated exposure.

No adverse effects were seen in dominant lethal and single-generation reproduction studies in rats exposed to 132 mg m^{-3} red phosphorus/butyl rubber smoke for 8 min day^{-1}, 5 days $week^{-1}$, for 10 weeks (Weimer et al., 1980). These authors also conducted a developmental toxicity study in pregnant rats exposed to 132 mg m^{-3} red phosphorus/butyl rubber smoke for 8 min day^{-1} from gestation days 5 through 15. No dose-related increases in variations or malformations were seen compared with a control group (Weimer et al., 1980). A clastogenicity (micronucleus) study was conducted in rats exposed to red phosphorus/butyl rubber smoke at 1000 mg m^{-3}. A significant effect on the micronucleus count was observed in bone marrow and peripheral blood erythrocytes after 2 weeks of exposure, but not after 4 weeks of exposure or following a 2 week recovery period (Aranyi, 1984). The findings indicate that the smoke is a weak clastogen.

Human Toxicology. Animal studies indicate that acute exposure to red phosphorus smoke will result in chemosensory irritant, inflammatory, and corrosive effects on the respiratory tract, and longer term repeated exposures could result in chronic pulmonary disease. Exposure of human volunteer subjects to concentrations of 100–700 mg m^{-3} red phosphorus smoke caused significant but reversible symptoms of respiratory distress with eye irritation (Uhrmacher et al., 1985). It has been estimated that exposure of humans to about 2000 mg m^{-3} for more than 15 min could cause mortality, that concentrations of 1000 mg m^{-3} are probably intolerable, and that 700 mg m^{-3} is the minimum harassing concentration (Li et al., 1988). Red phosphorus does not produce the severe and penetrating skin and subcutaneous burns that the white phosphorus allotrope is capable of inflicting.

Exposure Guideline Values. (1) EEGL—15 min, 40 mg m^{-3}; 1 h, 10 mg m^{-3}; 6 h, 2 mg m^{-3}; values based on studies indicating that exposure of rats and dogs to 1200 mg m^{-3} for 1 h causes respiratory distress, and applying uncertainty factors of 10 for interspecies differences and conversion of a LOAEL to an NOAEL; NRC, 1997, (2) PEGL—5 mg m^{-3}; 6 h d^{-1}, 5 d $week^{-1}$; based on ACGIH value for phosphoric acid, (3) SPEGL—15 min, 4 mg m^{-3}; 1 h, 1 mg m^{-3}; 6 h, 0.2 mg m^{-3}, (4) PPEGL—0.1 mg m^{-3}; 8 h d^{-1}, 5 d $week^{-1}$.

2. White Phosphorus Smoke

Chemistry and Generation. White phosphorus (WP) is highly flammable and pyrophoric, and it is more reactive than the red phosphorus allotrope. It has an auto-ignition temperature of about 30°C in moist air and 35°C–46°C in dry air (National Safety Council, 1990). On exposure to air, WP ignites spontaneously in an exothermic reaction resulting in yellow flaming and the production of a dense white smoke, the particles of which scatter light and absorb infrared radiation. The smoke contains phosphorus pentoxide (P_4O_{10}) and phosphorus trioxide (P_4O_6), which are hygroscopic and form liquid droplets of polyphosphoric acids, which transform into *o*-phosphoric acid (H_3PO_4), pyrophosphoric acid ($H_4P_2O_7$), and *o*-phosphorus acid (H_3PO_3), hypophosphorus acids (H_3PO_2), polyphosphoric acid of the general formula $H_{n+2}P_nO_{3n+1}$ (where $n = 2–8$), and a homologous series of linear and cyclic P_6–P_{16} polyphosphates (Spanggord et al., 1983; Tolle et al., 1988). The droplets continue to increase in size until they are large enough to result in light scattering. The smoke is usually generated from explosive munitions such as grenades, mortar bombs, artillery shells, and incendiary bombs. Because white phosphorus is pyrophoric most munitions have a simple burst charge to split open the canister and spray fragments of WP through the air, where they ignite spontaneously. In artillery shells, the WP is contained in felt wedges that ignite on exposure to air. Up to 15% of the WP remains within the charred wedge and can reignite if the felt is crushed and the unburned white phosphorus exposed to the atmosphere (RAMC, 2002). It is an effective smoke screening agent with high weight efficiency and develops rapidly. However, one disadvantage is ''pillaring''; in which the conditions of generation are such that cloud particles are hot and as a consequence the screen tends to rise off the ground and form aerial ''pillars'' of smoke.

White phosphorus has been used for the production of a screening smoke for signaling and for screening purposes, and when fired with a fuse time to obtain an airburst for battlefield illumination to aid target location and navigation. Additionally, there are reports of the use of WP for offensive

antipersonnel incendiary purposes (AOL, 2005; BBC, 2005a, b, 2006; Cobb et al., 2005; Popham, 2005; Rayment, 2005), a practice that has raised some concerns (AOL, 2005; BBC, 2005c).

General Toxicology. The acute peroral LD_{50} values for WP given by gavage to rats are 3.03 mg kg^{-1} for females and 3.76 mg kg^{-1} for males, and with mice 4.82 mg kg^{-1} for females and 4.85 mg kg^{-1} for males (Lee et al., 1975). Rats receiving acute cutaneous applications of WP and developing skin burns (26–200 mg kg^{-1} day^{-1}) had necrosis and vascular degeneration of renal proximal convoluted tubules with ischemic changes in the glomerulus, resulting in increased blood urea nitrogen levels, oliguria, decreased creatinine clearance, and renal failure (Ben-Hur et al., 1972; Ben-Hur and Appelbaum, 1973; Appelbaum et al., 1975). Following a single or a few repeated exposures to WP smokes, the lowest lethal concentrations that have been identified are 1742 mg *o*-phosphoric acid equivalents m^{-3} for pregnant rats, 1794 mg *o*-phosphoric acid equivalents m^{-3} for nonpregnant rats, 310 mg phosphorus pentoxide equivalents m^{-3} (428 mg *o*-phosphoric acid equivalents m^{-3}) for mice, 264 mg *o*-phosphoric acid equivalents m^{-3} for guinea pigs, and 6230 mg phosphorus pentoxide equivalents m^{-3} (8599 mg *o*-phosphoric acid equivalents m^{-3}) for goats (cited in ATSDR, 1997a). The respiratory tract is a prime target for WP smoke, causing congestion, edema, hemorrhage, and inflammatory change. WP in water was not genotoxic in a *S. typhimurium* reverse mutation study (TA1535, TA1537, TA1538, TA98, and TA100) with or without metabolic activation (cited in ATSDR, 1997a). An extensive and detailed review of the toxicology of WP can be found in ATSDR (1997a).

Human Toxicology. Men inhaling WP smoke for 2–5 min at a concentration of 187 mg phosphorus pentoxide equivalents m^{-3} had throat irritation and at 514 mg phosphorus pentoxide equivalents m^{-3} they developed cough and nasal irritation (White and Armstrong, 1935). Skin contact with WP particles causes severe skin irritation and chemical burns which may penetrate deep into subcutaneous tissue, the latter being facilitated by its lipophilicity. Eschar is produced surrounded by vesication. Thus, WP can cause second or third-degree burns, and there may be delayed wound healing (Global Security, 2005). In accidental explosions from ignited phosphorus in munitions factories a high mortality rate was reported (12/27). Workers who died had third-degree burns over 30%–90% body surface, and those surviving had burns affecting $\leq$19% body surface (Walker et al., 1947). WP skin burns are a combined consequence of local heat generated by ignition, the corrosive action of phosphoric acid, and the hygroscopic properties of phosphorus pentoxide; they tend to heal more slowly than third-degree burns resulting from other causes (ATSDR, 1997a). Anemia, hemolysis, and leukocytosis have been noted in individuals with WP skin burns (Summerlin et al., 1967). Also, jaundice, hepatomegaly, and elevated bilirubin have been recorded (Song et al., 1985). There may be evidence of renal failure from WP burns, seen as increased blood urea (Summerlin et al., 1967) and proteinuria with increased urea nitrogen (Walker et al., 1947). Management of skin burns from WP includes immersion or flushing with water to extinguish or retard phosphorus ignition, debridement of phosphorus particles, and topical application of bicarbonate solution to neutralize phosphoric acids. Some authorities recommend rinsing with freshly prepared dilute (ca. 1%–2%) copper sulfate, which combines with phosphorus on the surface of particles to form a blue–black cupric phosphide covering which impedes further oxidation and also facilitates the identification of retained particles (Jelenko, 1974; Eldad and Simon, 1991); to avoid potential copper systemic toxicity, the affected area should be lavaged with saline following copper sulfate rinse (Summerlin et al., 1967; Bowen et al., 1971). Contaminated skin wounds should be covered with a saline-soaked dressing and kept moistened to prevent reignition of any retained particles. Absorption of phosphorus from burned surfaces may contribute to mortality following white phosphorus skin burns; this may result in hepatorenal failure, hyperphosphatemia, hypocalcemia, and ECG abnormalities (ST-segment depression, QT elongation, QRS microvoltage, and bradycardia) (Bowen et al., 1971; Eldad and Simon, 1991). Contact of WP particles with the eye results in severe local ocular damage. WP smoke can cause moderate eye injury and irritation of the respiratory tract. Susceptible individuals at risk are those with bronchitis, pneumoconiosis, asthma, pulmonary tuberculosis, and upper respiratory tract disease.

Exposure Guideline Values. (1) A minimal risk level (MRL) of 0.02 mg m^{-3} is suggested for acute exposure (ATSDR, 1997a); although developed for 5 min exposure, it was stated that expansion of the value for 24 h is reasonable. (2) A TWA concentration over 8 h (TWA_8) of 0.1 mg m^{-3} is recommended by ACGIH (2004).

B. Titanium Tetrachloride Smoke

Chemistry and Generation. Titanium tetrachloride (CAS No. 7550-45-0), also known as titanic chloride and has the military code of FM, is a colorless liquid of vapor pressure 10.0 mm Hg (20°C; Whitehead, 1983), that fumes strongly when exposed to moist air, forming a persistent dense white cloud (Lewis, 1993). The exothermic reaction products are oxychlorides and hydrogen chloride (Ballantyne, 1982) and finally titanium hydroxide and hydrochloric acid (Lee et al., 1986). One dissemination mode is from aircraft for the production of smoke curtains extending down to ground/sea level (RAMC, 2002).

General Toxicology. Liquid titanium tetrachloride causes skin irritation and burns (Sanotskii, 1960; Lawson, 1961), and plashes of titanium tetrachloride cause corneal injury (Sanotskii, 1960).

LC_{50} values have been cited as 108,000 mg m^{-3} for 2 min and 460 mg m^{-3} for 4 h (DuPont, 1980). Female rats were exposed to titanium tetrachloride for 10 min at concentrations of 1,466, 5,112, 7,529, and 11,492 mg m^{-3} (Karlsson et al., 1986); signs were nasal discharge, swollen eyelids, and respiratory distress, but there were no moralities. These signs resolved within 48–72 h post-exposure, and lung histology at 7 days showed minor changes of discrete inflammatory residues, thickened alveolar septa, and a sparse accumulation of phagocytes. Ballantyne (1982) exposed rabbits, rats, mice, and guinea pigs to titanium tetrachloride smokes at the following inhalation exposure dosages (CT, in mg min m^{-3}) for the specified exposure times; 14,800–58,800 (over 30 min), 5,000–10,000 (over 10 min), and 400 (over 1 and 10 min). CTs of 5,000 mg min m^{-3} and greater produced the following acute inflammatory effects in the respiratory tract. Congestion, neutrophil infiltration, and focal necrosis of laryngeal and tracheal mucosa; scattered foci of necrotizing bronchiolitis; alveolar capillary congestion with scattered areas of intraalveolar hemorrhage and edema. These effects were most marked in the 24 h following exposure, resolved in the subsequent 7–14 days, and in general were dosage related. No exposure-related respiratory tract histopathology was seen in animals exposed to 400 mg min m^{-3} for 1 or 10 min.

In a 4 week inhalation study, male rats were exposed to titanium tetrachloride at concentrations of 0, 5, 10, or 40 mg m^{-3} for 6 h day^{-1}, 5 days $week^{-1}$ (DuPont, 1979). Two rats died on days 15 and 23. Necropsy revealed tracheal obstruction, acute obliterative bronchiolitis, interstitial pneumonitis, pulmonary edema, and hemorrhage. For a chronic study, rats were exposed to titanium hydrolysis products at aerosol concentrations of 0, 0.1, 1.0, and 10 mg m^{-3} for 6 h day^{-1}, 5 days $week^{-1}$, for 2 years (Lee et al., 1986). There were no abnormal clinical signs, body weight effects, or excess mortality in any group. Only mild rhinitis was seen at 0.1 mg m^{-3}. At 1.0 mg m^{-3}, there was mild rhinitis and tracheitis and the presence of dust-laden macrophages with slight Type II pneumocyte hyperplasia in alveoli adjacent to the alveolar ducts; the response was sufficient to classify the titanium tetrachloride effects as a nuisance dust response. At 10 mg m^{-3}, lung weights were significantly increased compared with the controls (males + 32%, females + 54%; $p < 0.05$). In this group, particle deposition occurred in the tracheobronchial lymph nodes, liver, and spleen without any tissue response. There was an increased incidence of rhinitis, tracheitis, and dust cell response with Type II pneumocyte hyperplasia, alveolar bronchiolization, foamy dust cell accumulation, alveolar proteinosis, cholesterol granuloma, and focal pleurisy were seen. It was believed that the dust cell response, which developed in the alveolar duct region, provoked a chronic tissue response. Free lipids from degenerative foamy macrophages and hyperplasic Type II pneumocytes are probably the main source of cholesterol esters in the granulomas. The absence of a significant fibrotic reaction accords with the fact that titanium tetrachloride did not cause collagen synthesis, based on ^{14}C-proline incubation in vivo in embryonic rat calvaria (Srivastava et al., 1976). A few

well-differentiated, cystic keratinizing squamous carcinomas developed from alveoli showing broncialization with squamous metaplasia in the alveolar duct region, but without metastasis. They were probably a consequence of chronic irritation from dust-laden macrophages and cellular debris. These tumors were described as unique to rats and have not been seen in other animals or man, and therefore their relevance to humans is questionable. It was subsequently determined that some of the tumors should be rediagnosed as squamous metaplasia and others as proliferative keratin cysts (ATSDR, 1997b).

A CHO forward gene mutation study (HGPRT locus) and Rec assay with *Bacillus subtilis* did not give any evidence for mutagenicity (Hsie et al., 1979; Kada et al., 1980; Kanematsu et al., 1980). Titanium tetrachloride was not mutagenic in a *S. typhimurium* assay using strains TA1537, TA2637, TA98, and TA102 (Ogawa et al., 1987).

Human Toxicology. Liquid titanium tetrachloride causes severe irritation and burns of the skin in humans (Lawson, 1961). Cases of acute exposure to the liquid should not be initially treated with a water rinse to avoid hydrolysis products (HSDB, 1995). Wiping the skin with towels or cotton gauze is regarded as the best first action to minimize effects of exposure, after which cool water may be used to completely decontaminate the skin. Splash contamination of the eye, depending on the degree of exposure varies from transient minor corneal epithelial injuries to a combination of hypopyon, elevated intraocular pressure, entropion and symblepharon, corneal injury with vascularization, uveitis, lens opacities, and corneal perforation (Chitkara and McNeela, 1992; Grant and Schuman, 1993). As with the initial management of skin contamination, it is suggested that dry wiping of the eye area should be followed by water washing (HSDB, 1995). Respiratory tract symptoms of mild overexposure include cough, chest tightness, and breathlessness; these irritant symptoms usually resolve quickly and do not leave abnormalities on chest radiography (Ross, 1985). More severe inhalation exposures may cause difficulty with breathing, dyspnea, hypoxia, retrosternal pains, pulmonary congestion and edema, and respiratory distress syndrome. An epidemiological study in an industrial setting found no association between titanium tetrachloride exposure and lung cancer mortality for 969 male workers occupationally exposed to concentrations ranging from <0.5 to >3.0 mg m^{-3} for <5 years; odds ratio 1.1 (Fayerweather et al., 1992). Also, there was no association with chronic respiratory disease, and no cases of pulmonary fibrosis were observed. Other epidemiological studies, however, suggested that long-term exposure to titanium tetrachloride might cause pulmonary impairment. Analysis of the findings with 209 occupationally exposed workers suggested that pulmonary impairment may have resulted. Chest radiographic information indicated that pleural thickening was strongly related to the duration of work, and initial estimates of loss of pulmonary function, taking smoking into account, was 45 mL $year^{-1}$ leading to a deficit 1.8 L per 40 year employment (NIOSH, 1980). Further analysis based on job analysis and duration of employment also showed decreases in FVC in workers employed in titanium tetrachloride for at least 10 years (Garabrant et al., 1987). Regression analysis of the data adjusted for age, height, and smoking revealed the rate of loss of FVC was 24 mL per year. The results suggest that chronic exposure to titanium tetrachloride may result in restrictive pulmonary changes. Susceptible individuals are those with bronchitis, pneumoconiosis, asthma, pulmonary tuberculosis, and upper respiratory tract disease (Mezentseva et al., 1963).

Exposure Guideline Values. (1) Inhalation MRLs that have been developed are 0.01 mg m^{-3} for intermediate exposure (up to 364 days) and 0.0001 mg m^{-3} for chronic inhalation exposures for 365+ days (ATSDR, 1997b). (2) A PEGL has not been formally established. However, the inhalation studies would indicate a PEGL of 0.1 mg m^{-3} as appropriate.

C. Zinc–Hexachloroethane Smoke

Chemistry and Generation. This smoke, sometimes referred to as HC smoke or Zn/HCE smoke, is produced by burning a mixture of equal parts of hexachloroethane (HCE, a chlorine donor) and zinc oxide with approximately 6% granular aluminium. The mixture is usually ignited by a pyrotechnic

starter mixture resulting in a self-perpetuating exothermic reaction, and the generation of a dense white smoke containing $ZnCl_2$, zinc oxychlorides, carbon dioxide, carbon monoxide, hydrogen chloride, tetrachloroethylene, and traces of phosgene (RAMC, 2002). $ZnCl_2$ leaves the reaction zone as a hot vapor, and on cooling below the condensation point it nucleates to form an aerosol that is hygroscopic. Hydrated $ZnCl_2$ particles then scatter light and obscure vision. In view of the affinity of $ZnCl_2$ for water, the aerosol probably consists of the hydrated forms of $ZnCl_2$ under most atmospheric conditions (Katz et al., 1980). Some formulations may contain a small amount of (ca. 10%) calcium silicide and liberate silicon on combustion (Cullumbine, 1957). Increasing the proportion of calcium silicide raises the reaction temperature and the burning rate (Jarvis and Wart, 1971). Results from a study of Zn/HCE smoke characteristics during a military exercise gave MMAD measurements of the particles in the range 0.4–2.8 μm, and thus respirable (Young et al., 1989). In extensive field studies, DeVaull et al. (1989) found MMADs ranged from 0.77 to 1.05 μm (gsd 1.78–2.35). Simulated combat training during a military operation indicated that trainees were exposed to $ZnCl_2$ in concentrations ranging 0.02–0.98 mg m^{-3} during a 225 min period (Young, 1992). Zn/HCE smoke may be generated by grenades, candles, smoke pots, cartridges, and air-delivered bombs (RAMC, 2002). Zn/HCE smoke has also been used in fire-fighting exercises (ATSDR, 1994).

General Toxicology. The major toxicity of Zn/HCE smoke is often attributed to $ZnCl_2$, which is a potent irritant. Dogs exposed to $ZnCl_2$ smoke had radiological changes suggestive of pulmonary edema (Ardran, 1950). The $L(CT)_{50}$ for mice was stated as 11,800 mg min m^{-3}, but guinea pigs exposed to the smoke died rapidly from bronchospasm (Cullumbine, 1957). Marrs et al. (1983) exposed rats and rabbits acutely (30 min) to $ZnCl_2$ smoke generated from pyrotechnic mixtures containing HCE and zinc oxide, with observations for up to 14 days postexposure. For two differing exposures, the concentrations in the inhalation chamber were 0.60 and 0.81 g m^{-3} (expressed as Zn). Rabbits dying within 24 h of exposure showed histological evidence of acute inflammation and necrosis of the larynx and trachea together with alveolitis; those exposed to the higher concentration also had pulmonary edema. Survivors showed less marked pulmonary effects and no laryngotracheal necrosis. Rats dying within 24 h postexposure showed pulmonary edema, congestion, and petechiae. Rats surviving till sacrifice had mild-to-moderate laryngotracheal inflammatory change and alveolitis. The upper respiratory tract necrosis was suggested by the authors to be possibly caused by a direct cytotoxic effect of $ZnCl_2$, based on the observation that $ZnCl_2$ is highly toxic to mammalian cells (lymphocytes; toxic at 0.003 mol L^{-1}; Deknudt and Deminatti, 1978). Acute intratracheal instillation studies in Wistar rats were conducted by Richards et al. (1989) to assess the relative contributions of zinc oxide and $ZnCl_2$ in the pathogenesis of pulmonary histopathology produced by Zn/HCE smoke. $ZnCl_2$ produced edema, determined by histology and measurement of lavage fluid alveolar surface protein, with some evidence for a fibrogenic response. In contrast, zinc oxide did not produce an edematous reaction. The acute inhalation toxicity of Zn/HCE smoke (ZnO–HCE) has been compared with that of TiO_2–HCE smoke (Karlsson et al., 1991). ZnO–HCE smoke was significantly more acutely toxic than TiO_2–HCE smoke and caused mortalities with deaths due to pulmonary edema. Karlsson et al. (1991) also compared the acute inhalation toxicity of $TiCl_4$ vapor with that of $ZnCl_2$ aerosol. They found no mortalities with $TiCl_4$ up to 2900 mg m^{-3} with a 10 min exposure, compared with a 10 min LC_{50} of 2000 mg m^{-3} for $ZnCl_2$. A repeated inhalation exposure study of zinc oxide–HCE smoke was conducted by Marrs et al. (1988a) in which mice, rats, and guinea pigs were exposed to concentrations (as Zn) of 0, 1.3, 12.8, and 122 mg m^{-3}. Exposures were for 1 h day^{-1}, 5 days $week^{-1}$, for 20 weeks. At 122 mg m^{-3}, there was excess mortality with mice and guinea pigs, and respiratory tract histopathology that included pulmonary edema, emphysema, macrophage infiltration, and (in mice only) an increase in the incidence of alveogenic carcinomas. The incidence of alveogenic carcinoma was as follows: 0 mg m^{-3} (control), 6/78; 1.3 mg m^{-3}, 7/74; 12.8 mg m^{-3}, 8/76; 122 mg m^{-3}, 15/50. Exposures to 1.3 and 12.8 mg m^{-3} showed normal survival rates and no adverse pulmonary histopathology. Thus, under the conditions

of this study, the NOAEL was 12.8 mg Zn m^{-3} (equivalent to 26.5 mg m^{-3} as $ZnCl_2$). Percutaneous toxicity from $ZnCl_2$ is unlikely to occur because of the slow rate of absorption of $ZnCl_2$ across the skin (Wahlberg, 1965), and skin irritation is probably mild.

Using apparatus to permit exposure to zinc oxide–HCE smoke, the mutagenic potential was studied in a *S. typhimurium* reverse mutation assay. There was weak mutagenic activity with TA1535 and TA1537 without metabolic activation (S9 liver homogenate) and in TA100 in the presence of S9 (Clode et al., 1991). An in vivo femoral bone marrow micronucleus test was conducted by exposing CD-1 mice to concentrations of 0, 22, 77, and 117 μg L^{-1} zinc oxide–HCE smoke for 1 h. The maximum tolerated dose of 117 μg L^{-1} was estimated as 50% of the 1 h LC_{50} to smoke-exposed mice (273 μg L^{-1}). Marrow was examined for counting of polychromatophilic erythrocytes (PCEs) and normochromic erythrocytes (NCEs) at 24, 48, and 72 h postexposure. Absence of toxicity was indicated by no alteration in NCE and PCE counts or PCE/NCE ratios compared with controls (Clode et al., 1991). This was followed by confirmatory in vivo and in vitro studies for unscheduled DNA synthesis (UDS; Anderson et al., 1996). Male F-344 rats were exposed to zinc oxide–HCE smoke for 1 h and hepatocytes were isolated for UDS measurement at 16 h postexposure. In vitro, rat hepatocytes were incubated with medium exposed to zinc oxide–HCE smoke. Neither the in vitro or in vivo studies showed any evidence for UDS.

The mouse study of Marrs et al. (1988a) indicated that Zn/HCE smoke causes alveolar carcinomas in mice. A generalized multistage linear dose–response model fitted to the data gave an upper limit of cancer risk of 0.086 mg $ZnCl_2$ kg^{-1} day^{-1}. NRC (1997) derived a cancer potency factor for Zn/HCE smoke and determined that the possible cancer risk associated with the EEGLs and PEGL was approximately 1 in 10^{-6}.

Human Toxicology. The lower limit for detection of Zn/HCE smoke is cited as 40 mg m^{-3} (Schenker, 1981). A threshold inhalation dosage for adverse effects (nausea with irritation of the nose, throat, and chest) is cited as 160–240 mg min m^{-3} (NRC, 1997). Cullumbine (1957) noted that 2 min after exposure to 120 mg m^{-3}, there was minor irritation of the nose, throat, and chest with cough, and after 2 min at 80 mg m^{-3}, there was nausea. Symptoms resulting from overexposure to HC smoke include breathlessness, dyspnea, retrosternal pain, cough, chest constriction, occasional hemoptysis, lacrimation, and blepharospasm (Allen et al., 1992; RAMC, 2002). Pulmonary congestion and edema may be present. Chronic effects following exposure to high smoke concentrations include focal atelectasis, bronchiolar–alveolar hyperplasia, and pulmonary fibrosis (RAMC, 2002). Hsu et al. (2005) investigated the possible correlation between high-resolution CT scan and pulmonary function tests in a retrospective study of 20 patients who had been hospitalized following $ZnCl_2$ smoke inhalation injury. The major CT scan findings were patchy or diffuse opacities with and without consolidation. The majority of patients showed a significant reduction in FVC, FEV_1, total lung capacity, and pulmonary CO diffusing capacity, but normal FEV_1/FVC ratio values. Changes in pulmonary function tests correlated well with CT scan scores. Thus the majority of patients with $ZnCl_2$ smoke inhalation injury presented with predominant pulmonary parenchymal injury that was consistent with a restrictive type of functional impairment and a reduction in CO diffusing capacity rather than with obstructive disease. The investigators concluded that high-resolution CT scanning and pulmonary function testing may reliably predict the severity of $ZnCl_2$ smoke inhalation injury. Deaths from inhalation of high concentrations of $ZnCl_2$ smoke in confined spaces have been reported; death is due to respiratory tract injury, including necrotizing tracheitis, bronchiolitis obliterans, bronchopneumonia, and pulmonary congestion, edema, hemorrhage, and necrosis (Evans, 1945; Johnson and Stonehill, 1961; Macaulay and Mant, 1964; Fischer, 1974; Hjortso et al., 1988). Systemic toxicity, notably hepatotoxicity, may on occasion occur with overexposure to Zn/HCE smoke. For example, Loh et al. (2006) described two men who had chemical pneumonitis and respiratory distress after inhalation of Zn/HCE smoke during military training. Hepatic injury was based on increased blood alanine aminotransferase (ALT) and γ-glutamyl transpeptidase (γ-GT) activities.

Exposure Guideline Values. (1) EEGL: 15 min, 10 mg m^{-3}; 1 h, 3 mg m^{-3}; 6 h, 0.4 mg m^{-3} (expressed as $ZnCl_2$). This was based on a CT threshold value of 160 mg min m^{-3} for nausea and respiratory tract irritation in humans. (2) PEGL: 0.2 mg m^{-3}; 8 h d^{-1}, 5 d $week^{-1}$. This value was based on a rodent study giving a NOAEL of 26.6 mg m^{-3} for $ZnCl_2$; uncertainty factors of 10 were applied for interspecies variations and the short (1 h) animal exposure conditions (NRC, 1997). ACGIH (2004) proposed a TWA_8 of 1 mg m^{-3} with a STEL of 2 mg m^{-3} for $ZnCl_2$ fumes. (3) SPEGL: 15 min, 1 mg m^{-3}; 1 h, 0.3 mg m^{-3}; 6 h, 0.04 mg m^{-3}. (4) PPEGL: 0.02 mg m^{-3}; 8 h d^{-1}, 5 d $week^{-1}$ (NRC, 1997).

D. Diesel-Fuel Smoke

Generation. Smoke from diesel fuel is generated by injecting the fuel into the exhaust manifold of a vehicle that results in vaporization of the fuel which subsequently condenses in the atmosphere to produce particles, 0.5–1.0 μm diameter, which form a dense white obscuring smoke to conceal personnel and equipment; such systems are referred to as Vehicle Engine Exhaust Smoke Systems (VEESS). Diesel fuels are classified as in the middle distillates from crude oil and consist of hydrocarbons with carbon numbers in the range C_9–C_{20}, and boiling in the range 163°C–357°C (IARC, 1989). This definition covers both diesel fuels No. 1 (DF1) and No. 2 (DF2). DF1 is basically kerosene and has hydrocarbons mainly in the range C_9–C_{16} with boiling range 150°C–300°C. DF2 is essentially similar to DF1 and boils in the range 160°C–360°C, but also includes olefins and mixed aromatic olefin-type compounds.

Toxicity. A proportion of the particles in the diesel-fuel smoke may be respirable. The material deposits and accumulates in the lung, being retained long enough to induce an inflammatory response. Dodecachlorodiphenyl has been used as a dosimetric tracer for aerosols of diesel fuel (Dalbey and Lock, 1982; Dalbey et al., 1982, 1987; Jenkins et al., 1983). The fraction of inhaled diesel-fuel aerosol retained by rats postexposure was 4%–8%, with the largest amount being found in lungs. Acute and repeated inhalation exposures of rats and mice to diesel-fuel smoke results in inflammation in the respiratory tract with pulmonary congestion, edema, and hemorrhage (NRC, 1997). Calahan et al. (1982, 1986) investigated the acute inhalation toxicity of DF1 and DF2 smokes in rats, guinea pigs, and mice that were exposed for various times in the range 15–300 min. The average concentration of DF2 smoke for 15 and 60 min exposures was 35,000 mg m^{-3}, and that for DF1 smoke was 15,000 mg m^{-3} for exposures from 15 to 300 min; MMAD of smoke particles was $\leq$0.23 μm. After the 15 min exposure to DF2, rats and guinea pigs had decreased mobility. The 1 h exposure caused lacrimation, increased oral and nasal secretions, nasal hemorrhages, and tremors in guinea pigs; rats demonstrated cyanosis, and mice had piloerection. With the 60 min exposure, 1/10 rats and 4/10 guinea pigs died. Mortality with DF1 smoke increased with increasing concentration, and for the 300 min exposure all but four mice died. Histopathological lesions found in the respiratory tract were congestion in nasal turbinates, bronchitis, peribronchiolitis, peribronchiolar lymphocyte infiltration, pulmonary histiocytosis, bronchopneumonia, and pulmonary congestion, edema, and hemorrhage. Dalbey and Lock (1982) exposed rats to diesel fluid aerosols at concentrations ranging 670–16,000 mg m^{-3} for 2, 4, or 6 h; MMAD was 0.43–0.75 μm (gsd 1.4–1.5). Mortality was correlated with inhalation exposure dosage (CT), and probit analysis indicated that 1% mortality would be at 12, 200 mg min m^{-3}.

Subchronic repeated exposure studies were also conducted by Callahan et al. (1986) who exposed mice and rats to DF2 smoke at a mean concentration of 2300 mg m^{-3} for 15 or 60 min each day for 5 days $week^{-1}$ for up to 13 weeks. The only clinical sign was hypoactivity. Mild-to-moderate pulmonary congestion was seen compared with unexposed controls, with minimal inflammatory changes in the nasal turbinates and trachea. Lock et al. (1984) conducted a 13 week study in which male and female rats were exposed to diesel-fuel smoke for 4 h, twice a week for 13 weeks. Exposure concentrations were measured as 170, 870, and 1600 mg m^{-3}. There were no signs and no mortality. Body weight decreased until the fourth exposure week, after which there were body weight gains.

Breathing rates and CO diffusing capacity were not affected, but residual volume was significantly reduced with the highest exposure concentration group. Functional residual capacity, vital capacity, and peak flow rate were not affected. No microscopic pathology was seen.

A developmental toxicity study was carried out by Starke et al. (1987) in which pregnant female rats were exposed to DF2 smoke (2340 mg m^{-3}) over gestational days 6–15 for daily periods of 15 min, and sacrificed on gestational day 20. There were no effects that could be attributed to DF2 smoke alone. A dominant-lethal study was conducted in male rats in which they were exposed to DF2 smoke for 15 or 60 min daily, 5 days $week^{-1}$ for 10 weeks (Starke et al., 1987). There were no indications of dominant-lethal mutations. Diesel fuel was not mutagenic, with and without metabolic activation, in *S. typhimurium* and mouse lymphoma assays. Intraperitoneal dosing of rats with diesel fuel was clastogenic to bone marrow cells, inducing a 1% increase in chromosomal abnormalities at 2 mL kg^{-1} (but not 0.6 mL kg^{-1}) (Conway et al., 1982). DF2 concentrates were not mutagenic in *Drosophila melanogaster* (Calahan et al., 1986). DF2 was a promoter in a SENCAR mouse skin tumorigenesis assay, but did not show activity as a complete carcinogen in the same test (Slaga et al., 1983).

Exposure Guideline Values. The following values were developed by the U.S. National Research Council (NRC) using NRC guidelines developed for military personnel:

1. EEGL—15 min, 300 mg m^{-3}; 1 h, 80 mg m^{-3}; 6 h, 15 mg m^{-3}. These values were derived from a 1% lethal CT in rats of 8200 mg h m^{-3}. These figures were divided by an uncertainty factor of 10 for nonpermanent health impairment, and a further uncertainty factor of 10 for interspecies variations. Assuming the applicability of Haber's law to the resultant CT of 80 mg h m^{-3}, the cited exposure guideline concentrations were calculated for the defined periods.
2. PEGL—10 mg m^{-3}, 8 h d^{-1}, 2 d $week^{-1}$; 5 mg m^{-3}, 8 h d^{-1}, 1 d $week^{-1}$, as C values. These values were based on a LOAEL (focal pneumonitis) of 8000 mg h m^{-3}, which was divided by an uncertainty factor of 10 to estimate a NOAEL and a further uncertainty factor of 10 for interspecies variation and the resultant PEGL CT of 80 mg m^{-3} then converted to permissible exposure concentrations for exposures of 8 h day^{-1} for 1 or 2 days $week^{-1}$.
3. SPEGL—15 min, 30 mg m^{-3}; 1 h, 8 mg m^{-3}; 6 h, 1.5 mg m^{-3}.
4. PPEGL—1 mg m^{-3}, 8 h day^{-1}, 1 day $week^{-1}$; 0.5 mg m^{-3}, 8 h day^{-1}, 2 days $week^{-1}$.

E. Fog-Oil Smoke

Generation. Fog-oil smoke is generated by injecting mineral oil into a heated manifold, and the vapor so produced condenses in the atmosphere into a cloud of suspended obscuring particles. The MMAD of oil-smoke particles generally ranges 0.5–3.0 μm (Policastro and Dunn, 1985; Liljegren et al., 1988; Cataldo et al., 1989; Young et al., 1989). Before 1986, smoke was generated from oil that contained polycyclic aromatic hydrocarbons (PAHs) and related heterocyclic compounds. Following the conclusion by IARC (1984) that untreated naphthenic oils are carcinogenic, the U.S. military specification for fog oil was changed to exclude all carcinogenic or potentially carcinogenic components. This is achieved by solvent refining or extraction, or by hydrotreatment that converts the materials into less toxic saturated compounds (NRC, 1997).

Toxicity. Mineral oils produce slight-to-moderate irritation by standard rabbit skin irritation tests (Beck et al., 1982; Mayhew et al., 1985). Repeated applications of mineral oils to skin over a week resulted in epidermal hyperplasia, hyperkeratosis, and depilation; C_{14}–C_{19} hydrocarbons caused more damage than C_{21}–C_{23} hydrocarbons (Hoekstra and Phillips, 1963).

As with diesel-fuel smokes, a proportion of generated particulates are respirable, and the most sensitive toxic end point following acute or repeated exposures is respiratory tract toxicity. An acute exposure of rats for 3.5 h–1000 mg m^{-3} fog oil did not cause mortalities, but a 6 h exposure to this concentration produced 20% mortality. The 35 h LC_{50} was determined to be 5200 mg m^{-3} (Grose et al., 1986; Selgrade et al., 1987). Repeated exposure of mice to 4500 mg m^{-3} mineral-oil aerosol

caused localized foreign body reactions and lipoid pneumonia (Shoshkes et al., 1950). For a repeated exposure study, male Sprague-Dawley rats were exposed to concentrations of 0.5 and 1.5 mg L^{-1} fog-oil aerosol for 3.5 h day^{-1}, 4 days $week^{-1}$ for 4 or 13 weeks; average droplet size was 1.0–1.3 μm MMAD ± 1.5 μm gsd (Grose et al., 1985). After the 4 week exposure period to 0.5 mg L^{-1}, there was minor increase in lung weights and alveolar macrophages. At 1.5 mg L^{-1}, a multifocal pneumonitis was seen. Lung lavage had an increased number of polymorphonuclear leukocytes, alveolar macrophages, total cells, and increase in lavage fluid proteins. Pulmonary function tests revealed an increase in end expiratory volume. There were minimal systemic effects. Hematology showed a dose-related decrease in mean corpuscular volume and mean corpuscular hemoglobin that correlated with increased erythrocyte count. Behavioral studies, clinical chemistry, and immune function studies showed no significant effects. The subchronic (13 week) exposure study showed a decrease in body weight with increased lung weights at 0.5 and 1.5 mg L^{-1}. There was increased lavage fluid protein and end expiratory volume at 1.5 mg L^{-1}. There were no effects on clinical chemistry or immune function monitors.

Mineral oils, including the older preparations of fog oil, can produce cutaneous malignant neoplasms because of the presence of PAHs. In view of this, the military (army) altered the specification for screening smoke fog oil, requiring that it be solvent-treated or hydrotreated to remove potential carcinogenic hydrocarbons and analyzed to confirm the absence of such materials (NRC, 1997).

Exposure Guideline Values. The following exposure guideline values were developed similarly to those for diesel-fuel smoke (see above), using a 2 h LOAEL CT of 4500 mg m^{-3} in mice:

1. EEGL: 15 min, 360 mg m^{-3}; 1 h, 90 mg m^{-3}; 6 h, 15 mg m^{-3}.
2. PEGL: 5 mg m^{-3}, 8 h d^{-1}, 5 d $week^{-1}$.
3. SPEGL: 15 min, 36 mg m^{-3}; 1 h, 9 mg m^{-3}; 6 h, 1.5 mg m^{-3}.
4. PPEGL: 0.5 mg m^{-3}, 8 h d^{-1}, 5 d $week^{-1}$.

F. Dye-Based Smokes

Chemistry and Generation. Colored smokes are used for identification purposes (friendly units or targets), signaling, coordination purposes during operations, and simulation of explosions. Such smokes are generated by volatilizing and subsequently condensing a pyrotechnic mixture containing an organic dye. A variety of dyes, singly or in combination, are used or have been suggested for use in screening smokes (Table 18.3). Some examples of these are reviewed below with respect to the major factors relevant to use in screening smokes.

1-Methylaminoanthraquinone. Known commonly as Disperse Red 9, this dye comprises up to 40% of red smoke grenades. It is also used in a violet smoke grenade when mixed with 1,4-diamino-2,3-dihydroanthraquinone and as a screening smoke mixed with Solvent Yellow 33 and Solvent Green 3.

TABLE 18.3
Dyes Used or Proposed for Use in Colored Screening Smokes

Chemical Name	Common Name
1-Methylaminoanthraquinone	Disperse Red 9
Dibenzo[b,def]chrysene-7,14-dione	Vat Yellow 4
7H-Benz[de]anthracene7-one	Benzanthrone
1,4-di-*p*-toluidinoanthraquinone	Solvent Green 3
1,4-Diamino-2,3-dihydroanthraquinone	—

Disperse Red 9 can cause skin irritation and sensitization. The acute inhalation toxicity of red smoke generated pyrotechnically from the following composition was studied: Disperse Red 9, 40%; sodium bicarbonate, 25%; potassium chlorate, 26%; sulfur, 9% (Owens and Ward, 1974). Using monkeys, dogs, goat, swine, rabbit, rat, and guinea pigs exposed to smoke concentrations in the range 2,753–17,946 mg m^{-3} for exposure times ranging 10–240 min the combined species $L(CT)_{50}$ was 647,470 mg min m^{-3} (95% confidence limits 568,611–737,265). The majority of animals (80%–90%) died within 24 h of exposure. After exposure animals demonstrated signs of nasal irritation, salivation, gagging, and respiratory difficulty. The $L(CT)_{50}$ value of 647,470 mg min m^{-3} indicates a lethal inhalation toxicity about 10 times less than that for CS (Owens and Ward, 1974). An in vivo screen for carcinogenicity, based on the sensitivity of the mouse mammary gland to certain carcinogens, was conducted by Griswold et al. (1968) and showed no evidence that the material is carcinogenic.

A repeated exposure inhalation study to pyrotechnically generated smoke from a dye mixture of Disperse Red 9, Solvent Yellow 33, and Solvent Green 3 was conducted by Marrs et al. (1984). Exposures of mice, rats, and guinea pigs were to mean concentrations of 105.8, 309.6, and 1012.4 mg m^{-3} for 1 h day^{-1} for a total planned of 100 exposures. Excess mortality was seen with high-concentration exposure in mice and guinea pigs. Lungs of the high-dose mice and rats were heavier than unexposed controls. Rats had marked collections of macrophages, and this species also showed adenocarcinomas of the breast and biliary hyperplasia. Mice showed increased alveolar macrophages and fatty change was noted in the livers.

Dibenzo[b,def]chrysene-7,14-dione. This is a yellow dye (Vat Yellow 4), often combined with 7-H-benz[de]anthracene-7-one for a yellow smoke, or with benzanthrone and Solvent Green 3 to give a green smoke. No tumors were reported in several studies in which dibenzo[b,def]chrysene-7,14-dione was given by subcutaneous injection and by cutaneous dosing to mice (Hartwell, 1957).

7H-Benz[de]anthracene-7-one. This dye, also known as benzanthrone, is often combined with dibenzo[b,def]chrysene-7,14-dione to produce a yellow dye smoke, or with Solvent Green 3 and Vat Yellow 4 for a green screening smoke. It is not irritating to the skin of guinea pigs (Owens and Ward, 1974), but may cause irritant dermatitis and skin pigmentation in humans (Uebelin and Buess, 1951; Trivedi and Niyogi, 1968). Benzanthrone did not show dominant-lethal activity in a mouse test (Epstein et al., 1972). Benzanthrone is not carcinogenic by skin painting or subcutaneous injection (Hartwell, 1957; Owens and Ward, 1974).

Concentrations of benzanthrone (in mg L^{-1}) in reservoir water have been noted to result in the following effects (Owens and Ward, 1974); 0.05, noticeable yellow color; $\leq$5.0, Daphnia activity normal; 20–50, noticeable taste; 50, noticeable odor; 50–100, inhibition of biological oxygen demand (BOD) but not $>$18%.

1,4-di-p-Toluidinoanthraquinone. Also known as Solvent Green 3, it is used in screening smokes with Solvent Yellow 33 and with benzanthrone/Vat Yellow 4. Studies have indicated that inhaled Solvent Green 3 has a long residence time in the lung. Thus, in a study by Sun et al. (1987) a $t_{1/2}$ of 277 days was calculated for male rats and 289 days for female rats.

2-(2′-Quinolyl)-1,3-indandione. Known also as Solvent Yellow 33, this dye, sometimes in combination with Solvent Green 3, is used in colored screening smokes. It has been shown to produce allergic contact dermatitis in both laboratory animals and humans (Larsen, 1974; Calnan, 1976; Noster and Hausen, 1978). Solvent Yellow 33 was mutagenic in *S. typhimurium* strain TA15,537R+ with and without metabolic activation (Marrs et al., 1988b). The repeated inhalation potential toxicity of Solvent Yellow 3 was investigated in rats exposed to an aerosol of the material for 6 h day^{-1}, 5 days $week^{-1}$ for 4 or 13 weeks. Monitors for adverse effects included body weights, respiratory function, biochemical estimation of lung injury, and histology (Sun et al., 1987). For the 4 week study, the average exposure concentrations were 10, 51, and 230 mg m^{-3}. The MMAD for these exposures were measured as 3.2, 3.5, and 4.4 μm. At the end of the exposure period, the body weights of the 230 mg m^{-3} group were 8% lower than unexposed controls. Little dye was retained in the lungs,

but that present was related to the exposure concentration. After exposure lung compliance, functional residual capacity, and forced vital capacity were significantly increased for the high exposure concentration group, indicating loss of elastic recoil without airflow obstruction. Analysis of BAL fluid did not show any changes to indicate cytotoxicity or pulmonary inflammation, and total lung tissue collagen and BAL fluid hydroxyproline were not altered. There were no biologically significant changes in serum biochemistry and no abnormal histology. The mean exposure concentrations for the 13 week study were 1.0, 10.8, and 100 mg m^{-3}, with MMADs of 2.1, 2.9, and 4.0 μm. At the end of the 13 week exposure period, the high-concentration group had body weights 5% lower than unexposed controls. Little dye was recovered from the lungs, but that detected did not increase linearly with increasing aerosol concentration, probably due to lower deposition efficiency for the larger sized particles. There were no physiologically significant effects on respiratory function, no abnormal findings in BAL fluid, and lung hydroxyproline was unchanged. At the highest concentration, 100 mg m^{-3}, histologically there was minimal focal accumulations of vacuolated alveolar macrophages, minimal hypertrophy of type II pulmonary epithelial cells, and deposition of pigment in the nasal epithelium. The macrophages accumulated in alveoli adjacent to terminal bronchioles and alveolar ducts, and the pigment was present as irregular masses ranging from <1 to >15 μm diameter. Overall, the findings indicate that a 4 week exposure to 230 mg m^{-3} resulted in a toxic response where there was decreased lung elastic recoil and increased resting lung volume, consistent with some breakdown of pulmonary connective tissue leading to more compliant lungs. However, similar effects were not seen in the 13 week study where the highest exposure concentration was 100 mg m^{-3}. At this concentration, there were effects on body weights (reduction) and accumulation of vacuolated alveolar macrophages. Thus, in the subchronic study, the NOAEL was 11 mg m^{-3}. Sun et al. (1987) also investigated the repeated exposure inhalation toxicity over 4 and 13 weeks of a combined dye mix of Solvent Yellow 33 and Solvent Green 3 (30:70 w/w). The respective mean exposure concentrations (and MMAD) were 11 (3.2), 49 (3.7), and 210 mg m^{-3} (4.9 μm) for 4 weeks and 1.1 (2.8), 10.2 (3.0), and 101 mg m^{-3} (4.2 μm) for 13 weeks. Compared to unexposed controls, body weight was 7% lower for 4 weeks and 9% less after 13 weeks. Little Solvent Yellow 33 was retained in the lungs after 4 and 13 weeks, but a significantly larger proportion of Solvent Green 3 was still present in the lungs. In view of the higher lung retention of Solvent Green 3, the clearance of the dye was determined for the 13 week high-concentration exposure group. It was calculated that the $t_{1/2}$ values for Solvent Green 3 clearance from the lungs of male and female rats were 277 ± 67 and 289 ± 40 days, respectively. Respiratory function studies for the 4 week high-concentration study showed a reduced residual volume, slightly lower CO diffusing capacity, and decreased forced expiratory flow rates, indicating impaired gas exchange and airflow obstruction. No physiologically significant effects were noted for the 13 week study. Analysis of BAL fluid from the 4 week study indicated an inflammatory response. There was a dose-related increase in neutrophils, macrophages, and total proteins in the mid- and high-concentration exposure groups; the high-concentration exposure group had increased cytoplasmic enzymes (lactate dehydrogenase, glutathione reductase, and glutathione peroxidase) and the lysosomal enzyme β-D-glucuronidase. These findings indicate a pulmonary inflammatory response in the 210 mg m^{-3} exposure group and a lesser response in the 49 mg m^{-3} group. For the 13 week exposure, there was an inflammatory response at 101 mg m^{-3}, as indicated by BAL fluid analysis. No change in lung collagen or BAL fluid hydroxyproline was seen in the 4 week study, but in the 13 week study BAL fluid collagen was doubled after exposure to 101 mg m^{-3}. Histology of lungs from the 4 week 210 mg m^{-3} rats revealed a mild reaction around the terminal airways: there was slight proliferation of vacuolated alveolar macrophages and slight hyperplasia of Type II pulmonary epithelial cells. Rats exposed for 13 weeks to 101 mg^{-3} had slight-to-moderate accumulations of vacuolated alveolar macrophages with slight-to-moderate hyperplasia of Type II cells. In the 13 week study, effects were seen at 101 mg m^{-3} (body weight and pulmonary inflammatory response) and 10 mg m^{-3} (pulmonary histological lesions), and therefore the NOAEL for this subchronic inhalation study with combined exposure to Solvent Green 3 and Solvent Yellow 33 was 1 mg m^{-3}.

G. Cinnamic Acid Smoke

Chemistry and Generation. Cinnamic acid (CAS No. 621-82-9) has chemical synonyms of trans-β-phenylacrylic acid and 3-phenyl-propenoic acid ($C_6H_5{\cdot}CH = CH{\cdot}COOH$). Cinnamic acid smoke is generated pyrotechnically from grenades and has a particular potential for use as an obscuring smoke with a reasonable degree of obscuration and low toxicity that can be used as a simulant in firefighting training. A typical pyrotechnic composition is lactose 26%, potassium chlorate 26%, aluminium silicate 15%, and cinnamic acid 33%.

General Toxicity. There were no mortalities, signs of toxicity, or histopathology (larynx, trachea, liver, kidney) in rats and guinea pigs exposed acutely to pyrotechnically generated cinnamic smoke at concentrations of 920 mg m^{-3} for 30 min or 1630 mg m^{-3} for 45 min; a few animals had minimal pulmonary alveolar capillary congestion (Ballantyne and Clifford, 1978). Also, repeated 1 h exposures to 660 mg m^{-3} cinnamic acid smoke for 5 consecutive days (cumulative CT 198,000 mg min m^{-3}) did not result in mortality, signs, or histopathology (larynx, trachea, lung, liver, and kidney). Mice, rats, and guinea pigs exposed to cinnamic acid smoke for 1 h day^{-1} for 5 days $week^{-1}$ for a total of 40 weeks showed only minimal and nonspecific histological changes in the respiratory tract. Renal lesions were seen, but these were specific to mice and not present in a clearly graded manner (Marrs et al., 1989b).

Human Toxicology. Male volunteer subjects acutely exposed to 70 mg m^{-3} cinnamic acid for 10 min had only transient minimal irritant symptoms (slight excess lacrimation, mild rhinorrhea, and bouts of nonproductive coughing) with minor increases in breathing rate, tidal volume, and minute volume, which returned to control values within 5–10 min of the end of exposure; peak flow rate, forced vital capacity, and forced expiratory volume were not significantly altered. Pre- and post-exposure chest radiographs were normal (Ballantyne and Clifford, 1978).

The acute inhalation studies conducted in animals at concentrations of 920 and 1630 mg m^{-3} were, respectively, 12 and 22 times higher than the 70 mg m^{-3} concentration that is likely to be used in practical situations (Ballantyne and Clifford, 1978). The inhalation exposure dosage for the 45 min to 1,630 mg m^{-3} concentration was 73,350 mg min m^{-3}, and thus if exposure to a 70 mg m^{-3} concentration of cinnamic acid is restricted to 10 min, the inhalation exposure dosage (700 mg min m^{-3}) is about 1% that is shown not to produce toxicity in the acute rodent studies.

VII. OVEREXPOSURE TO SCREENING SMOKES AND ITS MEDICAL MANAGEMENT

The likelihood for the development of symptoms following inhalation exposure and the nature and severity of respiratory tract injury depends on a number of factors, which include the chemical nature of the smoke, concentration and toxic potency of inhaled materials, particle size and vapor proportion, duration of exposure, water solubility, respiratory minute volume, and personal characteristics (e.g., differential susceptibility, exertion). During training and operational use, exercise will result in an increased respiratory minute volume (effect of tachypnea and increased tidal volume) and thus a greater inhalation exposure dose. Most of the more soluble inhaled material will tend to predominantly affect the upper airways, and the less soluble materials affect mainly the peripheral airways and alveoli.

Symptomatic overexposure to irritant screening smokes such as phosphorus, titanium tetrachloride, and Zn/HCE usually presents with effects on the respiratory tract. There may be chest discomfort, difficulty in breathing, dyspnea, retrosternal pain/discomfort, cough, and nausea. Bronchoconstriction may be a consequence of bronchospasm or bronchiolitis. However, exposure to such smokes in a confined space, particularly if airflow is restricted, may lead to more severe respiratory inflammation and damage, including necrotizing bronchiolitis and pulmonary edema, with the possible development of cyanosis and hemoptysis. Arterial hypoxemia can result from obliterating bronchiolitis and by disturbed gas exchange due to alveolar and interstitial edema.

Arterial hypoxemia can also be provoked by alveolar collapse as a result of reduced production of surfactant by alveolar Type II cells (Meulenbelt, 2004). High-concentration exposures in confined spaces with limited airflow may be fatal with some irritant smokes; for example, Zn/HCE, phosphorus, titanium tetrachloride. Some overexposed subjects may develop acute respiratory distress syndrome (ARDS) due to damage to the alveolar epithelium and vascular endothelium resulting in increased permeability to plasma and inflammatory cells into the interstitium and alveolar space. Damage to Type II pneumocytes and the presence of protein-rich fluid in the alveoli disrupts the production and functioning of pulmonary surfactant leading to microatelectasis and impaired gas exchange. ARDS has been defined as the acute onset of bilateral infiltrates on chest radiography, a PaO_2/fraction of inspired oxygen (FIO_2) ratio <200 mm Hg, and pulmonary occlusion artery pressure <18 mm Hg. Some patients with ARDS have an uncomplicated recovery, but others may progress to fibrosing alveolitis leading to poor lung compliance. Subjects with pre-existing pulmonary disease, such as chronic bronchitis or asthma, may be at greater susceptibility, particularly for the production of bronchospasm and increased mucus.

Ocular effects of overexposure to irritant smokes may include discomfort in the eyes, excess lacrimation, blepharospasm, and conjunctoblepharitis. With the exception of titanium tetrachloride, where water contact can lead to chemical burns, contaminated eyes should be copiously washed with water. Those exposed to titanium tetrachloride should have dry wiping of eye and skin initially. Management of inhalation overexposure to irritant smokes, and depending on the clinical status of the patient, may necessitate the use of corticosteroids (aerosol inhalation and systemic therapy), oxygen, and prophylactic antibiotic and antimycotic cover. The appearance of pulmonary fibrosis may indicate the need for D-penicillamine (Ministry of Defence, 1972). In view of the latency to development of symptomatic lung injury with some inhaled chemicals, all exposed subjects should be kept under observation for a period of time even in the absence of normal objective evaluation of the patient. Some have suggested a postexposure period of 6 h may be sufficient (Meulenbelt, 2004); however, we consider a period of 24 h more appropriate for a postexposure observation period, and particularly with those individuals having increased susceptibility due to pre-existing disease. Exposure to some smokes may, on occasion, result in the development of systemic toxicity; for example, hepatotoxicity with HC/ZnO (Loh et al., 2006).

Investigation of those overexposed to screening smokes should include, at least, chest radiograph, pulmonary function tests, arterial oxygen tension measurement, blood clinical chemistry, sputum culture, ophthalmic examination with slit-lamp biomicroscopy, and possibly measurement of intraocular pressure. If available, CT scan may be used to assess the severity of lung injury (Hsu et al., 2005). With some smokes, notably white phosphorus, there may be skin contamination with severe irritation and penetrating burns; the management of white phosphorus skin burns is discussed in detail in Section VI.A.2.

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19 Clinical Detection of Exposure to Chemical Warfare Agents

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CONTENTS

I. INTRODUCTION

Past attacks with chemical warfare agents (CWAs) on military and civilian personnel underscore the need to rapidly identify exposed and nonexposed personnel. Identifying the exposed facilitates appropriate treatment, whereas identifying the nonexposed avoids unnecessary psychological stress on those who are worried and avoids burdening the medical system. In addition to medical issues, the political and legal ramifications of CWA use by rogue nations or terrorist organizations can be enormous. Accurate, sensitive, and rapid analytical techniques enable the appropriate medical, political and military actions.

Monitoring for the presence of CWAs in humans generally involves assays of biomedical samples. Chemical warfare agent verification assays rarely target the intact agent due to its limited longevity in vivo. In living species, the body attempts to rapidly rid a foreign substance by metabolizing it to a more water-soluble state, in which it is then quickly excreted in the urine. These urinary metabolites serve as markers of CWA exposure. However, the opportunity to identify them is limited by urine clearance time course (usually requiring timely urine collection within a few days). In the late 1980s, the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) was tasked by the Department of Defense (DoD) to develop methodologies that could confirm a potential exposure to CWA. Afterward, a number of procedures for the verification of exposures to nerve agents and sulfur mustard using urinary markers were published. These methods primarily focused on gas chromatographic–mass spectrometric (GC–MS) analysis of hydrolysis products excreted in the urine following exposure. Subsequently, the methods were compiled together as part of Technical Bulletin Medical 296 (TB Med 296), entitled "Assay Techniques for Detection of Exposure to Sulfur Mustard, Cholinesterase Inhibitors, Sarin, Soman,

The opinions or assertions contained herein are the private views of the authors and are not to be construed as reflecting the view of the Department of the Army or the Department of Defense.

GF, and Cyanide'' (Department of the US Army, 1996; TB MED 296). The publication was intended to provide the clinician with laboratory tests to detect exposure to chemical warfare agents in urine or blood samples. More long-lived markers tend to be those that result from the agent interacting with large molecular weight targets, such as proteins and DNA. In these cases, the agent covalently binds to macromolecules, such as albumin, to form macromolecular adducts. As such, the protein acts as a depot for the adducted agent, and the residence time can be in terms of days to weeks.

Typical matrices for biomedical samples used for CWA verification include blood, plasma, urine, and tissues. However, any sample derived from the exposed individual may be considered as a potential matrix to include blister fluid, tears, and saliva. The collection of urine is considered to be noninvasive and does not require highly trained medical personnel or specialized equipment. From an analytical standpoint, urine is more complex than aqueous solutions, but less problematic than blood, plasma, or tissues. Although biomarkers present in urine are usually short-lived metabolites (hours to days), they can be present in relatively high concentrations if the sample is obtained shortly after exposure.

By comparison, collection of blood/plasma is more invasive and should be performed by trained medical personnel. These samples offer potential benefits in that both unbounded metabolites and the more long-lived macromolecular adducts can be assayed. However, blood is more difficult to prepare for analysis.

Tissue sample collection is obviously the most invasive and usually limited to a deceased casualty. Consequently, tissue collection is not normally used for diagnosis to medically respond but for forensic analysis, such as the formalin-fixed brain tissues from the Tokyo subway attack victims, to verify GB as the agent employed (Matsuda et al., 1998).

A variety of analytical methods have been developed to analyze biomedical samples. The ultimate objective would be to develop analytical methods that are simple to use, lightweight, field portable, accurate, sensitive, precise, inexpensive, and comprehensive for all CWAs. However, in general, the more sensitive the methods need to be, the more labor- and instrument-intensive the method becomes. The transition of analytical techniques developed in the laboratory to a field (incident) for forward setting has the potential to generate valuable information for the military or civilian clinician. The transition also has the potential for a vast array of problems, but the application of advanced digital telecommunication technologies could play a significant role in reducing or correcting these problems while in the field. These include, but are not limited to, data analysis, interpretation of complex spectra, and instrument troubleshooting and repair. As analytical methods are developed and refined and sent farther from the laboratory setting for which they were originally designed, the need for advanced telecommunication to provide a direct link between research scientists and field operators could become critical in the confirmation of patient exposure and for tracking patient recovery and treatment.

This chapter on medical diagnostics provides a basic outline and references for the state-of-the-art analytical methods presently available in the literature. First, we describe the collection, handling, storage, and shipping of biological samples for CWA analysis, as well as sample submissions requiring a chain-of-custody. Then we discuss specific agents: nerve agents, vesicants, cyanide, phosgene, and 3-quinuclidinyl benzilate.

II. COLLECTION, SHIPMENT, AND STORAGE OF BIOMEDICAL SPECIMENS

General. The following section describes the field collection, shipment, and storage of biomedical samples according to the guidance from the Centers for Disease Control and the United States Army Medical Research Institute of Chemical Defense. In most instances, blood (serum, plasma) and urine are commonly collected samples. Samples to be collected are dependent upon the

specific methodology required by the assay. The information provided in this section is intended to provide general guidelines for sample collection, shipment, and storage and is not intended to be comprehensive. In all cases, questions regarding specific procedures to obtain and ship samples should be addressed directly to the receiving laboratory by phone or by consulting the respective website before collecting the sample. However, extenuating circumstances may not allow for prior laboratory coordination. In such cases, follow the general guidance below.

Urine. The collection of urine samples should be done under the close supervision of a health care provider or an unbiased observer to preclude the possibility of sample tampering. Care should be taken to ensure appropriate handling so as to minimize chances for contamination from the environment or handling personnel. The urine should be collected immediately following suspected exposure or at the earliest possible time. A midstream urine collection is desirable. If follow-up is anticipated, additional samples should be obtained at 24 h. Urine should be collected in clean urine cups or screw-capped plastic containers that can withstand freezing temperatures without splitting. A minimum of 25–30 mL should be collected. Urine samples should be frozen immediately (−70°C or dry ice preferred).

Whole Blood. It is recommended that the samples be cautiously handled from the start of the collection to maintain integrity and preclude the possibility of contamination, tampering, or mislabeling. All samples should be collected under the close supervision of a health care provider/physician, and if possible, witnessed by an unbiased observer. Samples should be obtained as soon as possible following the suspected exposure. Additional follow-up samples may be obtained. Blood should be collected in 5 or 7 mL blood tubes. Specific methods may require specific types of tubes, such as purple-top (EDTA) tubes for plasma, or the red-top tubes for serum and vacuum-fill only (unopened).

For methods that analyze serum or plasma, it is useful to process whole blood samples by centrifugation, followed by separation of the plasma/serum from the red blood cell (RBC) pellet. The plasma or serum components can then be frozen (−70°C or dry ice preferred) and stored or shipped on dry ice. As on-site blood processing may not be convenient, whole blood samples that are not processed should be stored immediately at 4°C. Packed RBCs may be stored at 4°C or frozen depending on the properties of the analyte and the needs of the analytical method to be performed.

Labeling. Label specimens in accordance with the standing operating procedures of the receiving laboratory to ensure forensic integrity. The labels should be as comprehensive as possible and double-checked for accuracy. Label samples with facility of origin and clear markings that will be resistant to water and refrigeration/freezing temperature conditions. Labels should also include patient identification information (name or other specific identifiers), date and time of collection, specimen identity, and some identification of the collector. A list of samples with corresponding names of individuals should be maintained at the facility of origin and should also be included with the samples if they are shipped. Wrap each sample top with waterproof, tamper-evident, forensic evidence tape, being careful not to cover the sample identification labels.

Packaging. For blood, separate each tube from others or wrap individually to prevent direct contact. Tubes should be placed in secondary packages such as a divided box wrapped with absorbent material and sealed inside a plastic bag, other sealable containers, or individually wrapped tubes sealed inside a plastic bag. Place absorbent material between the primary receptacle and the secondary packaging. Use enough absorbent material to absorb the entire contents of primary receptacles. To facilitate processing and identification, package blood tubes so that similar tubes are packaged together (e.g., all purple-tops together) and not mixed (i.e., purple-tops and green/gray-tops in the same package). For urine, wrap frozen cups with absorbent material and place them into sealable secondary packaging, such as described for blood. Do not ship frozen urine and blood in the same package; they should be shipped in separate containers.

Shipping Container. The shipping container should be a sealable Styrofoam or other insulated container capable of maintaining the contents at the preferred temperature for the specimens. Place additional absorbent material in the bottom of the outer containers for cushioning. For samples that require refrigeration conditions, such as whole blood, add a layer of frozen cold packs, and place the secondary containers on top of the cold packs. Place additional cold packs or absorbent material between the secondary containers to reduce movement within the container. Lastly, place a layer of frozen cold packs on top of the secondary containers. For shipment of frozen samples (plasma, serum, urine), add a layer of dry ice on top of the cushioning material in the bottom of the shipping container. Do not use large chunks of dry ice for shipment because they have the potential for shattering items during transport. Place additional absorbent material between wrapped urine cups to reduce their movement within the outer container. Finally, add an additional layer of dry ice.

Documentation (Shipping Manifest, Incident Report, Chain-of-Custody). Prepare separate documentation for each container shipped; blood and urine should be shipped separately as previously described. Prepare and place a shipping manifest designating sample identification numbers, number of samples, and type of samples in a zippered, plastic bag on top of the specimens before closing and sealing the container. Maintain a copy of the manifest at the point of origin. Enclose an incident report form with as much information as possible with respect to description of incident, date/time of suspected exposure, onset and description of symptoms, sample collection time, and suspected agent involved. Include patient information, such as name, social security number, age, and gender, as well as a point of contact. The incident report form should be stored in a zippered plastic bag on top of the specimens before closing and sealing the container. A chain-of-custody form must also be included. Prepare a separate chain-of-custody form for samples in each container. Indicate sample identity, any pertinent descriptors, and number of samples. Place the completed chain-of-custody forms in a plastic, zippered bag on the outside of the shipping container.

Shipping. Close and secure outer container with filamentous shipping/strapping tape. Affix labels and markings. Place a label on the outer container that indicates the proper name, ''Diagnostic Specimens.'' A class 9 label should be placed on the outer container of all containers with dry ice. This label must indicate the amount of dry ice in the container, the address of the shipper, and the address of the recipient. This label must be placed on the same side of the container as the ''Diagnostic Specimens'' label.

Storage. Upon arrival, the receiving laboratory should maintain a proper chain-of-custody. If the samples are not processed immediately upon arrival, they should be stored as soon as possible. Storage of samples either before or after they are shipped should be in accordance with conditions dictated by the sample type. Blood should be stored at 4°C. Plasma or serum should be stored frozen at −70°C. RBCs can be stored by refrigerating at 4°C or frozen at −70°C; freezing is preferred for long-term storage. Avoid repeated freeze/thaw or refrigerated/room temperature cycles for samples.

III. NERVE AGENTS

Numerous assays for determining nerve age exposure have been developed and reported in the literature. Most of the assays are based upon determination of either levels of nerve agent metabolites or activity of cholinesterase (ChE) enzymes. Nerve agents inhibit ChE by forming a covalent bond between the phosphorus atom of the agent and the serine residue of the enzyme active site. For the agents GB, GD, and GF, that interaction results in the displacement or loss of fluorine from agents. Binding of GA and VX is different in that the leaving group is cyanide and the thiol group, respectively (Figure 19.1) (Degenhardt et al., 2004). Spontaneous reactivation of the enzyme or hydrolysis reactions with water can occur to produce corresponding alkyl methylphosphonic acids. Alternatively, the loss of the *O*-alkyl group while bound to the enzyme produces a highly stable organophosphoryl–ChE bond, a process referred to as aging. Once aging has occurred, the enzyme is considered to be resistant to reactivation by oximes or other nucleophilic reagents (Sidell, 1997).

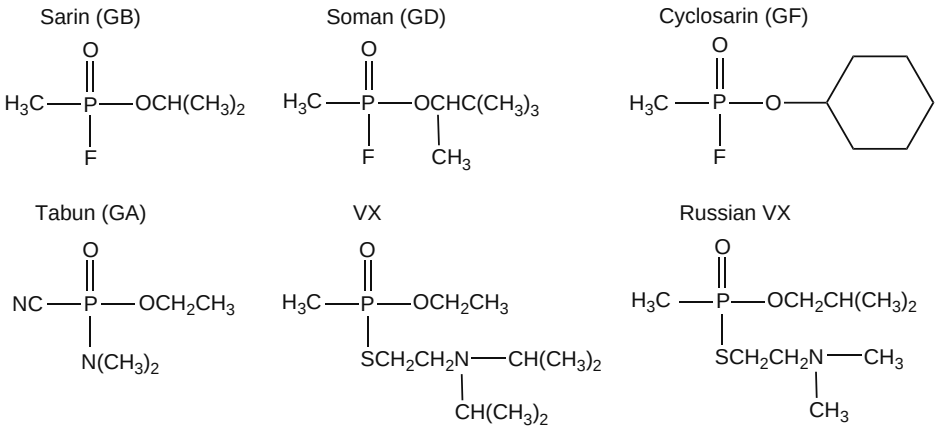

FIGURE 19.1 Chemical structures of nerve agents: the nerve agents sarin (GB), soman (GD), and cyclosarin (GF) lose fluorine subsequent to binding to cholinesterase. The agents tabun (GA), VX, and Russian VX lose CN, and the thiol groups, respectively.

The spontaneous reactivation and aging rates of the agents vary depending on the *O*-alkyl group. For example, VX-inhibited RBC–ChE reactivates at an approximate rate of 0.5%–1% h^{-1} for the first 48 h with minimal aging. On the other hand, GD-inhibited ChE does not spontaneously reactivate and has a very rapid aging rate with a half-time of approximately 2 min (Sidell, 1997).

General Clinical Tests. With the exception of ChE analysis, there are no standard or routine clinical assays that specifically test for nerve agent exposure. However, over the years numerous laboratory-based non-ChE analytical methods have been developed and several successfully used to verify exposure to nerve agents. For the most part, these employ MS detection techniques with GC or liquid chromatographic (LC) separations. The tests are relatively labor intensive requiring trained personnel and sophisticated instrumentation not common to clinical settings. In terms of application of the techniques, most experience has come from animal exposure models. These assessments allow for the determination of sensitivity and longevity of the biomarker in the particular experimental model. There is some experience in humans with accidental exposure or terror-related events. This chapter will review assays that have been published in the open literature for CWA exposure and how they have been applied in potential exposure situations.

Assay of Parent Compounds. Analysis of the parent nerve agents from biomedical matrices such as blood or urine is generally not a viable option for a diagnostic technique or retrospective detection of exposure (Noort et al., 2002). The parent agents are relatively short-lived due to rapid hydrolysis, and their rapid binding to plasma/tissue proteins imposes unrealistic time restraints on sample collection. The short residence time is especially profound with the G-agents relative to VX. Results from toxicokinetic studies of GB and GD support the above contention (Benschop and De Jong, 1991, 2001). The administration of $2 \times LD_{50}$ intravenous (i.v.) soman indicated that the parent agent can be detected at toxicologically relevant levels for 104 and 49 min in guinea pigs and marmosets, respectively, following exposure; rapid elimination was reflected in terminal half-life rates (16.5 min, guinea pigs; 9 min, marmosets) (Benschop and De Jong, 1991). Inhalation experiments using nose only exposure of guinea pigs to ($0.8 \times LCt_{50}$) demonstrate terminal half-life of approximately 36 and 9 min for GB and GD, respectively (Benschop and De Jong, 2001). In contrast, similar studies with VX in hairless guinea pigs (HGP) and marmosets indicate that it is more persistent than the G-agents (van der Schans et al., 2003). These authors indicate that VX could be found at acutely toxic levels for 10–20 h following the i.v. administration

(1, 2 × LD_{50}) with terminal elimination rates of 98 min (1 × LD_{50}, HGP), 165 min (2 × LD_{50}), and 111 min in marmosets (at a dose equivalent to 1 × LD_{50} in HGP). Percutaneous administration of 1 × LD_{50} VX to HGP demonstrated relatively low blood levels (140 pg/mL), which reached a maximum after approximately 6 h (van der Schans et al., 2003). Because the route of exposure for VX would most likely be percutaneous, the timeframe of 6 h may be the more relevant assessment of its persistence in blood and as such, offers only limited ability for detection in terms of time for sample collection and concentration. Others have demonstrated that VX can be assayed from spiked rat plasma (Bonierbale et al., 1996). These authors noted that 53% of the VX had disappeared from spiked plasma after 2 h. The disappearance was attributed to organophosphorus hydrolase cleavage of the S–P bond to form diisopropyl aminoethanethiol and ethyl methylphosphonic acid (EMPA).

Analytical Methods for Hydrolysis Compounds. An alternative approach to the direct assay of the parent nerve agents is to look for metabolic or hydrolysis products that may appear in biomedical samples. The compounds are produced in vivo as a result of hydrolysis with water or split off following spontaneous regeneration of the acetylcholinesterase (AChE) enzyme. Studies with radiolabeled parent nerve agents (^{32}P or ^{3}H) in animals suggest that they are rapidly metabolized/hydrolyzed in the blood and appear in the urine as their respective alkyl methylphosphonic acids (Harris et al., 1964; Polak and Cohen, 1970; Reynolds et al., 1985; Little et al., 1986). This observation led to the development of the initial assay for alkyl methylphosphonic acids in biological samples (Shih et al., 1991), the applicability of which was subsequently demonstrated in animals exposed to nerve agents (Shih et al., 1994). The products and the respective parent agents from which they are derived are presented in Figure 19.2. The common products found are isopropyl methylphosphonic acid (IMPA), pinacolyl methylphosphonic acid (PMPA), cyclohexyl methylphosphonic acid (CMPA), and ethyl methylphosphonic acid (EMPA) derived from GB, GD, GF, and VX, respectively. Additionally, for VX, hydrolysis of the S–P bond occurs to yield diisopropyl aminoethanethiol (DAET) and EMPA. The formation and assay of DAET has been reported in rat plasma spiked with VX (Bonierbale et al., 1996). Over time, numerous variations on the assay of alkyl methylphosphonic acids in biological fluids, such as plasma, urine, and saliva, have been developed. These include GC separations with MS (Fredriksson et al., 1995; Tsuchihashi et al., 1998; Miki et al., 1999), tandem MS (MS–MS) (Fredriksson et al., 1995; Tsuchihashi et al., 1998; Driskell et al., 2002; Barr et al., 2004) or flame photometric detection (FPD) (Minami et al., 1997; Nakajima et al., 1998).

Other methods involving LC with MS–MS (Noort et al., 1998) and indirect photometric detection (Katagi et al., 1997) have also been reported. Various methods on the assay of alkyl methylphosphonic acids in biological fluids are presented in Table 19.1.

Application to Human Exposures. The utility of some methodologies has been demonstrated in actual human exposure incidents. Most involve the assay of urine and plasma/serum. Tsuchihashi et al. (1998) demonstrated the presence of EMPA in the serum of an individual assassinated with VX in Osaka, Japan in 1994. As mentioned above, these authors also reported the presence of DAEMS, which results from the in vivo methylation of DAET subsequent to cleavage of the P–S bond. Reported concentrations in serum that was collected 1 h after exposure were 143 ng/mL DAEMS and 1.25 μg/mL for EMPA. The terrorist attacks by the Aum Shinrikyo cult in Japan used GB on two separate occasions. The first was in an apartment complex in Matsumoto City where approximately 12 L of GB were released using a heater and fan. According to police reports, 600 inhabitants in the surrounding area were harmed and seven died. In the second attack, GB was released into the Tokyo subway resulting in greater than 5000 casualties and 10 deaths (Suzuki et al., 1995). Numerous reports using the assay of hydrolysis products as a definitive marker have been employed to verify GB as the agent employed in these events. Minami et al. (1997) and Nakajima et al. (1998) demonstrated the presence of IMPA or MPA in the urine following GB exposure in the Tokyo and Matsumoto attacks, respectively. These methods used GC separ-

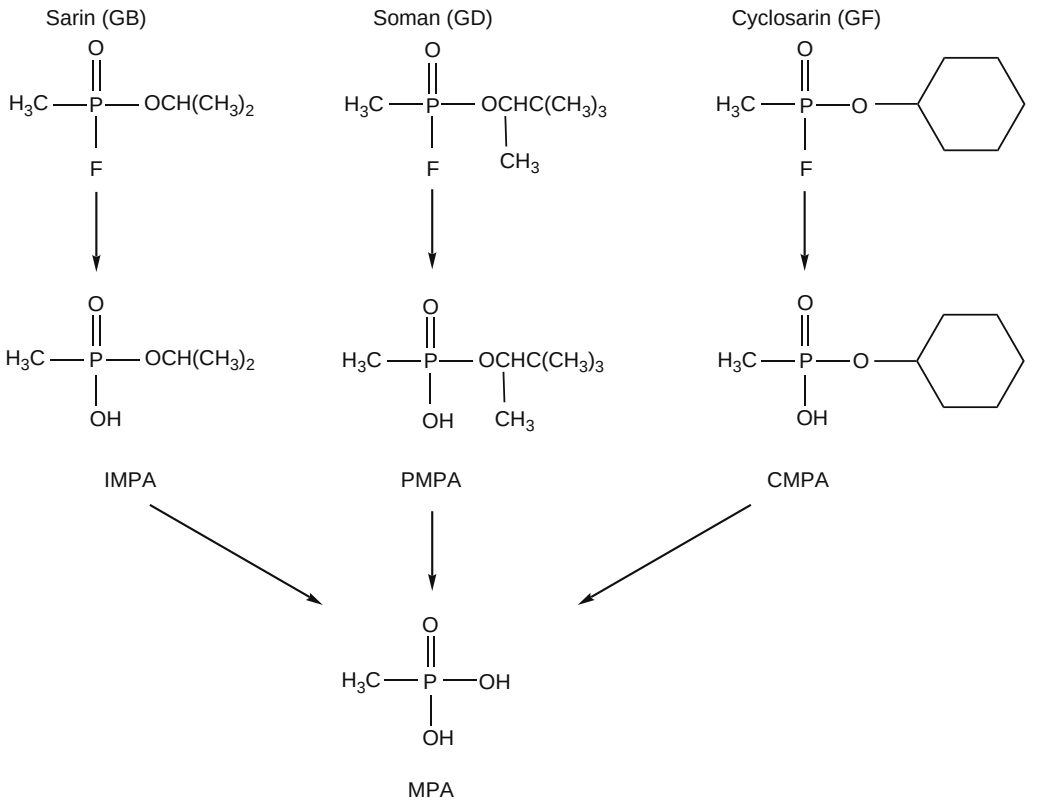

FIGURE 19.2 Hydrolysis pathway of sarin (GB), soman (GD), and cyclosarin (GF): hydrolysis pathway of nerve agents proceeds through the alkyl methylphosphonic acids, isopropyl methylphosphonic acid (IMPA), pinacolyl methylphosphonic acid (PMPA), and cyclohexyl methylphosphonic acid (CMPA) to methylphosphonic acid (MPA). Analysis of the alkyl methylphosphonic acids allows identification of the parent agent, while assay of MPA is nonspecific.

ations of the prepared urine matrix coupled with FPD detection. In the Matsumoto incident, urinary concentrations of IMPA and MPA, as well as the total dose of the sarin exposure, were reported. Methyl phosphonic acid concentrations were 0.14 and 0.02 μg/mL on the first and third days after exposure, and 0.76, 0.08, and 0.01 μg/mL for IMPA on the first, third, and seventh days, respectively, after exposure (Nakajima et al., 1998). The individual was estimated to have been exposed to 2.79 mg of sarin. Although the report on the Tokyo (Minami et al., 1997) incident did not directly indicate urinary concentrations, calculations were made on estimates of total sarin exposure. Estimates on the two individuals involved were 0.13–0.25 mg/person in an individual described as comatose and 0.016–0.032 mg/person in the less severely exposed casualty (Minami et al., 1997). These numbers are approximately 10-fold less that that reported by Nakajima et al. (1998) for the severely intoxicated patient. Consistent with the rapid elimination of these compounds, the maximum urine concentration was reported to be within 12 h of exposure. Noort et al. (1998) and Polhuijs et al. (1999), using LC–MS–MS methods, detected IMPA in serum of individuals poisoned in both the Tokyo and Matsumoto incidents. This assay involves fairly sophisticated instrumentation but allows a simplified sample work-up procedure. Reported serum concentrations ranged from 2–127 ng/mL and 2–135 ng/mL in the Tokyo and Matsumoto incidents, respectively. Samples were obtained 1.5 h after the incident. In some patients, a second

TABLE 19.1
Analytical Methods for Assay of Nerve Agent Hydrolysis Products

Sample Matrix	Product Identified	Analytical Method	References
Blood, plasma, urine, lung	IMPA, CMPA, PMPA	GC–MS	Shih et al. (1991, 1994)
Serum, urine	EMPA, IMPA, PMPA	GC–MS, GC–MS–MS	Fredriksson et al. (1995)
Plasma	Diisopropyl aminoethanethiol	GC–MS	Bonierbale et al. (1996)
Urine	EMPA, IMPA, MPA	GC–FPD	Minami et al. (1997)
Urine	IMPA, MPA	GC–FPD	Nakajima et al. (1998)
Serum	EMPA, diisopropyl aminoethyl methyl sulfide	GC–MS, GC–MS–MS	Tsuchihashi et al. (1998)
Serum, urine, saliva	EMPA, IMPA, PMPA	GC–MS	Miki et al. (1999)
Urine	EMPA, IMPA, CMPA, PMPA, GA acid	GC–MS–MS	Driskell et al. (2002)
Urine	IMPA	LC–MS–MS	Noort et al. (1998), Polhuijs et al. (1999)
Serum	EMPA, IMPA, MPA PMPA	Indirect photometric detection ion chromatography	Katagi et al. (1997)
Urine, saliva	EMPA, IMPA, CMPA, MPA, PMPA	LC–MS–MS	Hayes et al. (2004)
Urine	EMPA, RVX acid, IMPA, PMPA, CMPA, GA acid, GA diacid	GC–MS–MS	Barr et al. (2004)

DAET, diisopropyl aminoethanethiol; DAEMS, diisopropyl aminoethyl methyl sulfide—resulting from the metabolic methylation of diisopropyl aminoethanethiol.

sample was obtained at 2–2.5 h after the incident; in those samples, the authors reported significantly lower IMPA concentrations consistent with the rapid elimination of these compounds (Noort et al., 1998). Calculation of sarin dose was reported to be 0.2–15 mg/person (Noort et al., 1998). These reported values for sarin exposure are in the range of those reported by Nakajima et al. (1998) and are approximately 10-fold greater than that reported by Minami et al. (1997).

The alkyl methylphosphonic acids provide a convenient marker for determining exposure to nerve agents. Numerous modifications for the assay of these compounds have been developed for blood or urine, and several have been applied to actual human exposure cases. Important factors in considering this test are extent of exposure and time following the event. One of the most severely poisoned victims of the Matsumoto sarin attack demonstrated measurable IMPA in the urine on the seventh day. To put the severity of this case in perspective, AChE values were in the range of 5%–8% of normal (Nakajima et al., 1998). However, in most cases, hydrolysis products should not be considered to be present for more than 24–48 h following exposure. The methods used to verify human exposure to nerve agents based on assay of hydrolysis products are presented in Table 19.2.

Assay of Adducts to Biomolecules. From the standpoint of exposure verification, the relatively rapid excretion and short-lived presence of urinary hydrolysis products imposes time restrictions for collecting a viable sample. It follows that methods which expand the window of opportunity for detection extend utility by allowing more time for sample collection. Efforts to increase the sampling window have taken advantage of interactions of CWAs with large molecular weight targets (adducts to biomolecules), such as proteins. The reaction of CWAs with large molecules provides a pool of the bound compound, which can be used to verify exposure. Theoretically, the longevity of the marker is consistent with the in vivo half-life of the target molecule, provided that the binding affinity is high enough that spontaneous reactivation does not occur. Binding

TABLE 19.2
Methods Used to Confirm Human Exposures to Nerve Agents Using Assay of Hydrolysis Products

Agent/Incident	Sample Matrix	Product Identified	Concentration Reported	Analytical Method	References
GB, Tokyo, Japan	Urine	EMPA, IMPA, MPA	NR	GC–FPD	Minami et al. (1997)
GB, Matsumoto, Japan	Urine	IMPA	0.76–0.01 μg/mL	GC–FPD	Nakajima et al. (1998)
		MPA	0.14–0.02 μg/mL		
GB, Matsumoto and Tokyo, Japan	Serum	IMPA	Matsumoto (2–135 ng/mL) Tokyo (2–127 ng/mL)	LC–MS–MS	Noort et al. (1998), Polhuijs et al. (1999)
VX, Osaka, Japan	Serum	EMPA, diisopropyl aminoethyl methyl sulfide	1.25 μg/mL, 143 ng/mL	GC–MS, GC–MS–MS	Tsuchihashi et al. (1998)

NR, not reported.

of nerve agents to ChE targets has been one of the primary interactions that can be leveraged to develop assays with an increased window of opportunity for detecting exposure. Several assays have been developed based on variations of this concept.

Analytical Methods. Polhuijs et al. (1997) developed an assay technique based on observations of earlier findings that ChE inhibited by GB could be reactivated with fluoride ions (Heilbronn, 1964, 1965; Albanus et al., 1965). The displacement of GB covalently bound to butyrylcholinesterase (BChE) was accomplished by incubation of inhibited plasma with fluoride to form free enzyme plus the parent agent (isopropyl methylphosphonofluoridate). Following isolation from the matrix with solid-phase extraction techniques, the agent was then analyzed using GC with MS or other appropriate detection systems. Other research has demonstrated conceptually similar approaches for GA (Polhuijs et al., 1999) and VX (Jakubowski et al., 2001). In the case of GA, the cyanide group, which is initially lost upon binding to the enzyme, is replaced with fluorine, leading to formation of *O*-ethyl *N*,*N*-dimethyl-phosphoramidofluoridate or a fluorinated analog of GA (Polhuijs et al., 1999). Similarly with VX, the thiol group, which is initially lost upon binding to the enzyme, is replaced by fluorine, resulting in a fluorinated analog of VX (ethyl methyl-phosphonofluoridate; VX-G) (Jakubowski et al., 2001). Variations and improvements of the fluoride regeneration procedure have evolved to enhance sensitivity by optimizing the agent extraction, increasing injection volumes (thermal desorption and large volume injector), and use of alternate detection formats (FPD, positive ion chemical ionization, high resolution electron impact MS) (Jakubowski et al., 2001; Degenhardt et al., 2004). Additionally, Jakubowski et al. (2004) have successfully applied the procedure to RBCs.

With regard to the ability of fluoride ion to regenerate GD bound to BChE, it is well known that the process of aging would preclude release from the enzyme. However, studies have indicated that the fluoride ion regeneration process as applied to GD poisoned animals has produced successful results (Adams et al., 2004). These studies suggest that soman can be displaced from sites where aging does not play a significant role. Black et al. (1999) have demonstrated that both GB and GD bind to tyrosine residues of human serum albumin. The observation that the alkyl group remained intact, in particular for GD, strongly argues that binding to this site does not result in aging, as seen with ChEs (Black et al., 1999). Similarly, carboxylesterase (CaE), known to exist in high quantities

in rats and mice, has been shown to form adducts with soman (Clement, 1984; De Jong and Van Dijk, 1984; Clement et al., 1987; Maxwell et al., 1987; Maxwell and Brecht, 2001). Moreover, the formation of soman has been demonstrated via fluoride-induced regeneration of soman-inhibited CaE in rat plasma (De Jong and Van Dijk, 1984) and purified human albumin (Adams et al., 2004). Although the presence of CaE in significant amounts is questionable in humans, the albumin provides a potential source of protein from which the agent can be regenerated. Currently the utility of the fluoride regeneration procedure in human exposures to GD is notclear. The implication from some of the above studies is that binding to other sites may offer potential for using this procedure. More studies are needed to clarify the utility of fluoride regeneration in humans with GD following confirmed exposure events.

Nagao et al. (1997a, 1997b) developed a different approach exploiting GB bound to AChE using blood as the matrix. This procedure detected IMPA, following its release from the GB–AChE complex, using an alkaline phosphatase digestion process. The analytical technique employed for the analysis is similar to numerous other GC–MS methods used to assay for hydrolysis products (see above).

Another approach based on OP binding to BChE has been reported by Fidder et al., 2002. This particular methodology involves digestion of BChE to produce nonapeptide fragments containing the serine-198 residue to which nerve agents are known to bind. Analysis of nonapeptides employs LC–MS–MS techniques. The utility of the method was demonstrated by analyzing two archived samples from the Tokyo subway terrorist attack. The authors reported similar results to previous analysis of those samples using the fluoride regeneration procedure. Reported advantages of this technique are that aged or nonaged OPs can be successfully identified. In the case of GB-inhibited enzyme, the serine-198 is conjugated to IMPA; and for GD following loss of the pinacolyl alkyl group (i.e., aging), MPA was found bound to the serine residue. In addition, the procedure was useful for detecting BChE adducted to OP pesticides, as well as non-OP anti-cholinesterases, such as pyridostigmine (Fidder et al., 2002). A limitation to the assay is that advanced information on the agent identity is needed for the MS analysis (Noort et al., 2006). For this reason, an extension of this procedure using a generic approach was developed (Noort et al., 2006). The methodology employed the chemical modification of the phosphyl group on the serine residue to a common nonapeptide regardless of the specific agent involved (Noort et al., 2006). Therefore, because a common nonapeptide is the outcome, a single MS method would be employed in the analysis (Noort et al., 2006). Various published methods on the assay of adducts to biomolecules are presented in Table 19.3.

Application to Human Exposures. The fluoride ion regeneration procedure (Polhuijs et al., 1997) was used to analyze serum from exposed individuals in the Aum Shinrikyo terrorist attacks at Matsumoto and in the Tokyo subway. As previously indicated, this procedure is based upon the use of fluoride ion to regenerate the parent agent and free BChE. The amount of regenerated GB from serum ranged from 1.8 to 2.7 ng/mL in the Matsumoto incident and 0.2 to 4.1 ng/mL from the Tokyo attacks. Although unable to detect MPA or IMPA directly from blood from victims of the Tokyo subway attack, Nagao et al. (1997a, 1997b) indicated more success with detecting these compounds following release from erythrocyte derived AChE following alkaline phosphatase digestion of the GB–AChE complex. The authors did not report MPA or IMPA concentrations. Although not relevant to diagnostic testing directly, a conceptually similar approach was also applied to formalin-fixed brain tissues (GB-bound AChE) from victims of the Tokyo subway attack (Matsuda et al., 1998). These authors indicated that the assays conducted with frozen cerebral cortex did not detect MPA or IMPA. However, similar studies with formalin-fixed cerebellum tissue resulted in detection of MPA only. The authors indicate that the inability to detect IMPA was due to hydrolysis during the 2 year storage period. The inability to detect hydrolysis products in the cerebral cortex as opposed to the cerebellum was reported to be consistent with the relative AChE activity detected in each tissue. These authors reported that this was the first verification of nerve agent exposure using formalin-fixed brains.

TABLE 19.3
Analytical Methods Using Nerve Agent Adducts to Biomolecules

Sample Matrix	Product Identified	Analytical Method	References
Plasma/serum	GA, GB	GC–NPD	Polhuijs et al. (1997, 1999)
Red blood cell	IMPA, MPA	GC–MS	Nagao et al. (1997a, 1997b)
Brain (cerebellum)	MPA	GC–MS	Matsuda et al. (1998)
Plasma/serum	VX-G	GC–FPD/GC–MS	Jakubowski et al. (2001)
Plasma/serum	Phosphylated nonapeptides from BChE	LC–MS–MS	Fidder et al. (2002)
Plasma/serum	GA, GB, GF, VX-G	GC–MS/GC–MS(HR)	Degenhardt et al. (2004)
Plasma/serum/red blood cell	GB	GC–MS	Jakubowski et al. (2004)
Plasma/serum	Phosphylated nonapeptides from BChE-derivatized	LC–MS–MS	Noort et al. (2006)

VX-G, ethyl methylphosphonofluoridate; HR, high resolution.

Because of the limited number of human exposures involving nerve agents, it is difficult to fully ascertain the advantages and disadvantages of the various definitive methodologies that have been developed. Numerous assays to detect hydrolysis products in blood or urine have been developed; some have been employed in exposure incidents. The drawback of the methods is the relatively rapid elimination and limited opportunity to obtain a viable sample. The advantage of using adducts formed with large molecular weight targets (AChE or BChE) is a longer window of opportunity to verify exposures relative to that of hydrolysis products. Some investigations have indicated that methods employing BChE provide benefits over those with AChE in that it is more abundant in blood. Assays involving digestion of BChE with subsequent assay of nonapeptide fragments facilitate identification of aged or nonaged adduct at the phosphylated serine-198 residue and are therefore potentially useful with agents such as GD. Methods used to confirm human exposures to nerve agent adducts to biomolecules are presented in Table 19.4.

TABLE 19.4
Methods Used to Confirm Human Exposures to Nerve Agents Adducts to Biomolecules

Agent/Incident	Sample Matrix	Product Identified	Concentration Reported	Analytical Method	References
GB, Matsumoto and Tokyo, Japan	Serum	GB	Matsumoto (1.8–2.7 ng/mL) Tokyo (0.2–4.1 ng/mL)	GC–NP	Polhuijs et al. (1997, 1999)
GB, Tokyo, Japan	Red blood cell	IMPA, MPA	NR	GC–MS	Nagao et al. (1997a, 1997b)
GB, Tokyo, Japan	Brain (cerebellum)	MPA	NR	GC–MS	Matsuda et al. (1998)
GB, Tokyo, Japan (selected samples)	Plasma/serum	Phosphylated nonapeptides from BChE	10–20 pmol inhibited BChE/mL	LC–MS–MS	Fidder et al. (2002)

NR, not reported

Measurement of ChE Activities. ChEs are highly polymorphic CaEs that display broad substrate specificity and are involved in the termination of neurotransmission in cholinergic synapses and neuromuscular junctions of the central nervous system (CNS). They are classified as AChE (EC 3.1.1.7) and BChE (EC 3.1.1.8) according to their substrate specificity and sensitivity to selected inhibitors (Silver, 1974). The activities of AChE in whole blood and, to a lesser extent, BChE in plasma, can be used as reliable surrogate biomarkers of suppressed or heightened central and peripheral nervous system activity. For AChE, this is due to the functional similarities between the erythrocyte AChE in particular and the synaptic enzyme (Mortensen et al., 1998). Exposure to organophosphorous (OP) nerve agents, pesticides, anesthetics, drugs such as cocaine, and some neurodegenerative disease states and their treatments selectively reduce AChE and BChE activity (Taylor, 2006). Thus, the early diagnosis of OP exposure or intoxication is crucial, and blood ChE activity (specifically RBC-AChE) can be exploited as a tool for confirming exposure to these agents and commencing antidotal (oxime) therapy (Wilson et al., 1997; Nigg and Knaak, 2000). Because these ChE inhibitors comprise a group of structurally diverse compounds with a wide range of relative specificities of ChEs found in blood (RBC-AChE and plasma-BChE), these observations suggest that a complete profile of inhibition would be more accurately reflected if both ChEs were measured.

Clinical tests demonstrating exposure to these nerve agents or pesticides uses measurement of the activities of AChE or BChE enzymes in the blood. If the activities are significantly lower than unexposed normal (baseline) values and the individual was exposed to nerve agents, it is likely that there will also be symptoms of poisoning. Exposure that results in inhibition of less than about 20% (especially if clinical symptoms are absent) may not easily be detected due to considerable inter- and intraindividual variations in AChE and (especially) BChE activities (Lotti, 1995). Moderate clinical symptoms of poisoning will be apparent at 50%–70% AChE inhibition, with severe toxicity seen at greater than 90% inhibition. While general clinical measurement of ChE activity in blood is not specific for exposure to any OP nerve agent, carbamate, or pesticide, laboratory measurements by mass spectrometry can positively identify OPs by evaluating their leaving group from fluoride reactivated proteins (Polhuijs et al., 1997). Thus, the determination of an individual's ChE status can be important prior to decisions regarding oxime therapy and confirmation of OP poisoning, particularly in the case of long lasting (e.g., VX), rapidly aging (e.g., soman) nerve agents, and the persistence of OP in blood and tissues (Wolthuis et al., 1981).

Several sensitive and specific assays for measurement of AChE activities in blood have been developed for use in clinical and toxicology laboratories. For routine use, however, a number of drawbacks are apparent, including time-consuming sample preparation, long turnaround times, and lack of standardization because of the difficulty in comparing results between alternate laboratories using different ChE assays that report values in different or nonstandard units. None of the widely used methods have been approved by the U.S. Food and Drug Administration. Clinical determination of AChE and BChE activities in blood commonly use several techniques (colorimetry, electrometry, and radiometry) and normally determine *either* RBC-AChE or plasma BChE concentrations, but not usually *both* (St. Omer and Rottinghaus, 1992). A summary depicting methods for measuring ChE activity in blood and plasma is presented in Figure 19.3.

Ellman Assay: The Ellman method (Ellman et al., 1961) is a popular colorimetric procedure for detecting and monitoring pesticide exposure in human and animal blood. The breakdown of thiocholine substrates (acetylthiocholine and butyrylthiocholine) by AChE and BChE is detected kinetically using the Ellman reagent DTNB (5,5′-dithio-bis-2-nitrobenzoate). This assay is accurate, reliable, and inexpensive. However, the absorption maxima (412 nm) of the resulting yellow TNB^- (3-carboxy-4-nitrobenzenethiolate dianion) coincides with the Soret band of hemoglobin, resulting in interference and reduced assay sensitivity. By increasing the wavelength from 412 to 436 nm, hemoglobin interference can be reduced by 75%, improving assay sensitivity without significant sacrifice of the indicator (TNB^-) absorption (Worek et al., 1999). It should also be noted that the

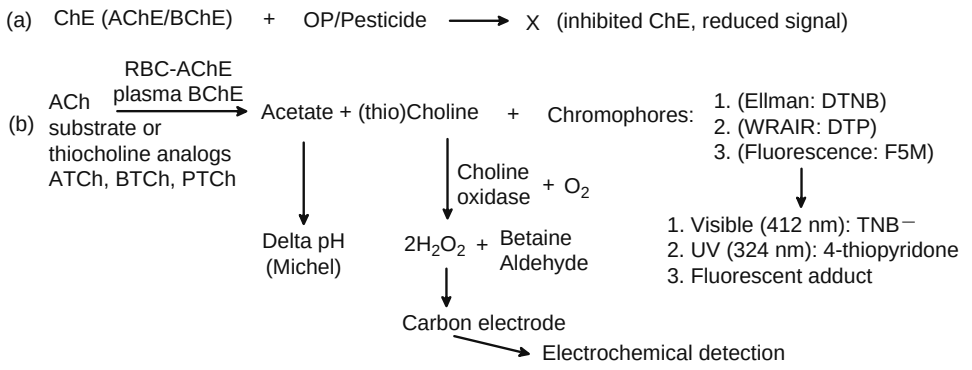

FIGURE 19.3 (a) Reaction depicting inhibition of AChE and BChE (in blood, RBC and plasma, respectively) by chemical warfare and pesticide organophosphorous compounds, yielding inhibited enzyme and reduced signal. (b) Summary of methods for measuring cholinesterase activity in blood (only colorimetric and electrometric are shown). See text for abbreviations.

molar extinction coefficient for TNB (13.6×10^3 M^{-1} cm^{-1}) used in the original Ellman assay (Ellman et al., 1961) has been almost universally used to calculate ChE activities, although this value has been shown to vary depending on temperature, wavelength, and buffer conditions (Eyer et al., 2003). There are many published variations of the original cuvette-based Ellman assay, including the now ubiquitous 96 well microtiter plate format (Doctor et al., 1987).

Walter Reed Army Institute of Research (WRAIR) Assay: An important variation of the Ellman ChE assay is the recently described whole blood assay (WRAIR), which uses 4,4′-dithiopyridine (DTP) instead of DTNB as a chromogenic indicator, and three thiocholine substrates (acetyl-, butyryl-, and propionyl-thiocholine). The absorption maxima in the UV range (324 nm) of the 4-thiopyridone formed yields a high signal-to-noise ratio because hemoglobin interference is minimal. The use of three substrates in this assay provides redundancy and independent measurement of the activities of RBC-AChE and plasma-BChE rapidly and *simultaneously* in a small sample of unprocessed whole blood using a 96 well microtiter plate spectrophotometer (Gordon et al., 2005). The method is not labor intensive and although it can be performed manually, it has been semiautomated using a Beckman–Coulter robotic platform for high sample throughput. A unique feature of the WRAIR method is that the blood is not treated prior to assay; whole blood, frozen blood, or lysed blood yield both AChE and BChE activities without centrifugation or the use of inhibitors.

Fluorescence Assays: In place of the visible or UV chromogens DTNB or DTP, respectively, there are a number of additional thiol-reactive reagents that covalently react with the thiocholine formed by ChE hydrolysis of the thio-substrate. These reagents, such as fluorescein-5-maleimide (F5M) or methylcoumarin maleimide analogs (Parvari et al., 1983), offer increased sensitivity over visible adducts, readily react at physiological pH, exhibit absorbance and emission spectra distant from most interfering compounds, and form stable adducts, but otherwise are equivalent replacements for DTNB. However, there is the requirement for a fluorescent spectrophotometer or plate reader in place of the more common and less expensive visible or UV-capable instrument.

Test-Mate™ Assay: In addition to the laboratory-based methodologies, a field deployable unit is commercially available, the Test-mate™ ChE system (EQM Research Inc., Cincinnati, OH). This method is also based on the Ellman procedure and is supplied as a kit containing reagents to *separately* measure erythrocyte AChE and plasma-BChE with a battery-operated, photometric analyzer (Taylor et al., 2003). A specific serum BChE inhibitor (As1397, or 10-(α-diethylaminopropionyl)-phenothiazone) is included in the kit and is required to measure AChE (after a period of incubation). Two capillary

whole-blood samples must be collected for AChE and BChE determination (and correction for hemoglobin content, which is also measured in the blood sample). The kit is easy to use by a relatively untrained operator and matches the sensitivity of the laboratory-based Ellman methods, but has relatively low throughput as it is performed manually and requires several processing steps. Although the Test-mate™ ChE kit is designed primarily for field use, it is widely used for monitoring pesticide exposure in agricultural workers where longer processing times are required for complete AChE and BChE screening and hemoglobin measurement.

Future Handheld ChE Assays: Field deployable and handheld medical diagnostic units, unlike conventional clinical tests described above, should provide full analysis of the soldier's/patient's AChE and BChE activities and not rely on the addition of selective AChE or BChE inhibitors, thereby simplifying the use while wearing protective gear in the field. The fielded device should use a single, noninvasive blood collection technique. It should not be labor intensive or otherwise complicated, and should produce results in minutes. These systems will provide the Army and general public health clinics with units capable of screening and confirming exposure of soldiers, agricultural workers, and first responders to CWAs, pesticides, or other such toxic chemicals that affect either AChE or BChE. These devices will use MEMS, microelectromechanical systems, an integration of mechanical fluidic elements coupled to electrochemical detection sensors in a handheld unit. Electrochemical detection has already been implemented in the research laboratory for on-line HPLC detection (Andreescu and Marty, 2006) and remote field sensing by an electronic biosensor comprising disposable carbon microelectrodes that detect electrochemical signals from H_2O_2 generated by a choline-coupled reaction (Gordon and Doctor, 2002), as shown in Figure 19.3.

Michel (ΔpH) ChE Assay. A manual method still widely used by the U.S. Department of Defense for the measurement of RBC-AChE is a modification (Ellin et al., 1973) of the end point delta pH method originally described by Michel (1949). This assay monitors the decrease in pH (using a simple pH electrode and pH meter) that occurs when AChE catalyzes the hydrolysis of acetylcholine into choline and acetic acid. The assay is initiated by the addition of substrate (acetylcholine) and the change in pH monitored for 17 min. This method is slow and laborious, although throughput can be increased by staggering the addition of substrate to each sample. Up to 51 blood samples can be analyzed in 35 min, and the RBC-AChE activity is reported as a change (delta) in pH units/h. Another drawback is that the delta pH method requires centrifugation of blood to pellet RBCs followed by removal of the plasma (containing BChE) prior to analysis of AChE activity. Lysis of the blood sample is precluded, so blood must be refrigerated (not frozen) before centrifugation and AChE analysis. Furthermore, because plasma-BChE is not determined, the complete spectrum of blood inhibition is unknown. Although this technique was developed nearly 60 years ago, it is reliable, and the U.S. Army (through the Department of Defense Cholinesterase Reference Laboratory) has a quality assurance testing program for primary RBC-AChE monitoring of more than 25,000 military personnel per year.

Radioactive Assays. Radioactive assays for AChE activity are very sensitive but require special handling and disposal. Although used in a research setting, radioactive assays have not been developed for general clinical laboratory or field use. These methods are based on the measurement of the production of [^{14}C]carbon dioxide or choline, or [^{3}H]acetate from the hydrolysis of appropriately ^{14}C- or ^{3}H-labeled acetylcholine, respectively. The radioactive species are separated by Reinecke salt precipitation, differential extraction into organic solvent (Johnson and Russell, 1975), and ion-exchange chromatography or ion-exchange disks (Gordon et al., 1982). The advantage of radioactive methods is that they overcome the potentially high background in some biological samples from SH groups that may react with the Ellman reagent. On the other hand, there are notable disadvantages, including disposal of the radioactive waste, and the assays are not readily adaptable for kinetic analysis because aliquots of the radioactive mixture must be removed for scintillation counting at specific time intervals.

IV. VESICANTS

Sulfur Mustard. Although human exposure incidents requiring analysis of biomedical samples such as blood or urine following a suspected CWA exposure have been a relatively rare event, those involving exposure to sulfur mustard have been the most frequent. A major portion of these samples were acquired from suspected sulfur mustard exposure casualties of the Iran–Iraq war in the 1980s that were transported to hospitals in Europe for medical treatment. Analytical methods for sulfur mustard verification prior to the early 1990s consisted of assays for the unmetabolized compound or the hydrolysis product thiodiglycol (TDG). Since 1995, a number of significant advances have occurred and are reflected in the published reports from that time to the present. In some instances, blood and urine specimens from past incidents were frozen and then later reanalyzed using more recently developed analytical methods. The newer methods generally had greater sensitivity than previous methods and used other biomarkers of exposure. Additionally, there have been several incidents where a laboratory or field exposure to sulfur mustard occurred and biomedical specimens were collected and analyzed using some of the more recently developed methods. The targeted analytes of sulfur mustard exposure result from the formation of a highly reactive sulfonium ion that is produced following cyclization of an ethylene group of sulfur mustard. The sulfonium ion readily reacts with nucleophiles, such as water, or can combine with a variety of nucleophilic sites that occur in macromolecules. The chemical reactions have the potential to produce a number of both free metabolites and stable adducts to macromolecules that can be exploited for analysis in blood, urine, or tissue samples (Noort et al., 2002; Black and Noort, 2005; Noort and Black, 2005). Although sulfur mustard will probably react with a wide range of compounds within the body, the focus in this section will be on metabolites that have previously been identified in biomedical samples obtained from human exposure to sulfur mustard.

Analysis of Urine Samples. As noted, earlier analysis of urine samples targeted either unmetabolized sulfur mustard or the hydrolysis product TDG. Since that time, the number of analytes has expanded to a total of five urinary metabolites that are of primary interest for sulfur mustard verification in cases of human exposure. Two of the metabolites, TDG and TDG-sulfoxide, are primarily derived from chemical hydrolysis reactions (see Figure 19.4). The other three products are formed following the reaction of sulfur mustard with glutathione (see Figure 19.5). Each of the five analytes has been identified in the urine of sulfur mustard exposed individuals.

Analytical Methods for Urine. Efforts to analyze for specific biomarkers in urine of sulfur mustard exposure prior to 1995 targeted either unmetabolized sulfur mustard or TDG. Vycudilik (1985) prepared urine samples using organic extraction with diethylether and analyzed them using GC–MS. The method was later modified (Vycudilik, 1987) by the addition of a strong acid to the urine samples to isolate possible conjugates of sulfur mustard. Vycudilik indicated that the methods could not distinguish between sulfur mustard and its hydroxyethyl metabolites that were present in the urine samples.

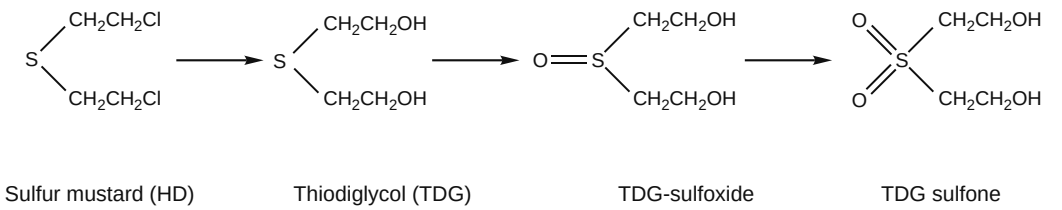

FIGURE 19.4 Hydrolysis of sulfur mustard to produce thiodiglycol followed by oxidation reactions.

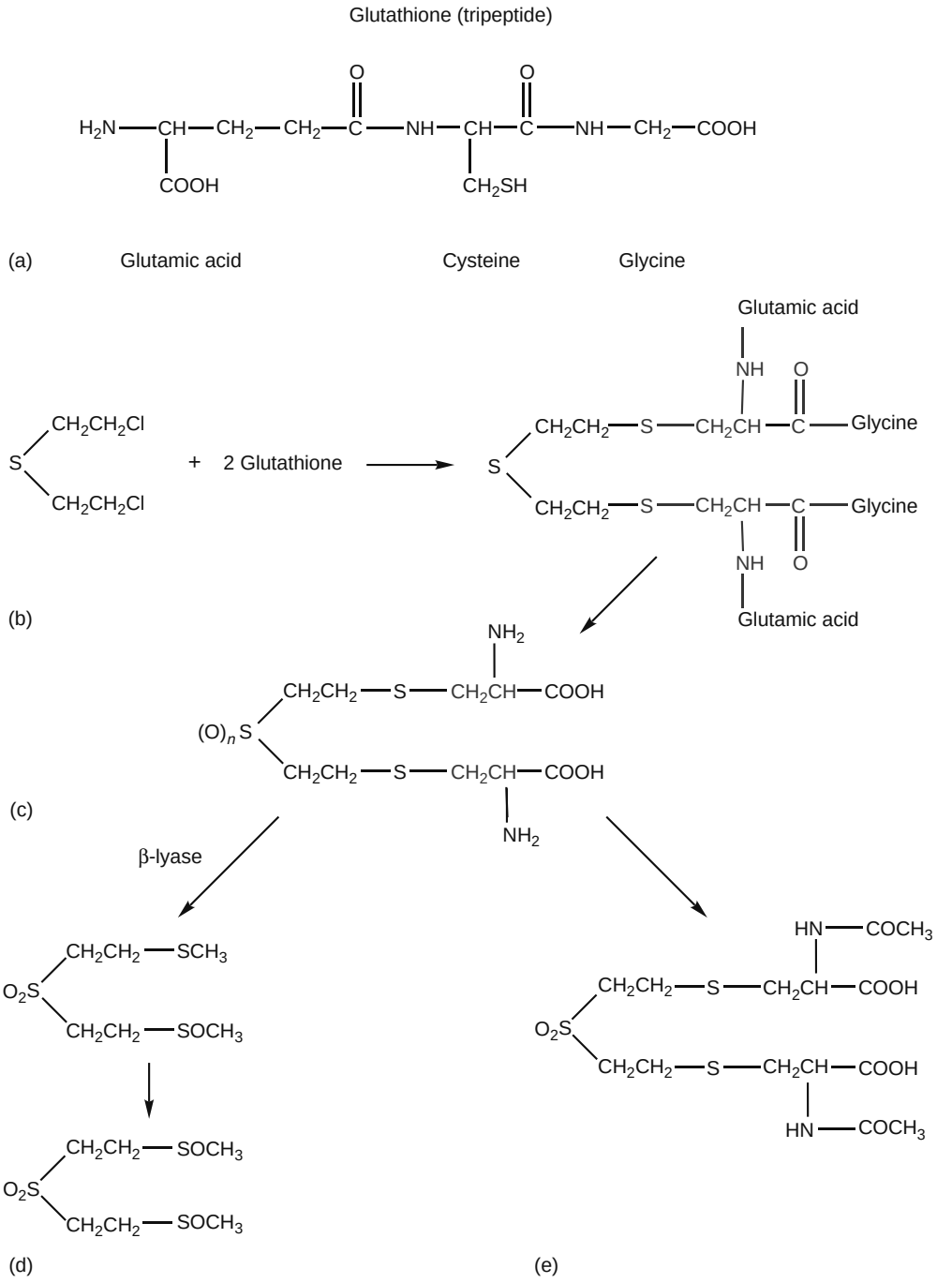

FIGURE 19.5 Reaction pathway proposed by Black et al., 1992a. (a) Structure of glutathione, (b) reaction of sulfur mustard and glutathione, (c) intermediate product, (d) β-lyase metabolites MSMTESE and SBMSE, and (e) bis-mercapturic acid conjugate of mustard sulfone.

Wils et al. (1985, 1988) treated urine with concentrated hydrochloric acid (HCl) to convert TDG back to sulfur mustard. Urine was passed through two C18 solid-phase extraction cartridges, mixed with concentrated HCl, and heated. Sulfur mustard was purged from the solution and trapped onto a Tenax-TA adsorption tube. Analysis was performed using a thermodesorption cold trap injector interfaced with a GC–MS. Analysis of urine samples obtained from a control group of patients found levels of TDG at low ng/mL concentrations. Although most of the control levels were approximately 5 ng/mL, two individuals had levels that exceeded 20 ng/mL. As will be explained below, these high background levels probably indicate that the method was also converting another analyte, in addition to TDG, into sulfur mustard.

Drasch et al. (1987) examined urine samples for unmetabolized sulfur mustard. Following organic extraction, thin-layer chromatography, and derivatization with gold, the extracts were analyzed using electrothermal atomic absorption spectroscopy.

More recently, a number of additional methods have been developed for the trace level analysis of TDG and TDG-sulfoxide in urine (Black and Read, 1988, 1995a; Jakubowski et al., 1990; Boyer et al, 2004). There are a number of characteristics that are common to all of the methods. They all use GC in association with some form of MS for the analysis, use a derivatizing agent to make the analyte more amenable to GC analysis and to increase sensitivity, and incorporate an isotopically labeled form of TDG as an internal standard. Most of the methods use a solid-phase extraction cartridge for sample preparation. Some of the methods incubate the urine samples with glucuronidase with sulfatase activity to release any glucuronide-bound conjugates. Some of the methods use titanium trichloride ($TiCl_3$) in HCl to reduce TDG-sulfoxide to TDG. The strong acid also appears to hydrolyze acid-labile esters of TDG and TDG-sulfoxide. Ultimately, each of the methods will convert all target analytes into the single analyte TDG for analysis. All of the methods have similar limits of detection, approximately 0.5–1 ng/mL. Although an assay has been developed for the analysis of TDG-sulfoxide separately without a conversion to TDG, the method is complicated by the high polarity of the analyte (Black and Read, 1991a). Consequently, the use of the reducing agent $TiCl_3$ has been the more common approach.

Unfortunately, regardless of the analytical method used, the TDG and TDG-sulfoxide methods all suffer from the observation that background levels have consistently been found in urine samples obtained from nonexposed individuals. In the most extensive study of background levels, urine samples from 105 individuals were examined for sulfur mustard metabolites using the assay that incorporates both a deconjugation process and a reduction step (i.e., the assay measures free and bound forms of both TDG and TDG-sulfoxide) (Boyer et al., 2004). Quantifiable background levels were observed in 82% of the samples that were evaluated. Nearly 60% of the samples had observed levels of TDG in the 0.5–2.0 ng/mL range whereas approximately 9% had levels in the 10–20 ng/mL range. When the urine samples with the higher background levels were reanalyzed without the reduction step, the TDG levels in all samples were less than 2.5 ng/mL. This indicates that the free and bound forms of the TDG-sulfoxide, rather than the free and bound forms of TDG, are responsible for a larger portion of the observed background levels in the nonexposed human urine samples. These results are consistent with those found in other, smaller studies of background levels (Black and Read, 1988, 1991a, 1995a; Jakubowski et al., 1990). Boyer et al. (2004) discovered that the storage of urine samples must also be considered when analyzing samples for TDG and TDG-sulfoxide. In a study of urine samples stored at −20°C for an 8 month period, they found that all free and conjugated TDG in the samples had oxidized to free and conjugated TDG-sulfoxide. Consequently, the use of a reducing agent was shown to be critical for the analysis of samples that had been frozen for any length of time.

A series of metabolites formed from the reaction of sulfur mustard with glutathione, a small molecular weight tripeptide that acts as a free radical scavenger, have been identified (Black et al., 1992a, 1992b; Black and Read, 1995b). Although a large number of metabolites were identified in

animal experiments, there are three reaction products that have been verified in urine samples obtained from sulfur mustard exposed individuals. One set of reaction products is believed to result from the metabolism of the sulfur mustard–glutathione conjugate by the β-lyase enzyme (see Figure 19.5). Two β-lyase metabolites have been identified in the urine from exposed individuals: 1-methylsulfinyl-2-[2-(methylthio)ethylsulfonyl]ethane (MSMTESE) and 1,1′-sulfonyl-bis-[2-(methylsulfinyl)ethane] (SBMSE). MSMTESE and SBMSE can be reduced using $TiCl_3$ and analyzed by GC–MS–MS as a single analyte: 1,1′-sulfonyl-bis-[2-(methylthio)ethane] (SBMTE) (Black et al., 1991b; Young et al., 2004). Black et al. (1991b) reported a limit of detection of 0.1 ng/mL, whereas Young et al. (2004) extended the lower limit of detection to 0.038 ng/mL. To date, no background levels of SBMTE have been found in the urine of unexposed individuals including studies where urine samples from over 100 individuals were analyzed using two different assay methods (Boyer et al., 2004; Young et al., 2004). Alternatively, MSMTESE and SBMSE can be analyzed individually without reducing the two analytes to a common analyte using electrospray LC–MS–MS (Read and Black, 2004a). Lower limits of detection were 0.1–0.5 ng/mL for each of the analytes.

The final sulfur mustard urinary biomarker to be discussed is also a reaction product of sulfur mustard with glutathione: 1,1′-sulfonyl-bis-[2-*S*-(*N*-acetylcysteinyl)ethane]. Using solid-phase extraction for sample cleanup and analyte concentration followed by analysis with negative ion electrospray LC–MS–MS, Read and Black (2004b) were able to achieve detection limits of 0.5–1.0 ng/mL. Methods for the analysis of urine samples for sulfur mustard verification are summarized in Table 19.5.

Assays for several other potential urinary analytes have been developed, but the analytes have yet to be confirmed in human exposed samples. N7-(2-hydroxyethylthioethyl) guanine is a breakdown product from alkylated DNA that has been observed in animal studies. Fidder et al. (1996a) developed both a GC–MS method that requires derivatization of the analyte and also a LC–MS–MS method that can analyze the compound directly. Other possible urinary analytes are an imidazole derivative formed from the reaction of sulfur mustard with protein histidine residues (Sandelowsky et al., 1992) and sulfur mustard adducts to metallothionien (Price et al., 2000).

Application to Human Exposure (Urine Samples). Vycudilik (1985, 1987) analyzed urine samples from multiple casualties of the Iran–Iraq War in the 1980s that were hospitalized for treatment of suspected sulfur mustard exposure. Urine samples from eight of the patients produced positive results for sulfur mustard using GC–MS. Concentrations found ranged from 1 to 30 ng/mL. The method could not distinguish between sulfur mustard and its hydroxyethyl metabolites that were present in the urine samples.

Wils et al. (1985, 1988) examined a large number of urine samples that were also obtained in the 1980s from Iranian casualties of the Iran–Iraq War for TDG concentrations. The majority of the urine samples were the first collected samples following admission to the hospital, between 5–10 days after the suspected exposure to sulfur mustard. The medical history of the patients has been detailed by Willems (1989). Urine samples were initially analyzed for intact sulfur mustard, but were found to be negative. Following acid treatment of the samples and analysis by GC–MS, the concentration of TDG that was found in the first set of samples collected at the hospital ranged between 5 and 100 ng/mL for the majority of the samples. The highest observed TDG concentration was 330 ng/mL from a casualty who died 1 day after admission. TDG background levels were determined from urine samples obtained from nonexposed individuals and were generally less than 12 ng/mL, although two of the control samples had levels of 21 and 55 ng/mL.

Drasch et al. (1987) analyzed urine samples for unmetabolized sulfur mustard that were obtained during an autopsy of a sulfur mustard casualty. The victim was an Iranian soldier who died of complications 7 days after the exposure. Samples were stored at −20°C for 1 year prior to analysis. Despite very high concentrations of sulfur mustard found in autopsy tissue specimens, sulfur mustard was not detected in the urine samples.

TABLE 19.5
Analytical Methods Used for the Verification of Exposure to Sulfur Mustard in Biomedical Samples

Sample Matrix	Biomarker	Sample Preparation	Analytical Method	LOD	References
Urine, blood, plasma	TDG	Enzyme incubation, derivatization	Negative ion CI GC–MS	~1 ng/mL	Black and Read (1988)
Urine	TDG	Enzyme incubation, derivatization	Electron impact GC–MS	~1 ng/mL	Jakubowski et al., (1990)
Urine	TDG, TDGO	$TiCl_3$ reduction, derivatization	Negative ion CI GC–MS–MS	<1 ng/mL	Black and Read (1995a)
Urine	TDG, TDGO	Enzyme incubation, $TiCl_3$ reduction, derivatization	Positive ion CI GC–MS	0.5 ng/mL	Boyer et al. (2004)
Urine	TDGO	Derivatization	Negative ion CI GC–MS	2 ng/mL	Black et al. (1991a)
Urine	SBMTE	$TiCl_3$ reduction	Positive ion CI GC–MS–MS	0.1 ng/mL	Black et al. (1991b)
Urine	SBMTE	$TiCl_3$ reduction	Positive ion CI GC–MS–MS	0.04 ng/mL	Young et al. (2004)
Urine	MSMTESE	SPE cartridge extraction	Positive ion electrospray LC–MS–MS	0.1–0.5 ng/mL	Read and Black (2004a)
Urine	SBMSE	SPE cartridge extraction	Positive ion electrospray LC–MS–MS	0.1–0.5 ng/mL	Read and Black (2004a)
Urine	Bis-(*N*-acetylcysteine) conjugate	SPE cartridge extraction	Negative ion electrospray LC–MS–MS	0.5–1 ng/mL	Read and Black (2004b)
Blood	Hemoglobin valine adduct	Globin isolation, valine cleavage by Edman degradation, derivatization	Negative ion CI GC–MS	100 nM whole blood exposure	Fidder et al. (1996b) Noort et al. (2004a)
Blood	Hemoglobin valine adduct	Globin isolation, valine cleavage by Edman degradation	High resolution negative ion CI GC–MS	0.5 pmol adduct/mL	Black et al. (1997b)
Blood	Hemoglobin histidine adduct	Acid hydrolysis of globin, derivatization	Positive ion electrospray LC–MS–MS	Not reported	Black et al. (1997b)
Blood	Hemoglobin histidine adduct	Acid hydrolysis of globin, derivatization	Positive ion electrospray LC–MS–MS	10 μM whole blood exposure	Noort et al. (1997)
Plasma	Albumin cysteine adduct	Albumin isolation, pronase digestion	Positive ion electrospray LC–MS–MS	10 nM whole blood exposure	Noort et al. (1999, 2004b)
Blood, plasma	Protein adducts	Protein precipitation, alkaline hydrolysis, derivatization, SPE extraction	Negative ion CI GC–MS	25 nM plasma exposure	Capacio et al. (2004)
Blood, skin	DNA adducts	Blood: WBC isolation, lysis, extraction, treatment with RNAse and proteinase K Skin: epidermal layer isolation, lysis, extraction, treatment with RNAse and proteinase K	Immunuslotblot assay	Blood: 50 nM whole blood exposure; skin: 1 s exposure to saturated vapor	van der Schans et al. (1994, 2004)
Skin	Keratin adducts	Alkaline hydrolysis, derivatization	LC/radiometric detector	Not reported	Noort et al. (2000a)

In 1990, urine samples from an accidental laboratory exposure to sulfur mustard were obtained (Jakubowski et al., 2000). The erythematous and vesication areas of the individual were estimated to be less than 5% and 1%, respectively, of the total body surface area. The assay measured both free and conjugated TDG using GC–MS (Jakubowski et al., 1990). The maximum TDG urinary excretion rate was 20 (μg/day on day 3. TDG concentrations of 10 ng/mL or greater were observed in some samples for up to a week after the exposure. A first-order elimination was calculated from days 4 through 10 and found to be 1.2 days. A great deal of intraday variability was noted for the TDG urine concentrations. Attempts were made to estimate the total amount of sulfur mustard on the skin of the patient. The estimate was based on two assumptions: (1) that the assay for the free and conjugated TDG represents approximately 5% of the total amount of sulfur mustard related products in the blood, and (2) that the bioavailability factor from skin to blood is 10. A total of 0.243 mg of TDG was recovered over a 2 week period. This would represent 4.86 mg in the blood or 48.6 mg on the skin.

Only recently have a small number of reports been published in which urine samples obtained from victims of sulfur mustard exposure were subjected to several different assays to target multiple urinary metabolites. The first report used a small subset of the samples previously analyzed by Wils et al. (1985) for TDG concentrations. The urine samples had been obtained 10 days after the suspected sulfur mustard exposure. Four of the five patients were discharged approximately 1 month after hospitalization, but the fifth patient (labeled patient C1 in graphs 19.6 and 19.7) died 5 days after hospital admission. Wils et al. (1985) found concentrations of TDG at 90 (patient C1), 45, 40, 40, and 15 ng/mL for the five individuals. The samples were reanalyzed by Black and Read (1995a) after being stored at −20°C for a 5 year period. Using a GC–MS–MS method that measures both TDG and TDG-sulfoxide as a single analyte, Black and Read (1995a) analyzed urine samples from three of the individuals. They found TDG plus TDG-sulfoxide levels of 69 ng/mL for the patient who died after admission (patient C1) and approximately 30 ng/mL for the other two individuals. Control urine samples analyzed using the assay produced background levels of 11 ng/mL. Urine samples from all five casualties were analyzed for β-lyase concentrations with the highest concentration found in patient C1. The concentrations found in the urine from four of the casualties ranged between 0.5 and 5 ng/mL, while the individual that died had a β-lyase concentration of 220 ng/mL.

Once again using the GC–MS–MS method that measures both TDG and TDG-sulfoxide as a single analyte, Black and Read (1995a) analyzed urine samples from two casualties of an alleged sulfur mustard attack on the Kurdish town of Halabja in 1988. The patients had been transferred to London for medical treatment. Urine samples were collected 13 days after the alleged incident. They found combined TDG plus TDG-sulfoxide levels of 11 ng/mL for both patients, but also found similar concentration levels in control samples. Urine samples were also analyzed for β-lyase concentrations using GC–MS–MS. Although the concentration of the β-lyase metabolites found in both patients was near the limit of detection for the assay, the analytes were clearly detectable. The urine samples were later analyzed using the LC–MS–MS assay that can distinguish the individual β-lyase metabolites (Read and Black, 2004a). The mono-sulfoxide (MSMTESE) was only detected from one of the casualties and was near the limit of detection for the assay. The bis-sulfoxide (SBMSE) was detected in the urine from both casualties, but for each sample was near the limit of detection of the assay (0.1–0.5 ng/mL).

Some of the most extensive testing of urine samples for sulfur mustard related metabolites involved two individuals that were accidentally exposed to a World War I (WWI) munition containing sulfur mustard. The injuries were described as a cutaneous (predominately) exposure with both individuals suffering extensive blistering of the skin. Urine samples were collected 2–3 days after the individuals were exposed. Black and Read (1995b) analyzed the urine using three different methods to detect sulfur mustard hydrolysis metabolites. In addition, the urine samples were examined for reaction products between sulfur mustard and glutathione. The first assay measured TDG (free and conjugated together) and found concentrations of 2 ng/mL for each

individual. The second assay targeted only free TDG-sulfoxide and concentrations of 69 and 45 ng/mL were found for the two individuals. Using the GC–MS–MS assay that measures TDG and TDG-sulfoxide as a single analyte, concentrations of 77 and 54 ng/mL were found. Control samples analyzed along with the patient samples for the second and third assays gave levels of 4–5 ng/mL, therefore the patient results were significantly higher than control values. The β-lyase metabolites were measured using both the GC–MS–MS method (Black et al., 1995b) and using the LC–MS–MS method (Read and Black, 2004a). When the β-lyase metabolites were analyzed individually by LC–MS–MS, their concentrations ranged from 15 to 17 ng/mL and from 30 to 34 ng/mL for the mono-sulfoxide and bis-sulfoxide, respectively. When analyzed as the single, reduced form SBMTE using GC–MS–MS, observed concentrations were 42 and 56 ng/mL. Samples were also analyzed for a bis-(*N*-acetylcysteine) conjugate using LC–MS–MS (Read and Black, 2004b). This biomarker is also a reaction product between sulfur mustard and glutathione. Although it is a major metabolite in rats exposed to sulfur mustard, it had not been previously reported to be found in urine samples from exposed individuals. The metabolite was found in urine samples from both exposed individuals, but concentrations were near the lower limit of detection of 0.5–1 ng/mL.

The most recently reported exposure incident involving sulfur mustard involved two individuals that were accidentally exposed to a WWI munition. Following the demolition of an old munition, two ordnance technicians came into contact with liquid that was found leaking from the remnants of the munition. The next day one of the individuals (patient D1) had extensive erythema and blistering (approximately 6% body surface area) and was admitted to a regional burn center (Carroll, 2005). The second individual (patient D2) had a small, single blister. Urine samples from patient D1 were collected on days 1 through 10 and days 28, 34, and 41 after exposure and from patient D2 on days 1, 3, and 6 after exposure. Urine from both individuals was analyzed for hydrolysis metabolites and glutathione reaction products (Barr et al., 2005). Hydrolysis metabolites were determined using two different methods. The first GC–MS–MS assay measured both free and glucuronide-bound TDG. The individual with the single blister (patient D2) did not have detectable levels in any of the urine samples. Patient D1 had the highest observed TDG concentration at day 1 (24 ng/mL). TDG concentrations ranged between 6 and 11 ng/mL over the next 5 days, decreased to a range of 1–2 ng/mL for the next 4 days, and were undetected after day 10. The second GC–MS–MS assay for hydrolysis metabolites targeted free and glucuronide-bound TDG, free and glucuronide-bound TDG-sulfoxide, and acid-labile esters of TDG and TDG-sulfoxide. The observed concentrations for patient D2 (2–4 ng/mL) fell within the range of concentrations previously observed in urine samples of unexposed individuals. The highest observed levels found in unexposed individuals using this assay were approximately 20 ng/mL (Boyer et al., 2004). Although observed concentrations were much higher for patient D1, only days 1, 4, and 5 (50, 28, and 24 ng/mL, respectively) produced concentrations that were greater than the highest observed background control levels. β-lyase metabolites were measured as the single analyte SBMTE using GC–MS–MS. Levels for both patients decreased dramatically by day 2 after exposure. Patient D2 urine SBMTE concentrations were 2.6, 0.8, and 0.08 ng/mL for samples taken 1, 3, and 6 days after exposure, respectively. Patient D1's concentrations decreased from 41 ng/mL at day 1 after exposure to 7, 3.3, and 1.3 over the next 3 days. For days 5 through 10, concentrations ranged between 0.07 and 0.02 ng/mL and were not detected beyond day 10. The urine from days 1 and 2 of patient D1 was also examined for the presence of the bis-(*N*-acetylcysteine) conjugate using LC–MS–MS. It was detected at a concentration of 3.1 ng/mL in the urine sample collected 1 day after exposure, but was not detected in the day 2 sample.

Currently, the method of choice for assessment of potential exposure to sulfur mustard in urine samples would be the two β-lyase metabolites. This is based on the fact that it has been verified in human exposure cases, sensitive and selective assays have been developed using both GC–MS–MS and LC–MS–MS techniques, and to date, no known examples of background levels have been found in the urine from unexposed individuals. A summary of urine samples that have been

analyzed to verify human exposure to sulfur mustard is presented in Tables 19.6 and 19.7. Table 19.6 contains verification results using methods for the analysis of hydrolysis products, while Table 19.7 contains results based on glutathione reaction products.

Analysis of Blood Samples. Urinary metabolites undergo relatively rapid elimination from the body, whereas blood components offer biomarkers that have the potential to be used for verification of sulfur mustard exposure long after the exposure incident. Three different approaches have been used for blood biomarker analysis. The intact macromolecule such as protein or DNA with the sulfur mustard adducts still attached can be analyzed. To date, this approach has only been demonstrated for hemoglobin using in vitro experiments. For proteins, an alternate approach is to enzymatically digest them to produce a smaller peptide with the sulfur mustard adduct still attached. Methods of this type have been developed for both hemoglobin and albumin. A third approach has been to cleave the sulfur mustard adduct from the macromolecule and analyze in a fashion similar to that used for free metabolites found in the urine. The later two approaches have both been successfully used to verify human exposure of sulfur mustard.

Analytical Methods for Blood. Methods to measure sulfur mustard adducts to DNA in white blood cells have been developed using LC with fluorescence detection (Ludlum et al., 1994) and using an enzyme-linked immunosorbent assay (ELISA) (van der Schans et al., 1994, 2004). The DNA adduct that appears to be the most abundant results from sulfur mustard attachment to the N7 position of deoxyguaninosine (Fidder et al., 1994). The immunochemical method developed by van der Schans used monoclonal antibodies that were raised against N7-(2-hydroxyethylthioethyl)-guanosine-5′-phosphate.

Hemoglobin is an abundant and long-lived protein in human blood. Alkylation reactions between sulfur mustard and hemoglobin have been shown to occur with six histidine, three glutamic acid, and two valine amino acids of hemoglobin (Noort et al., 1996; Black et al., 1997a). Methods for the analysis of several of the adducts have been developed. Although the histidine adducts appear to be the most abundant type, their analysis using mass spectrometry techniques is problematic and the method does not appear to be as sensitive as the method for the analysis of the N-terminal valine adducts (Noort et al., 1997). Adducts to the N-terminal valine amino acids represent only a small fraction of the total alkylation of the macromolecule, but their location on the periphery of the molecule allows them to be selectively cleaved using a modified Edman degradation. Following isolation of the globin from the RBCs, the globin is reacted with pentafluorophenyl isothiocyanate to form a thiohydantion compound which is further derivatized prior to analysis. The derivatized compound can then be analyzed using negative ion chemical ionization GC–MS (Fidder et al., 1996b; Black et al., 1997b). The lower limit of detection for the assay was determined using in vitro exposures of sulfur mustard in human whole blood and was determined to be equivalent to a 100 nM exposure level (Fidder et al., 1996b; Noort et al., 2004a). Following the administration of a single dose of sulfur mustard to a marmoset (4.1 mg/kg; i.v.), the valine adduct was still detected in blood taken 94 days later (Noort et al., 2002). Intact hemoglobin with the sulfur mustard adducts attached have been examined using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, but to date the technique has only been used for in vitro experiments at relatively high concentrations of sulfur mustard (Price et al., 2000).

Human serum albumin was found to be alkylated by sulfur mustard at the cysteine-34 position. Following isolation of the albumin from the blood, the albumin can be reacted with Pronase enzyme to digest the protein. One of the resulting peptide fragments is a tripeptide of the sequence cysteine–proline–phenylalanine which contains the sulfur mustard alkylated cysteine-34. After SPE extraction, the tripeptide was analyzed using LC–MS–MS (Noort et al., 1999). The lower limit of detection for the assay (1 nM) was once again reported as an equivalent exposure level as determined using in vitro exposures of sulfur mustard in human whole blood. Recently, a modification to the isolation of the albumin from blood was reported using affinity chromatography rather than the precipitation procedure used previously (Noort et al., 2004b). This modified procedure reduced the sample preparation time significantly.

TABLE 19.6
Published Reports (1995–2006) of Laboratory Analysis of Human Urine Samples for Hydrolysis Metabolites Following a Suspected Exposure to Sulfur Mustard

Patient Sample Information: (Incident/Sample Collection Time after Suspected Exposure)	Glucuronidase Incubation: TDG (Free + Glucuronide-Bound)	$TiCl_3$ Reduction: TDG (Free); TDG-Sulfoxide (Free); Acid-Labile Esters of Both	TDG-Sulfoxide	Glucuronidase Incubation and $TiCl_3$ Reduction: TDG (Free + Bound); TDG-Sulfoxide (Free + Bound); Acid-Labile Esters of Both	References
Iranian casualties, 3 of 5 individuals, treated at Ghent hospital, collected 10 days after incident (09 March 1984)		Patient C1 = 69 ng/mL Patient C2 = 28 ng/mL Patient C5 = 33 ng/mL Control = 11 ng/mL			Black and Read (1995a)
Kurdish casualties, 2 individuals, treated at London hospital, collected 13 days after incident (17 March 1988)		Patient L1 = 11 ng/mL Patient L2 = 11 ng/mL Control = 11 ng/mL			Black and Read (1995a)
Accidental exposure to WWI munition, 2 individuals, collected 2–3 days after incident (1992)	Patient S1 = 2 ng/mL Patient S2 = 2 ng/mL	Patient S1 = 77 ng/mL Patient S2 = 54 ng/mL Control = 4.5 ng/mL	Patient S1 = 69 ng/mL Patient S2 = 45 ng/mL Control = 5 ng/mL		Black and Read (1995b)
Accidental laboratory exposure, 1 individual, collected 2–14 days after incident (1990)	Maximum excretion rate = 20 μg/day on day 3; concentration >10 ng/mL for 1 week postexposure				Jakubowski et al. (2000)
Accidental exposure to WWI munition, 2 individuals, collected 1–41 days after incident (19 Jul 2004)	Patient D1: 24, 9, 5, 14, 11, 6, 2, 2, 1.5, 1.2 ng/mL for days 1 through 10 after exposure, respectively			Patient D1: 50, 17, 11, 28, 24, 14, 4.5, 9, 5, 6 ng/mL for days 1 through 10 after exposure, respectively	Barr et al. (2005)
	Patient D2: not detected days 1, 3, 6			Patient D2: 1.8, 3, 4.4 ng/mL for days 1, 3, 6, respectively	

TABLE 19.7
Published Reports (1995–2006) of Laboratory Analysis of Human Urine Samples for Glutathione Reaction Products Following a Suspected Exposure to Sulfur Mustard

Patient Sample Information: (Incident/Sample Collection Time after Suspected Exposure)	β-Lyase Metabolites: GC–MS–MS Analysis	β-Lyase Metabolites: LC–MS–MS Analysis	Bis-(*N*-Acetylcysteine) Conjugate: LC–MS–MS Analysis	References
Iranian casualties, 5 of 5 individuals, treated at Ghent hospital, collected 10 days after incident (09 March 1984)	Patient C1 = 220 ng/mL Patient C2 = 0.5 ng/mL Patient C3 = 1 ng/mL Patient C4 = 5 ng/mL Patient C5 = 1 ng/mL			Black and Read (1995a)
Kurdish casualties, 2 individuals, treated at London hospital, collected 13 days after incident (17 March 1988)	Patient L1 = 0.1 ng/mL	Patient L1: MSMTESE = <0.1 ng/mL SBMSE = ~0.1 ng/mL		Black and Read (1995a) Read and Black (2004a)
	Patient L2 = 0.3 ng/mL	Patient L2: MSMTESE = 0.1 ng/mL SBMSE = ~0.2 ng/mL		
Accidental exposure to WWI munition, 2 individuals, collected 2–3 days after incident (1992)	Patient S1 = 42 ng/mL	Patient S1: MSMTESE = 15 ng/mL SBMSE = 30 ng/mL	Patient S1 = 1 ng/mL	Black and Read (1995b) Read and Black (2004a) Read and Black (2004b)
	Patient S2 = 56 ng/mL	Patient S2: MSMTESE = 17 ng/mL SBMSE = 34 ng/mL	Patient S2 = 1 ng/mL	
Accidental exposure to WWI munition, 2 individuals, collected 1–41 days after incident (19 July 2004)	Patient D1: 41, 7, 3.3, 1.3 ng/mL for days 1–4 after exposure, respectively; 0.07–0.02 ng/mL for days 5–10 after exposure Patient D2: 2.6, 0.8, 0.08 ng/mL for days 1, 3, 6, respectively		Patient D1 = 3.1 ng/mL (unpublished Data)	Barr et al. (2005)

The final method for the analysis of blood samples to be discussed targets blood proteins in a more general approach. It was previously shown that sulfur mustard adducts of glutamic and aspartic acids to keratin could be cleaved using base (Noort et al., 2000a). Using a similar approach, precipitated proteins from plasma, whole blood, or RBCs were treated with base to liberate the sulfur mustard adduct (hydroxyethylthioethyl) from the protein. Upon release, the adduct (in the form of TDG) was derivatized and analyzed using negative-ion chemical ionization GC–MS. The lower limit of detection for the assay in plasma was 25 nM as determined using in vitro exposures of sulfur mustard in human plasma (Capacio et al., 2004).

A cautionary note regarding the limit of detection or exposure level reported for the various assays. Most of the assays for blood products report amounts of the sulfur mustard adducts that are found in samples relative to the amount of adducts that are found from in vitro exposures of whole blood or plasma at various known concentrations of sulfur mustard. The choice of whole blood versus plasma for the generation of the in vitro standard curve will obviously produce very different results. Additionally, the technique used for generation of the in vitro standards can have significant effects. For example, approximately a 30% difference was observed for the generation of two in vitro standard curves according to how the incubation of the sulfur mustard was performed in blood (Noort et al., 2004a). Higher adduct levels were observed when the sulfur mustard was allowed to react with the blood for 2 h at 37°C as opposed to 4 h at room temperature. Methods for the analysis of blood samples for sulfur mustard verification are summarized in Table 19.5.

Application to Human Exposure (Blood Samples). Blood samples following a suspected human exposure to sulfur mustard have only rarely become available for laboratory analysis. Three of the five known reports involve the analysis of samples that were taken from casualties of the Iran–Iraq War, frozen for several years and then analyzed to verify exposure as methods were developed. The other two published reports are on the analysis of blood samples obtained from three individuals that were casualties of accidental exposures to WWI munitions.

The blood from two Iranian casualties that were believed to have been exposed to sulfur mustard in 1988 was analyzed using both the ELISA method for DNA adducts and the GC–MS method for the analysis of the N-terminal valine of hemoglobin (Benschop et al., 1997). Samples were collected 22 and 26 days following the suspected exposure to sulfur mustard. One of the casualties had injuries to the skin that were consistent with an exposure to sulfur mustard, but the second casualty had injuries that were described as only ''vaguely compatible'' with sulfur mustard exposure. Both individuals had approximately the same level of hemoglobin valine adduct that was equivalent to the amount observed from a 900 nM in vitro sulfur mustard exposure in whole blood. ELISA DNA adduct levels observed in the granulocytes were also similar for both individuals, 150–160 nM. The individual with the skin injuries consistent with sulfur mustard exposure had observed ELISA DNA adduct levels in the lymphocytes that were only about half that observed in the individual with injuries that were less pronounced, 220 and 430 nM, respectively.

Blood samples obtained in 1986 from a group of Iranian casualties that were treated at a hospital in Ghent for injuries believed to have been caused by sulfur mustard were examined years later using mass spectrometry methods by Black et al. (1997b) for both valine and histidine adducts of hemoglobin. The four individuals had blood samples collected at 5 or 10 days following the suspected exposure event. Levels of the valine adduct ranged between 0.3 and 0.8 ng/mL. Observed levels of the histidine adduct were greater than the amount of valine adduct and ranged between 0.7 and 2.5 ng/mL. Using the same methodologies, Black et al. (1997b) also examined blood from one of the two individuals who were accidentally exposed to a WWI sulfur mustard munition. Several urinary metabolites indicating exposure to sulfur mustard were detected from this individual and were detailed earlier in this section. The blood sample was obtained 2 days after the exposure. The valine and histidine hemoglobin adduct levels were 0.3 and 2.5 ng/mL, respectively.

Blood samples obtained from nine Iranian casualties of sulfur mustard exposure were analyzed for the N-terminal valine adduct of hemoglobin using GC–MS (Benschop et al., 2000) and for the albumin cysteine adduct using LC–MS–MS (Noort et al., 1999). All nine individuals were

hospitalized and had skin injuries that were consistent with sulfur mustard exposure. Several of the casualties also suffered from respiratory difficulties. Blood samples were collected between 8 and 9 days after the exposure incident. Exposure levels of the patient blood samples were correlated with human whole blood that was exposed to sulfur mustard in vitro. Adduct levels for both the hemoglobin valine adduct and for the albumin cysteine adduct were in very close agreement with each other. Observed exposure levels were between 0.3 and 2 μM and 0.4 and 1.8 μM for the hemoglobin and albumin adducts, respectively.

The final exposure incident to be discussed involved the two individuals who were accidentally exposed to a WWI munition in 2004. Details of the exposure were given in the *Application to Human Exposure* (*Urine Samples*) section. This particular human exposure to sulfur mustard differed from nearly all other previous reported incidents in several important aspects. The two individuals are only the second and third casualties of a sulfur mustard exposure to have both urine and blood samples made available for laboratory testing. Generally, urine or blood samples collected and made available for sulfur mustard verification analysis are from a single time point after the exposure. In this instance, the patient with the more severe injuries (patient D1) had blood and urine collected almost daily for the first 10 days after the exposure and then again on days 28, 34, and 41. The incident also provided an opportunity to examine blood and urine metabolite levels from individuals with very different levels of injuries. Patient D1 had extensive vesication of the arm and leg, while patient D2 only suffered a single, small blister. The urinary metabolite results were detailed earlier in this section. As expected, the observed concentrations of both urine hydrolysis metabolites and glutathione reaction products were much greater in patient D1. Sulfur mustard metabolite concentrations in the blood were also much greater in patient D1. Blood metabolites were assayed using two different methods. The first assay targeted the sulfur mustard adduct to cysteine-34 of albumin using Pronase digestion of the protein followed by LC–MS–MS analysis (Noort et al., 2004b). Based on in vitro exposures of sulfur mustard in human whole blood, concentrations of albumin adducts found in the plasma of patient D1 were 350 nM on day 1 after the exposure and had decreased by 74% (90 nM) on day 41 (Barr et al., 2005). The rate of decrease over that time period was consistent with the reported half-life of human albumin of 21 days. Albumin adduct concentrations for patient D2 over the sample collection period of 1–6 days after exposure remained stable and ranged between 16 and 18 nM. The second assay targeted plasma protein adducts by cleaving the adduct with base followed by analysis of the derivatized adduct using negative ion chemical ionization GC–MS (Capacio et al., 2004). This was the first reported use of this assay in the verification of a human exposure to sulfur mustard. Concentrations of the plasma protein adducts were 97 nM on day 1 and decreased by 76% (23 nM) by day 41 based on in vitro exposures of sulfur mustard in human plasma instead of whole blood (Korte et al., 2005). The assay could not detect plasma protein adducts in patient D2. The assay was modified slightly to lower the reported lower limit of detection of 25 nM, but the limited amount of plasma received did not permit reanalysis for patient D2 using the modified method.

Blood provides several options for assessing potential exposure to sulfur mustard because a variety of different metabolites have been verified in human exposure cases. Most of the assays are sensitive and selective, and the majority of the methods use gas or liquid chromatography combined with mass spectrometric techniques. Background levels have not been found in the blood from unexposed individuals. The time periods between exposure and sample collection, along with the severity of the injury, which will impact sensitivity requirements of the assay, are probably the most important considerations when considering the appropriate assay. Currently, the most sensitive assay targets the alkylated cysteine of albumin, but alkylated hemoglobin should offer a biomarker of greater longevity. A summary of published reports for the analysis of blood samples to verify human exposure to sulfur mustard is presented in Table 19.8.

Analysis of Other Biomedical Sample Types (Tissue, Hair, Skin). Urine and blood have been the traditional biomedical samples of choice for sulfur mustard verification. There have only been a limited number of reports on the analysis of other types of biomedical samples.

TABLE 19.8
Published Reports (1997–2006) of Laboratory Analysis of Human Blood Samples Following a Suspected Exposure to Sulfur Mustard

Patient Sample Information: (Incident/Sample Collection Time after Suspected Exposure)	DNA Adduct: N7-(2-HETE)-2′-Deoxyguanosine	Hemoglobin Adduct: (HETE)-N-Terminal Valine	Hemoglobin Adduct: (HETE)-Histidine	Albumin Adduct: *S*-[2-(HETE)]-Cys-Pro-Phe	Blood Protein Adducts: (HETE)-Aspartic and Glutamic Acids	References
Iranian casualties, 2 individuals, collected 22 days (P1) and 26 days (P2) after incident (1988)	P1: Lymphocytes = 220 nM Granulocytes = 160 nM P2: Lymphocytes = 430 nM Granulocytes = 150 nM	P1: 900 nM P2: 900 nM				Benschop et al. (1997)
Iranian casualties, 4 individuals, treated at Ghent hospital; collected 5 and 10 days after incident (1986)		Range: 0.3–0.8 ng/mL	Range: 0.7–2.5 ng/mL			Black et al. (1997b)
Accidental exposure to WWI munition; 1 individual, collected 2 days after incident (1992)		0.3 ng/mL	2.5 ng/mL			Black et al. (1997b)
Iranian casualties, 9 individuals, treated at Utrecht hospital; collected 8–9 days after incident (1986)		Range: 0.3–2 μM		Range: 0.4–1.8 μM		Noort et al. (1999) Benschop et al. (2000)

(*continued*)

TABLE 19.8 (Continued)
Published Reports (1997–2006) of Laboratory Analysis of Human Blood Samples Following a Suspected Exposure to Sulfur Mustard

Patient Sample Information: (Incident/Sample Collection Time after Suspected Exposure)	DNA Adduct: N7-(2-HETE)-2′-Deoxyguanosine	Hemoglobin Adduct: (HETE)-N-Terminal Valine	Hemoglobin Adduct: (HETE)-Histidine	Albumin Adduct: *S*-[2-(HETE)]-Cys-Pro-Phe	Blood Protein Adducts: (HETE)-Aspartic and Glutamic Acids	References
Accidental exposure to WWI munition; 2 individuals, collected 1–41 days after incident (2004)				Patient D1: 350 and 90 nM for days 1 and 41 after exposure, respectively Patient D2: 16–18 nM for days 1, 3, 6 after exposure	Patient D1: 97 and 23 nM for days 1 and 41 after exposure, respectively	Barr et al. (2005) Korte et al. (2005)

HETE, hydroxyethylthioethyl.

Analytical Methods for Tissue, Hair, Skin. Drasch et al. (1987) analyzed several tissue types for unmetabolized sulfur mustard using the same method as previously described in the *Analytical Methods for Urine* section. Hair specimens have been analyzed for unmetabolized sulfur mustard using a methylene chloride extraction of the hair sample followed by analysis with GC–MS (United Nations, 1986). Immunochemical detection of sulfur mustard adducts have been developed for keratin in human skin (van der Schans et al., 2002) and for DNA in skin (van der Schans et al., 2004).

Application to Human Exposure (Tissue, Hair, Skin). Tissue samples taken during the autopsy of an Iranian casualty to sulfur mustard and stored at −20°C for 1 year were analyzed by Drasch et al. (1987) for unmetabolized sulfur mustard as described above. Sulfur mustard was found in the highest concentrations in fat, skin, brain, and kidney; concentrations ranged from 5 to 15 mg/kg. Lesser amounts were found in muscle, liver, spleen, and lung and ranged from approximately 1 to 2 mg/kg. Hair obtained from two casualties of the Iran–Iraq War was examined by a United Nations inspection team as part of an alleged use investigation. One of the hair samples (0.58 g) produced a positive response for sulfur mustard estimated to be between 0.5 and 1.0 μg/gram. The second patient's hair sample tested negative for sulfur mustard. To date, skin samples have not been analyzed for sulfur mustard adducts to keratin following a human exposure.

Lewisite. Lewisite is a small molecular weight arsenical vesicant that is primarily found in the trans isomeric form, although it also exists in cis and geminal forms (Smith et al., 1995). Stockpiles of lewisite or lewisite mixed with sulfur mustard reportedly exist in a number of countries and present a potential risk of accidental exposure. Most analytical methods that have been reported in the open scientific literature regarding lewisite or related compounds are for the sample preparation and analysis of environmental samples. In the past 10 years, there have only been a handful of reports regarding the analysis of biomedical samples to measure lewisite exposure in animals. Much like the other CWAs, lewisite is readily hydrolyzed in aqueous solutions, including biological fluids. Therefore, the likelihood of finding the parent compound in a biomedical sample, such as blood or urine, would be minimal. Consequently, method development has focused on the breakdown compounds of lewisite or on products formed from its interaction with biomolecules. Until recently, most assays for lewisite have involved the analysis of elemental arsenic using techniques such as atomic absorption spectroscopy. A drawback of this approach is the lack of specificity because arsenic is ubiquitous throughout the environment. In addition to naturally occurring sources, arsenic is also found in some commercial products and food items (particularly marine organisms). Arsenic is also a byproduct of several industrial processes. Consequently, analytical methods designed to measure arsenic levels lack the specificity required for verification of lewisite or one of its related compounds.

Lewisite will rapidly react with water to form chlorovinylarsonous acid (CVAA). CVAA will slowly convert to the arsinoxide form and polymerization reactions can also occur. Earlier studies indicated that animals (species not specified) exposed to lewisite either topically or via injection were found to have measurable levels of CVAA in the urine and throughout the digestive system (Waters and Williams, 1950).

Analytical Methods for Urine and Blood. Specific biomarkers of lewisite exposure are currently based on a very limited number of in vitro experiments (Jakubowski et al., 1993; Wooten et al., 2002) and animal studies (Logan et al., 1999; Fidder et al., 2000). Wooten et al. (2002) developed a solid-phase microextraction (SPME) headspace sampling method for urine samples followed by GC–MS analysis. It is the most sensitive method reported to date with a lower limit of detection of 7.4 pg/mL. Animal experiments have been limited in number and in their scope. In one study of four animals, guinea pigs were given a subcutaneous dose of lewisite (0.5 mg/kg). Urine samples were analyzed for CVAA using both GC–MS and GC coupled with an atomic emission spectrometer set for elemental arsenic (Logan et al., 1999). The excretion profile indicated a very rapid elimination of CVAA in the urine. The mean concentrations detected were 3.5 μg/mL, 250 ng/mL, and 50 ng/mL for the 0–8, 8–16, and 16–24 h samples, respectively. Trace level concentrations

(0–10 ng/mL) of CVAA were detected in the urine of the 24–32 and 32–40 h samples. The second animal study also used a subcutaneous dose of lewisite (0.25 mg/kg) given to four guinea pigs (Fidder et al., 2000). Using GC–MS, CVAA was observed in urine samples up to 12 h following exposure. In this same experiment, blood from the animals was also analyzed using GC–MS for CVAA. The amount of measured CVAA was the sum of CVAA that was displaced from hemoglobin along with free CVAA in the blood. The assay was able to detect the analyte at 10 days after the exposure, although the concentration was only 10% of that found at 24 h after exposure. Following the incubation of human blood with radiolabeled lewisite, Fidder et al. (2000) found that 90% of the radioactivity was associated with the RBCs, and 25%–50% was found with the globin. Due to the reactive nature of CVAA, derivatization using a thiol compound has generally been applied as part of the sample preparation process (see Figure 19.6).

Application to Human Exposure (Urine and Blood). To date, there have been no reports of the collection of biomedical samples from individuals with suspected lewisite exposure. Samples from such an incident will be critical for confirming the validity of assaying for the biomarkers observed in animal models. Additionally, the biomarkers that have been investigated in animal studies to date have indicated a rapid clearance in urine and less so for blood. This will obviously create severe problems for the retrospective determination of lewisite exposure beyond a few days at most when analyzing urine samples. The blood assay for both bound and free CVAA will potentially provide a longer opportunity for retrospective confirmation of exposure (based on one animal study), but also indicates a substantial decrease (90%) in concentration levels observed over a 10 day period.

V. CYANIDE

Cyanide is a common industrial chemical that has many uses, and it is produced in large quantities across the world as described in Chapter 14. Synonyms for HCN include hydrogen cyanide, prussic acid, formic anamminide, and fromonitrile (Agency for Toxic Substances and Disease Registry, 1997). Analytical methods are based upon the physiochemical state of cyanide and its interaction in the body.

Cyanide is the CN ion. At physiological pH, it exists as HCN in the body. The mechanism of cyanide toxicity is believed to be the inactivation of iron III (ferric) enzymes in the body. The inhibition of cytochrome oxidase, which disrupts mitochondrial oxidative phosphorylation, is thought to be the most important mechanism for cyanide toxicity (Warburg, 1911; Keilin, 1929; DiPalma, 1971; Agency for Toxic Substances and Disease Registry, 1997). Cyanide also binds to the hemoglobin in erythrocytes (Farooqui and Ahmed, 1982). The binding of cyanide to Fe(III) enzymes and proteins is reversible (DiPalma, 1971; Way, 1984).

At low concentrations, 93%–99% of total cyanide is bound to methemoglobin (metHb) in the erythrocytes (Agency for Toxic Substances and Disease Registry, 1997). The metHb is the deoxygenated Fe(III) form of Hb. Cyanide has a very high affinity to metHb and a low affinity to the oxygenated form of Hb. Usually less than 1% of the total Hb is in the metHb form, so at increased concentrations of cyanide in the blood, a larger amount is found in the serum. In tissue, cyanide binds to the heme group in mitochondrial cytochrome oxidase and inhibits electron transport (DiPalma, 1971). All tissues are affected by this enzymatic inhibition, but especially those that require high amounts of oxygen and ATP. Other proteins and enzymes are also affected by cyanide, such as superoxide dismutase and xanthine oxidase, and may also contribute to its toxic affects (Agency for Toxic Substances and Disease Registry, 1997).

Cyanide metabolism in the body is very rapid and can occur at 0.017 mg/min/kg (Agency for Toxic Substances and Disease Registry, 1997). The most common form of metabolism is the conversion to thiocyanate (SCN^-), which is then excreted via the kidneys (Blakley and Coop, 1949; Wood and Cooley, 1956). The mitochondrial enzyme rhodanese (thiosulfate sulfur transferase) is thought to be the main catalyst for the formation of thiocyanate; but β-mercaptopyruvate-cyanide

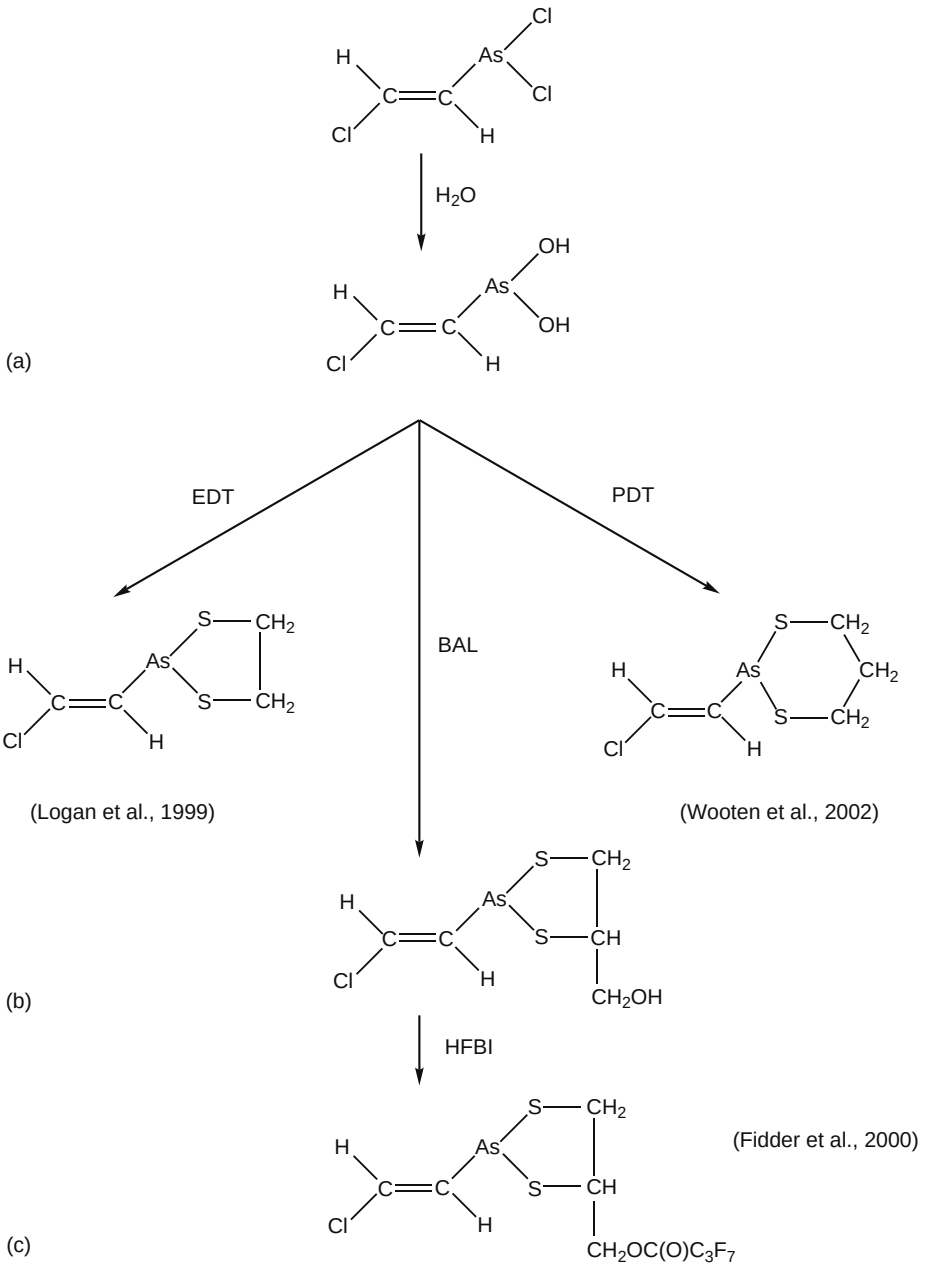

FIGURE 19.6 Published analytical approaches for the analysis of CVAA in urine (a) reaction of lewisite (trans isomer shown) with water to form CVAA, (b) reactions of CVAA with various thiols, and (c) derivatization using HFBI.

transferase can transform cyanide to thiocyanate through a different route (Way, 1984; Smith, 1995). The conversion to SCN^- is thought to be limited by the amount of thiosulfate. Cysteine, cystine, glutathione, and β-mercaptopyruvic acid can also be sulfur sources (Way, 1984; Smith, 1995; Agency for Toxic Substances and Disease Registry, 1997). The reaction with cystine to form 2-iminothiozoline-4-carboxylic acid is also an important pathway leading to 15% of the urinary excretion (Wood and Cooley, 1956). Other elimination pathways are the exhalation of HCN

and oxidation to cyanate (OCN^-) and reactions with vitamin B_{12} to form cyanocobalamin (Ansell and Lewis, 1970).

There are several matrices that have been used to assess cyanide exposure. Whole blood is the most common, but measurements have been made in serum, plasma, saliva, tissues, gastric aspirate, and urine. Whole blood has been the matrix of choice, thus far, to determine cyanide exposure in humans. There are problems associated with cyanide and thiocyanate measurements in blood. The sample collection, storage, and preparation are very important. Varied levels of cyanide have been found in samples collected from different vessels (venous, arterial, and ventricular). Different whole blood samples can contain different amounts of metHb, which has a high affinity to cyanide. Also, free HCN may be more important because it is the form that reacts with cytochrome oxidase and causes the most significant adverse health effects (Agency for Toxic Substances and Disease Registry, 1997). Storage of the whole blood is also critical but not well understood. Some studies have shown an increase in cyanide of up to 40% upon storage of whole blood for 1 week and a 14% increase after 1 day at 4°C, while others have shown a decrease of 20%–30% within 1 day at 4°C and continued decreases over a 2 week period. A decrease in cyanide levels was also seen when whole blood was stored at 2°C. Storage at −20°C has also been disputed with some studies showing up to 3.5 times the original cyanide levels and others showing no change (Seto, 1996). These differences appear to be independent of the original cyanide concentrations (Ballantyne, 1983).

Prior to detection, cyanide must first be separated from hemoglobin. This separation is most often done with sulfuric acid, followed by microdiffusion in a Conway cell. There have been some problems associated with the Conway cell. There are other methods that use acid to release the cyanide followed by a variety of techniques. Most of these methods are colorimetric and rely on the König reaction to produce a dye that is quantified using spectrophotometry. Cyanide and thiocyanate both react and must be separated using microdiffusion or distillation. Additionally, most of these methods are time consuming and suffer from lack of specificity or sensitivity. Detection limits using this type of approach are generally in the high parts-per-billion (ppb) range. There have also been assays developed that acidify the solution and then sample the head space for HCN followed by GC–NPD or derivatization followed by GC–ECD detection. Detection limits by NPD and ECD have been in the high ppb range. Cyanide has been measured in RBCs by HPLC after derivatization with fluorescence detection.

Methods to detect cyanide exposure in human urine, saliva, and either serum or plasma have concentrated on thiocyanate. These methods include derivatization with HPLC/UV detection, derivatization with spectrophotometric detection or GC with ECD detection. Some urine methods have measured the urinary metabolite, 2-aminothiozoline-4-carboxylic acid.

Unfortunately, reference range values for cyanide in various matrices tend to vary greatly on the study and on the method of analysis. Thus, a reference range will need to be established for any given method. A statistically relevant population will need to be established, and a reference method will need to be run on a group of individuals. In some of the studies, the range of blood cyanide levels in normal populations is less than 150 ppb and urinary thiocyanate less than 1.0 mg/mL (Agency for Toxic Substances and Disease Registry, 1997). Smokers have much higher cyanide levels than nonsmokers; blood cyanide levels in some smokers as high as 500 ng/mL have been reported, which is 50 times higher than that typically reported for nonsmokers (Agency for Toxic Substances and Disease Registry, 1997). See Table 19.9 for analytical methods used to determine cyanide in biological samples.

VI. PHOSGENE

Phosgene, also known as carbonyl chloride ($COCl_2$), was used extensively in WWI and caused more deaths than any other agent (Mathur and Krishna, 1992). It is now a widely used toxic industrial chemical (TIC). In 2002, the global production of phosgene was estimated to be over 5 million metric tons (Malveda, 2003), most of which is consumed at the site of production

TABLE 19.9
Analytical Methods for Determining Cyanide in Biological Samples (Modified from Table 6.1 in ATSDR's Toxicological Profile for Cyanide)

Sample Matrix	Preparation Method	Analysis Method	LOD	% Recovery	References
Blood	Separation in MDC; derivatization	Spectrophotometry	0.1 ppm	NR	Morgan and Way (1980)
Blood	Separation in MDC; derivatization	Spectrofluorometry (total CN)	0.025 ppm	NR	Ganjeloo et al. (1980)
Plasma	Deproteination with TCA; derivatization	Spectrophotometry (SCN–CN determination)	~0.07 ppm	96 (SCN)	Pettigrew and Fell (1972)
Erythrocyte suspension	Sample purged; absorption of HCN in NaOH; oxidation of SCN	Spectrophotometry (SCN–CN determination)	NR	93–97	McMillan and Svoboda (1982)
Blood cells	Centrifugation to separate cells; extraction; derivatization	HPLC-fluorescence detection	0.002 ppm	83	Sano et al. (1992)
Blood	Acidification	Headspace GC–NPD	~0.03 ppm	NR	Levin et al. (1990)
Blood	Acidification; derivatization	Headspace GC–ECD	0.1 ppm	NR	Odoul et al. (1994)
Blood	Separation in MDC; color development	Spectrophotometry	~0.07 ppm	NR	Laforge et al. (1994)
Blood	Incubation of acidified sample	GC–NPD	0.001 ppm	NR	Seto et al. (1993)
Blood	Separation in MDC; absorption in methemoglobin	Spectrophotometric (free CN)	0.4 ppm	~80	Tomoda and Hashimoto (1991)
Blood	Acidification	GC–NPD	0.014 ppm	86%–99%	Calafat and Stanfil (2002)
Blood	Microdiffusion, derivatization,	Isotope dilution LC–MS	5 ppb	N/A	Tracqui et al. (2002)
Blood and liver	Sample digestion; treatment with lead acetate; absorption with NaOH	Specific ion electrode (total CN)	0.005 ppm	100–109	Egekeze and Oehme (1979)
Blood and urine	Separation in MDC; derivatization	Spectrofluorometric	0.008 ppm	66–83 (blood) 76–82 (urine)	Sano et al. (1989)
Urine	Dilution; derivatization	Spectrophotometry (SCN–CN determination)	~0.07 ppm	88 (SCN)	Pettigrew and Fell (1972)
Saliva	Derivatization	HPLC-UV (SCN)	2 ng (on instrument)	95–99	Liu and Yun (1993)
Serum, urine, saliva	Extraction of buffered sample with isoamyl acetate	Flame AAS (SCN)	0.004 ppm	96–102	Chattaraj and Das (1992)
Serum	Addition of acetonitrile; centrifugation; separation	Spectrophotometry (SCN)	0.3 ppm	94	Li et al. (1993)
Urine, saliva	Basify; derivatization; extraction; back extraction	GC–ECD (SCN)	~0.033	83–106	Chen et al. (1994)

(*continued*)

TABLE 19.9 (Continued)
Analytical Methods for Determining Cyanide in Biological Samples (Modified from Table 6.1 in ATSDR's Toxicological Profile for Cyanide)

Sample Matrix	Preparation Method	Analysis Method	LOD	% Recovery	References
Urine, saliva	Dilution; filtration	Ion chromatography-UV (SCN)	0.02	95–101	Michigami et al. (1992)
Urine	Ion chromatography; acidification; derivatization	Spectrophotometry (SCN)	~0.145 ppm (lowest reported)	NR	Tominaga and Midio (1991)
Urine	Dilution; solid-phase extraction	Suppressed ion chromatography with conductivity detection	~0.011 ppm	NR	Miura and Koh (1991)
Urine (2-aminothiozoline-4-carboxylic acid)	Cation exchange; reduction; derivatization	Suppressed ion chromatography with fluorescence detection	~0.03 ppm	NR	Lundquist et al. (1995)
Urine (2-aminothiozoline-4-carboxylic acid)	Solid-phase extraction and derivatization	Isotope dilution GC–MS	25 ppb	N/A	Logue et al. (2005)

LOD, limit of detection; NR, not reported; MDC, mircodiffusion cell; TCA, trichloroacetic acid; AAS, atomic absorption spectrometry; UV, ultraviolet absorbance detection; ECD, electron capture detection; NPD, nitrogen phosphorus detection; GC–MSD, gas chromatography–mass selective detection; GC, gas chromatography; ATC, 2-amino-thiazoline-4-carboxylic acid.

(Chemistry Business, 2002). The first synthesis of phosgene was performed in 1812 by exposing a mixture of chlorine and carbon monoxide to sunlight (Sartori, 1939). During WWI, it was produced in bulk by the reaction of chlorine and carbon monoxide in the presence of activated carbon catalyst (Sartori, 1939). Phosgene is produced generally in the same way today but with higher efficiency due to newer high surface area catalysts (Parshall, 2002). Phosgene is an important intermediate in many industrial products including the production of insecticides, isocyanates, plastics, dyes, and resins (Malveda, 2003). Additionally, phosgene is formed during the combustion of chlorinated hydrocarbons during fires (Diller, 1978) and by the photoxidation of chlorinated solvents in the atmosphere (National Research Council (US) Committee on Toxicology, 1984).

At normal room temperatures (25°C), phosgene is a fuming liquid with a boiling point of 47°F and a vapor pressure of 1.6 atm at the boiling point. The gas is heavier than air with a relative density of 4.39 at 20°C. This density leads to collection of phosgene gas in low-lying areas. The odor of phosgene is somewhat sweet and resembles that of freshly cut grass or hay. At higher concentrations, the odor of phosgene becomes pungent or burning and causes rapid olfactory fatigue (Borak and Diller, 2001).

Exposure to phosgene gas causes irritation of the eyes, nose, throat, and respiratory tract. Higher phosgene gas exposures can lead to pulmonary edema and death. Exposure to liquid phosgene by direct skin or eye contact is rare but is thought to produce localized severe burns (Borak and Diller, 2001). Inhalation is the most toxic exposure route for phosgene and the route thought to cause most of the injuries and deaths during WWI. Inhalation of phosgene would require advanced treatment techniques.

Phosgene is a powerful acylating agent that reacts with nucleophiles, such as amines, sulfides, or hydroxyls. The toxicity of phosgene was originally thought to be due to the generation of HCl during the reaction of phosgene with moisture in the body. Later, acylation reactions were found to be responsible for a majority of phosgene's toxic effects. Phosgene is greater than 800 times more toxic than HCl. Free amines protect against phosgene poisoning but not against the toxic effects of HCl, phosgene inhibits coenzyme I whereas HCl does not, and chemically similar compounds, such as ketene, that do not have chlorine to generate HCl, have similar toxicity to phosgene (Diller, 1985). Furthermore, the rate of reaction of phosgene with free amines has been found to be much higher than the reaction of phosgene with water. In a solution of aniline in water, phosgene reacts almost exclusively with the aniline (Sartori, 1939).

Most of the data on health effects from phosgene is from inhalation exposure. Regulatory threshold limit value (TLV, 8 h time-weighted average) for phosgene has been established by National Institute for Occupational Safety and Health (NIOSH), an institute within the Centers for Disease Control and Prevention (CDC), as 0.1 ppm (NIOSH, 2004), with the 60 min and 24 h emergency exposure limits of 0.2 and 0.02 ppm, respectively (National Research Council [US] Committee on Toxicology, 1984). The estimated human 50% lethal exposure level (LCT_{50}) of phosgene concentrations in air related to exposure time through inhalation is 500 ppm/min (Borak and Diller, 2001). The LCT_{100} is estimated to range from 1300 ppm/min (Borak and Diller, 2001) to 1600 ppm/min (Cucinell, 1974). The nonlethal levels of phosgene are estimated to be less than 300 ppm/min, and 25 ppm/min is regarded as the threshold for lung damage (Borak and Diller, 2001). The recognition of the phosgene odor occurs at levels greater than 1.5 ppm/min, with irritation in the mucous membranes at 3 ppm/min or higher (Diller, 1985). Exposure limits that cause adverse health effects can be reached either by a longer exposure to lower concentration or a shorter exposure to higher concentration. In one study, however, workers exposed daily to phosgene concentrations above 1 ppm but less than 50 ppm showed no difference in mortality/morbidity as compared to workers in the same plant who were unexposed (Polednak, 1980).

The concentrations of phosgene in air that cause acute effects have been studied in many animal models. A review of previous animal studies in the literature was performed by Diller to estimate the approximate inhalation dose–toxicity relationship for many species of animals (Diller and Zante,

1982). In this report, the LCT_{50} of animals was approximated to range from 200 ppm/min for cats to 2000 ppm/min for goats. Guinea pigs and mice had the same approximate value as humans, 500 ppm/min. Dogs and rabbits had higher LCT_{50} values than humans (1000 and 1500 ppm/min, respectively) whereas nonhuman primates and rats had estimated lower LCT_{50} values than humans (300 and 400 ppm/min, respectively) (Diller and Zante, 1982).

Phosgene Metabolism and Markers for Phosgene Exposure. Phosgene is very reactive and is believed to be quickly transformed in vivo. Phosgene reacts with amino, hydroxyl, and thiol groups. In the blood, phosgene can react with a variety of proteins, including albumin and hemoglobin. It also reacts with glutathione and with cysteine. It is important to note that chloroform is metabolized to phosgene in the body. Thus, it is expected that low levels of the protein adducts and metabolites of phosgene will most likely be in the general background population due to low level incidental exposure to chloroform. Studies will be needed to accurately determine this reference range.

Glutathione and Glutathione Adducts. Phosgene is a highly reactive acylating agent and thus, is expected to interact with the antioxidant defense system of the body. Glutathione (GSH) is a tri-peptide thiol consisting of cysteine, glutamic acid, and glycine, which serves as both a scavenger of reactive compounds in protection of cells and as a store for cysteine moieties (Pastore et al., 2003). GSH is found in general concentrations in healthy adults in the millimolar range (Richie et al., 1996; Pastore et al., 2003). GSH can be oxidized to the glutathione disulfide (GSSG), a simple dimer of GSH joined by a disulfide linkage. The ratio of the GSH redox couple in vitro has been determined to be between 100:1 and 10:1 GSH to GSSG (Richie et al., 1996; Pastore et al., 2003). When depleted, glutathione fails to protect cellular oxidation and leads to irreversible oxidative damage (Reed and Fariss, 1994).

Phosgene was found to react with GSH to form an acylated dimer, diglutathionyl dithiocarbonate (GSCOSG, Figure 19.7). This marker for phosgene metabolism was first found by Pohl et al. (1981) in the metabolism of chloroform in the liver, where phosgene was believed to be generated by enzymatic action from chloroform. The bis-glutathione adduct GSCOSG was detected in vivo in the bile of rats exposed to chloroform and in vitro in rat liver microsomes. Pohl et al. (1981) also found a decrease in GSH levels in these rats. GSCOSG was also found directly by mixing phosgene with GSH in buffer solution (Fabrizi et al., 2001).

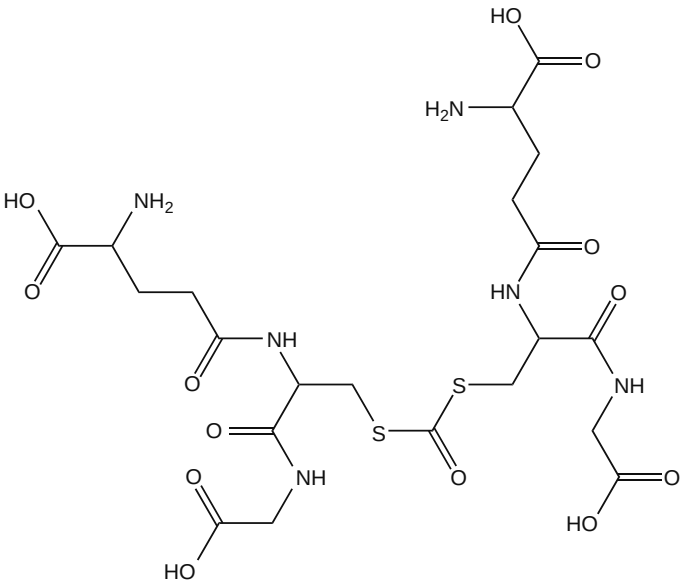

FIGURE 19.7 Structure of diglutathionyl dithiocarbonate.

The in vivo generation of GSSG and carbon monoxide has also been reported in the blood of mice exposed to haloforms (Anders et al., 1978). The observation of GSSG and CO may be linked to the further metabolism of GSCOSG after generation, but there are no studies that have identified this potential relationship.

Glutathione is also found in tissue and thus, similar reactions with GSH and phosgene are expected in the bronchoalveolar region. In the excised lung tissue from rabbits and mice inhalationally exposed to phosgene, there was little change in tissue total GSH concentration, but the reduced GSH levels fell significantly and GSSG levels increased significantly (Sciuto et al., 1997; Sciuto and Hurt, 2004). In these studies, the reduced GSH as percent of total was 41% less than the control subject. The collection of lung tissue samples from humans that are thought to be exposed to phosgene is impractical but the collection of bronchoalveolar lavage fluid is possible. Sciuto has reported on the concentrations of GSSG, GSH, and total GSH in bronchoalveolar lavage fluid of rodents with severe changes in the GSH redox state following inhalation of phosgene (Sciuto, 1998; Sciuto et al., 2003). In both these studies, increases in total GSH levels were observed, but the redox state of the GSH was not reported. It could be anticipated that increased levels of the oxidized form of glutathione could be a better indicator of phosgene exposure, but at this time only total GSH has been correlated with phosgene inhalation in lavage fluid. It should also be noted that in the studies reported by Sciuto (1998), there was no discrimination determined for GSSG and GSCOSG. Because these two species are similar in structure, it is unclear whether the reported detection of GSSG includes a component from GSCOSG.

The detection of GSCOSG, increased GSSG levels, and decreased GSH levels following phosgene exposure may hold promise for the detection of phosgene exposure, but there are considerable obstacles in some of these approaches. The levels of total GSH and changes in GSH redox state in animal models were determined shortly after exposure to phosgene. The lifetime of GSCOSG is not known, and even though the levels of total GSH in mice exposed to phosgene were significantly higher than controls up to 24 h postexposure, the GSH levels were only marginally above controls at times up to 7 days (Sciuto, 2003). Furthermore, there are large variations in levels of GSH and GSSH in human subjects, which would require predetermined baseline levels for each patient (Richie et al., 1996; Pastore et al., 2003). The formation of GSCOSG may be specific for phosgene (or chlorinated compounds that metabolize to phosgene). A reference range study of GSCOSG levels in people with no known exposure to phosgene would be required to use this biomarker for phosgene exposure.

Cysteine Adducts. Phosgene has also been reported to form an adduct with the thiol amino acid cysteine (Kubic and Anders, 1980). Cysteine is a building block of glutathione and is the rate-limiting step in generation of glutathione for antioxidant protection of tissue (Gwilt et al., 1998). As in the case of GSH, cysteine can be reduced to the disulfide cysteine dimer, cystine. Cysteine is generated in the body by the conversion of methionine through the cystathionine pathway. The average concentration of cysteine in humans is typically lower than glutathione and have been approximated in one report at 250 μM in plasma and 400 μM in urine (Pastore et al., 1998).

The reaction of phosgene and cysteine in vitro was first reported by Kubic and Anders (1980), and the product was identified as 2-oxothiazolidine-4-carboxylic acid (OTC, OTZ, or also known as procysteine). The structure of OTZ is shown in Figure 19.8. OTZ was formed by incubating hepatic microsomal fractions with chloroform, NADPH, and cysteine (Kubic and Anders, 1980). Isotopic studies showed that chloroform was first metabolized to phosgene by cytochrome P-450. The phosgene then reacted with cysteine to form OTZ. Synthetic routes to OTZ also have indicated the generation from cysteine and phosgene (Boettcher, 1984).

OTZ is a pro-drug of cysteine and is rapidly converted by 5-oxoprolinase to cysteine in vivo (Gwilt et al., 1998). Studies have indicated that the lifetime of OTZ in the human body varies from 3 to 8 h (Porta et al., 1991; Gwilt et al., 1998). The rapid elimination of OTZ limits its usefulness as a biomarker for phosgene unless samples are obtained quickly after a suspected exposure.

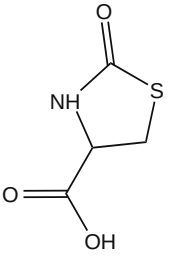

FIGURE 19.8 Structure of 2-oxothiazolidine-4-carboxylic acid.

Other possible markers for phosgene exposure may be the reduction of cysteine to its disulfide, cystine, upon exposure to phosgene. This reaction would not be specific to phosgene exposure, however. The formation of the acylated dimer Cys–CO–Cys has been shown in vitro when cysteine is treated with phosgene in solution, and this reaction also occurs in the presence of GSH (Fabrizi et al., 2001).

Small Molecule Adducts. Inhalational phosgene exposure has been reported to react with other components in the lungs. In 1933, treatment of lung pulp with phosgene was found to form the chlorocarbonic ester of cholesterol (Kling, 1933). Kling also indicated that there was a destruction of the hydrophilic character of the fats in the cells due the loss of free sterols and suggested this was the cause of acute pulmonary edema.

Phosgene has also been shown to form adducts with phospholipids. In a study of chloroform metabolism, a single phospholipid adduct was thought to be related to phosphatidylethanolamine (PE) because PE was the only phospolipid found to be depleted (Cowlen et al., 1984). In other studies on the metabolism of chloroform, a single phosgene adduct was found and identified as an adduct of PE with the phosgene carbonyl group bound to the amine of the head group of PE (DeBiasi et al., 1992; Guastadisegni et al., 1998). The adduct has been identified as a dimer of PE, the two subsistents linked at the amine of the head group by the carbonyl from phosgene (Guastadisegni et al., 1999), and this adduct has been implicated as the critical alteration leading to cell death by chloroform metabolism (Di Consiglio et al., 2001). Nonspecific increases in the phospholipid content of lavage fluid have been observed in rats 6 h after exposure to inhalational phosgene, but specific adducts were not monitored (Jugg et al., 1999).

Macromolecule Adducts. The damage of lung tissue after phosgene exposure that leads to permeability of the blood–air barrier and pulmonary edema is likely due, in part, to reactions of phosgene with various macromolecules, including proteins. The permeability of the blood–air barrier also raises the possibility of having phosgene enter the bloodstream and form adducts with blood proteins. Some work has been done to identify and develop methods for the identification and quantification of phosgene adducts with abundant blood proteins.

The initial evidence that phosgene reacts and forms an adduct with hemoglobin was from a study of the metabolism of chloroform. In this study, Pereira et al. (1984) allowed chloroform to be metabolized with liver microsomes. One of the initial metabolites of chloroform is believed to be phosgene. The authors then identified *N*-hydroxymethyl cysteine by GC–MS, resulting from the reaction of phosgene of cysteine moiety in hemoglobin (Pereira et al., 1984). Additionally, Fabrizi et al. (2001) identified adducts of phosgene with pentapeptides of lysozyme and the N-terminal peptide from human histone H2B even when these peptides were treated with phosgene in solution in the presence of a known phosgene scavenger, GSH (Sciuto et al., 1997). There is some evidence to suggest that phosgene reacts and forms blood protein adducts in vivo. Sciuto et al. (1996) reported spectrophotometric differences in plasma from mice, rats, and guinea pigs exposed to high doses of phosgene. This study also suggested that phosgene could directly attack RBCs by observation of the shifted fragility curve for erythrocytes (Scuito et al., 1996).

Noort et al. (2000b) at the TNO in The Netherlands have developed methods to detect and quantify phosgene adducts with both hemoglobin and albumin. Treatment of whole blood with ^{14}C-labeled phosgene showed that phosgene reacted with both albumin and hemoglobin and that there was a higher level of adduct formation with the albumin than with the hemoglobin. The analysis of the albumin-^{14}C-labeled phosgene adduct indicated that the major site of adduct formation was an intramolecular Lys–Lys adduct with a CO bridge (Noort et al., 2000b). Analysis of the hemoglobin–phosgene adduct showed the presence of a hydantoin function between the N-terminal valine and the NH of leucine in a fragment containing amino acids 1–5 (Noort et al., 2000b).

Noort et al. (2000b) were able to achieve detection of phosgene–hemoglobin at levels of 1 mM phosgene in whole blood. The phosgene–albumin adduct could be detected at three orders of magnitude lower concentrations of phosgene-treated whole blood (1μM). The higher detection limits of the globin adduct were reported to be in part due to an interference of natural hydantoin function in the same position as the phosgene adduct (Noort et al., 2000b). The method for the more sensitive albumin-based method included isolation of the albumin by affinity chromatography, carboxymethylation, dialysis, tryptic digestion, followed by micro-LC–MS–MS on either a high resolution MS on a Q-TOF or multiple reaction monitoring (MRM) on a triple quadrupole mass spectrometer. The MRM experiment employed monitoring of the fragmentation of the doubly charged ion at 861 to both the 747.5 and 773.6 transitions. Noort et al. (2000b) estimated that the method in vivo should be able to detect a 320 mg min/m^3 exposure, assuming a 10% absorption of the dose by the blood, resulting in an adduct concentration of 1.3 μM, just above the detection limit for the albumin adduct.

VII. BZ (3-QUINUCLIDINYL BENZILATE)

Background. 3-Quinuclidinyl benzilate (see Figure 19.9) is an anticholinergic glycolate that is commonly termed ''QNB'' and has been designated ''Agent BZ'' by the military. BZ is the only known incapacitating agent that has ever been weaponized for use by the U.S. military. That occurred in the early 1960s, and BZ was produced at the Pine Bluff Arsenal between 1962 and 1965. This agent was subsequently dropped from the chemical arsenal for several reasons, including concerns about variable and unpredictable effects.

The action of BZ is very similar to other anticholinergics, such as atropine and scopolamine, differing mainly in potency and duration of effect. The ID_{50} of BZ is about 6.2 μg/kg (about 500 ng/person). Centrally, BZ is about 25 times as potent as atropine, but only about threefold more active than scopolamine. However, the duration of action of BZ is typically much longer, and the uptake by an oral route is about 80% of i.v. or i.m. routes. Under optimal conditions, BZ is also about 40%–50% as effective by inhalation as by injection. Initial symptoms typically occur 30 min to 4 h after exposure. Full recovery may require 3–4 days.

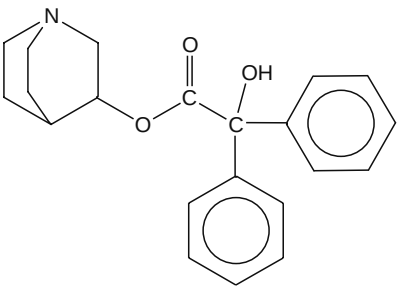

FIGURE 19.9 Structure of 3-quinuclidinyl benzilate (BZ).

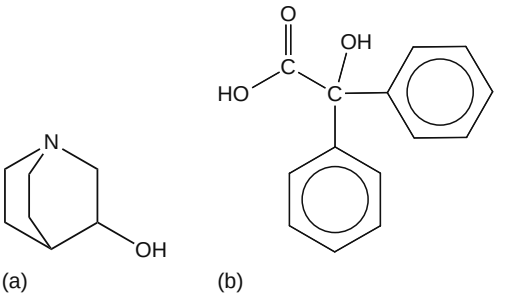

FIGURE 19.10 Structure of 3-quinuclidinol (a) and benzylic acid (b).

BZ has a molecular weight of 337 and a melting point of 168°C. Obviously, BZ is a solid at room temperature and has a negligible vapor pressure. BZ is relatively stable and moderately resistant to air oxidation and moderate temperatures. BZ will undergo hydrolysis in aqueous solution to produce benzylic acid (BA) and 3-quinuclidinol.

BZ in the Human Body. The information on BZ in the body is limited. It can take 3–4 days before symptoms of BZ intoxication disappear. Apparently, most BZ is excreted by the kidneys, and urine is the analysis matrix of choice. Information on the metabolism of BZ in humans is limited, but BA and 3-quinuclidinol are the probable main metabolites (see Figure 19.10a,b). It has been estimated that 3% of the BZ is excreted as the parent compound.

Analytical Methods. There are few references to the analysis of BZ in humans. BZ is used frequently by neuropharmacologists as a marker, but these methods are often not quantitative. In 1988, the United States began work on demilitarization of BZ stockpiles. As part of that work, a method was developed in collaboration between the National Institute of Standards and Technology and the U.S. Army Medical Research Institute of Chemical Defense at Aberdeen Proving Ground, MD for purposes of monitoring workers. That method was based on GC–MS of the TMS derivatives and monitored all three analytes (BZ, BA, and Q). Detection limits were 0.5 ng/mL for BZ and 5 ng/mL for the hydrolysis products (Byrd et al., 1992).

VIII. CONCLUSIONS

Studies on the toxicology of chemical warfare agents and on treatment of intoxications have predominantly focused on lethal and supralethal doses of the agent. However, the possible relationship between ''Gulf War Syndrome'' and accidental exposure to trace amounts of sarin and cyclohexyl sarin (Gardner et al., 2003) has made clear that knowledge on the acute and delayed effects of low or trace exposure to nerve agents, insecticides, and a variety of environmental chemicals is scarce (Ember, 1996), both in military and civilian environments. Several military and terrorist scenarios in which low or trace exposures become significant can be envisioned. Thus, given the potential increase in urban terrorism that may include the use of chemical warfare agents, Federal, State, and local authorities now have a variety of sensitive and accurate detection assays for appropriate containment, decontamination, and treatment measures.

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20 Personal Protective Equipment: Practical and Theoretical Considerations

Michael R. Jones

CONTENTS

Prehospital Setting. The most important care that the casualty receives is the care that is given within the first several minutes of a chemical attack. The conduct of the care given immediately after toxic chemical agent exposure, including the administration of antidotes, can literally mean the difference between life and death. This is not an overstatement (United States Army Medical Research Institute of Chemical Defense, June 2001). Since this care will be rendered in a warm zone, personal protective equipment (PPE) must be employed by the responder.

I. INTRODUCTION TO PERSONAL PROTECTIVE EQUIPMENT

A. Need for Protection and Routes of Exposure

Two extremely important points to remember about chemical, biological, radiological, nuclear, and explosives (CBRNE) warfare agent intoxication are: (1) all toxic effects of CBRNE agents are dose dependent and (2) the first obligation of a responder involved in a chemical weapons release is not to become a victim himself. In order to accomplish this latter point, PPE is employed. PPE surrounds the individual responder in a unique microenvironment with its own temperature, humidity, air currents, sounds, odors, and problems. Although contact with chemical warfare agents (CWAs) and toxic industrial chemicals (TICs) can occur with ingestion and injection, such exposures are extraordinarily rare. Common methods of exposure include respiratory (lungs) and dermal (skin) contact. Such contact theoretically can be with the agent in either its liquid or vapor (gas) state. Respiratory contact with liquid agent would occur only with aspiration of the liquid agent and is therefore almost never encountered. Consequently, the major routes of exposure are: (1) respiratory tract exposure to vapor (gas), (2) dermal (skin) exposure to liquids, and (3) dermal exposure to vapor. These three types of exposures are listed in order of descending importance of severity of injury and thus, the need for protection (Jones, 2006).

B. Anatomical and Physiological Considerations

So why is respiratory protection the most important? Let us examine the facts. The total surface area of the eye is 0.0002 m^2; the total surface area of the entire body of an average-sized individual is slightly less than 2 m^2; whereas the total surface area of the lungs of the average-sized individual is 50–100 m^2 (USAMRICD, CCCD, October 2001), with the average surface area being 75 m^2, roughly the size of a tennis court (Ross, 2006). Moreover, the type of surface covering of these areas (respiratory tract and skin) is extremely important. Skin is composed of multiple layers of stratified squamous cells whose purpose is to form a protective barrier. Although permeation of chemical agents through skin is possible, the process is generally slow compared with permeation through lung tissue. On the other hand, the surface of the lung alveoli is composed of a single layer of cells (pneumocytes types I and II) (Ross, 2006) whose purpose in life is to expedite the rapid exchange of gas between the alveolar sacs (location of respired air) and blood vessels (location of blood) within the lungs (Kimball, 2006). Clearly respiratory protection is by far the most important type of protection needed, since lung surface area is 25–50 times larger than the entire surface area of the skin, as well as the difference in the rate of transfer of gas and liquids across the two different cellular types. It should be noted that the necessity of dermal protection, though less important than that of respiratory protection, is nevertheless extremely vital.

C. Key Terms and Definitions

Several important terms must be defined at this time. Agent penetration is defined as the movement of a chemical agent through a material principally through partial nicks, cuts, cracks, zippers, or seams in the material. The movement of the chemical agent is through some defect in the chemical-protective material. Agent permeation is defined as the movement of a chemical agent through a material whereby the molecules of the agent pass between the molecules of the protective material; the material is free of nicks, cuts, or cracks. There is no defect in the chemical-protective material. Permeation time is defined as the time of permeation of a chemical, biological, radiological, or nuclear (CBRN) agent through chemical-protective material. All variables, including the CBRN agent, its physical state (solid, liquid, or gas), the chemical-protective material, and the conditions of the test (temperature, humidity, initial concentration of the agent, and final concentration of the permeated agent, etc.) must be specified. Generally speaking, materials that take greater than 480 min (8 h) are regarded as impermeable, since realistically no civilian is ever in PPE for more than 8 h. Adsorption is defined as the adsorbed particle remaining on the surface of the substrate; whereas in absorption the absorbed particle enters into the substrate. In both adsorption and absorption the particle is usually held in place by electrostatic forces. Sorption is defined as both adsorption and absorption occurring simultaneously. Fit factor (FF) is defined as the ratio of the number of particles of a particular contaminant outside the respirator (numerator of fraction), as compared with the number of particles of the same contaminant inside the respirator (denominator of fraction). This numerical ratio can only be obtained by employing quantitative (measuring with an electronic instrument such as TSI's Port-A-Count®), not qualitative (detecting the odor of banana oil, that is, isoamyl acetate, stannic chloride, bitrex, or C.S. gas), methods. Occupational Safety and Health Administration (OSHA) regulation 29 CFR 1910.134(f)(7) requires that tight-fitting full-face respirators have an FF of at least 500 in order to pass the quantitative fit testing requirements. LD_{50} (Lethal $Dose_{50}$) is defined as the amount of solid or liquid agent, usually expressed in milligrams, that would be lethal to 50% of the exposed individuals. LCt_{50} (Lethal $Concentration_{50}$) is defined as the concentration of vapor (gas) agent multiplied by the time of exposure, measured in minutes, which will be lethal to 50% of the exposed individuals. This represents total dose of vapor exposure and is expressed as mg-min per m^3; ppm: parts per million; and ppb: parts per billion. Vapor is defined as the gaseous component of a solid or liquid material below the critical temperature. Critical temperature is the temperature at which 100% of the solid or liquid phase of

a material changes to the gaseous phase of the material. Immediately dangerous to life or health (IDLH) means an atmosphere that poses an immediate threat to life, would cause irreversible adverse health effects, or would impair an individual's ability to escape from a dangerous atmosphere (OSHA 29 CFR 1910.120(a)(3)).

D. Atmosphere (Air)-Supplied Respirator

The respirator has its own supply of clean, dry, filtered air from a source independent of the ambient contaminated atmosphere. Frequently this air is carried in a high-pressure tank on the operator's back (Self-Contained Breathing Apparatus or SCBA), although it may also be supplied by a bank of large stationary air tanks or an air compressor with air-filtering capability connected to the facepiece of the respirator via a high-pressure hose of up to 300 feet in length. The air supplied to the respirator's user is from a source completely independent of the air present at the toxic agent release point. This air must meet NIOSH (National Institute of Safety and Health) grade D standards or better for compressed air purification, including all of the following parameters: oxygen = 19.5%–23.5% (by volume), carbon dioxide $\leq$1000 ppm, carbon monoxide less than 10 ppm, and hydrocarbons $\leq$5 mg per m^3 (as determined by oil mist) and possessing no pronounced odor (Woods, 1999). Should an atmosphere-supplied respirator (as opposed to an air-purifying respirator) be selected for use in HAZMAT or CBRN operations then only a SCBA-type respirator (as opposed to the air line system described above) must be employed.

Atmosphere-supplied respirators may also be classified as one of three types, depending on the type of air control valve within the respirator. In the continuous flow-type valve, air is continuously flowing within the respirator regardless of the phase of respiration (i.e., inspiration or expiration). With initiation of the inspiratory phase of respiration by the responder, a demand respirator valve allows creation of a negative pressure within the facepiece of the respirator, that is, the pressure within the facepiece will be lower than the surrounding atmospheric pressure; but after a preset trigger level of negative-pressure generation the demand valve will open to introduce positive-pressure air into the respirator's facepiece. This positive pressure is then maintained throughout the remainder of the inspiratory cycle. During the initial negative-pressure phase of inspiration, any leak in the faceseal of the respirator may entrain contaminated atmospheric air into the respirator's facepiece since air movement will always follow a pressure gradient from higher (positive) to lower (negative) pressure. Once a positive pressure is generated during the later inspiratory phase, this entrainment (or potential entrainment) of contaminated atmospheric air will stop, and the movement of air will reverse its flow, since the pressure gradient will be reversed. However, by the time positive pressure is achieved within the respirator's facepiece the responder may have already inhaled a fatal dose of contaminated air introduced when a leak occurred during the initial negative-pressure phase of inspiration. A spring-loaded exhalation valve creates a slight resistance to expiration and causes a demand-type respirator to maintain a positive pressure throughout the entire expiratory cycle with reference to atmospheric pressure. With a pressure-demand type valve, positive pressure within the facepiece of the respirator is maintained during every stage of inspiration and expiration. A very small continuous leak of air from the air supply into the facepiece of the respirator, coupled with a spring loaded expiratory valve (identical to that found in a demand-type respirator that creates resistance to expiration as mentioned above), maintains this positive pressure throughout the respiratory cycle. With the onset of inspiration, the positive pressure within the facepiece is reduced slightly, but it still remains positive when compared with the atmospheric pressure. At a predetermined level of reduction in inspiratory positive pressure, the respirator second stage air supply valve opens, and introduces additional positive-pressure air into the facepiece of the respirator. At no time during respiration is a negative pressure created within the facepiece of the respirator when referenced to atmospheric pressure (U.S. Army Center for Environmental Health Research TB 502, 1982; OSHA on line Course 2220a, unknown year). Consequently, should a break in the seal occur between the respirator's facepiece and the responder, the movement of air would always be from the area of higher air pressure (inside

the respirator's facepiece) to the area of lower air pressure (outside of the respirator's facepiece, i.e., atmospheric), and never the opposite. This method insures, as best as humanly possible, that the responder will never be breathing contaminated atmospheric air. When employing a pressure-demand regulator valve in the respirator's facepiece, should one suddenly experience a loud continuous hissing noise with a greatly expanded flow of cool dry air rushing across the responder's face, a leak in the respirator's faceseal has occurred. This loud hissing sound merely represents the pressure-demand valve rapidly introducing a large volume of air from the clean air supply into the respirator's facepiece. The valve is attempting to restore the positive-pressure gradient within the facepiece of the respirator which was reduced or destroyed by the faceseal leak. One should immediately attempt to find and fix the leak while exiting the contaminated area before the responder's supply of clean compressed air becomes exhausted.

One note of caution with use of pressure-demand respirators, as the respiratory workload increases as a result of heavy exercise, a greater ventilatory demand (due to increasing minute ventilation) is placed on the respirator and the more the pressure–flow response curve of the pressure-demand valve begins to resemble the pressure–flow response curve of the demand valve, that is, the positive pressure maintained in the respirator's facepiece throughout the respiratory cycle may become compromised, and a brief period of negative pressure may exist during each inspiration. Hence, the concept of increased protection as a result of pressuring the respirator's facepiece is seriously questioned during heavy exercise (Raven et al., 1982) with increasing levels of minute ventilation. Only pressure demand-type valves are allowed by OSHA for use in atmosphere-supplied respirators (i.e., SCBA) for HAZMAT or CBRN operations.

E. Self-Contained Breathing Apparatus

This device is a particular subtype of atmosphere-supplied respirators discussed above. Like all atmosphere-supplied respirators, it provides low-humidity, grade D (defined elsewhere) or better breathable air in a toxic or hypoxic (oxygen-deficient) environment. SCBAs are typically composed of multiple components. These include a high-pressure tank constructed of aluminum, steel or composite (fiber glass-wrapped aluminum), and having a shut-off valve at its exit. Under normal breathing conditions these tanks supply between 30 and 60 min of air. Filled-tank pressures vary from approximately 2200 to 4500 psi, and tank pressure gives a rough estimate of the total time clean air will be available to the responder. Obviously, this varies from responder to responder depending on respiratory patterns. Tank pressure is monitored with a tank pressure gauge connected by a high-pressure hose to the first stage (high-pressure reducing stage) of the regulator. The facepiece of this gauge is usually mounted on the front straps of the tank's carrying harness, in a position that is easily visible to the responder. A pressure regulator reduces the high pressure contained in the tank to low-pressure breathable air for the responder. This is usually accomplished in two stages that include both a high-pressure reducing and a low-pressure reducing regulator valve. The first stage (high-pressure reducing) is at the outlet of the tank, and characteristically this first stage reduces the air pressure from tank pressure (2200–4500 psi) to approximately 75 psi, which is then carried within a high-pressure hose to the second stage (low-pressure reducing) of the regulator located just before the entrance to the facepiece worn by the responder. In HAZMAT and CBRN operations this facepiece is a full-face facepiece. The second stage of the regulator further reduces the air pressure to a breathable pressure. This second stage of the regulator will have either a demand- or a pressure-demand valve. The difference between these two types of valves is discussed elsewhere in this chapter. In HAZMAT or CBRN operations only pressure-demand valves are permitted. Air enters the facepiece of the respirator from the second stage of the regulator. A carrying harness for the air tank is also part of the ensemble, and holds the air tank on the responder's back. Harness straps extend both in front of and behind the responder's torso. Newer-type mounting harnesses place the air tank orientated upside down, with the tank outlet valve and the first stage of the regulator at the most dependent position, thus making the shut-off valve easier

to reach by the responder than if this valve were located directly behind the responder's neck. Composite-type tanks are generally favored by responders since they have the least amount of weight, but they also have the shortest service life, usually approximately 15 years. The inside of tank must be visually inspected yearly, and pressure tested at regular intervals of 3–5 years (depending on regulations). Additionally, most regulators have a low-pressure warning device, which has a loud, auditory warning signal when pressure decreases to a preset level. Although the pressure within the tank will be low, the responder will have sufficient air within the tank to safely exit the hazardous environment. Moreover, in 2002, the National Fire Protection Association (NFPA) Standard 1981 required that SCBA contain a head-up display (HUD) on the facepiece of the respirator. Usually a light-emitting diode (LED) device is attached to the outside of one side of the respirator's facepiece, but is easily visible from within. This device continuously displays by sequentially arranged colored lights the amount of air remaining in the tank; that is, full, ¾, ½, ¼, and empty. Additional alerts are given when the tank is ½ full. If the HUD device is battery powered another alert signal must be displayed when the battery contains only enough power to operate the HUD device for an additional 2 hour (United States Department of Homeland Security, 2004). Newer models of SCBA equipment also have a ''lack of motion'' detector. If the responder stays totally stationary without any movement for a predetermined period of time, the detector will sound a loud, audible alarm to warn the responder's buddy of the lack of movement of the responder and possible difficulty with the responder.

In addition to being classified by the type of regulator valve (demand or pressure demand), SCBA may also be classified as open-circuit or closed-circuit (circle) types. In an open-circuit system, the airflow is from the tank, through the regulator, breathed only once by the responder, and then exited to the atmospheric air. There is no rebreathing of air. Hence, it makes rather inefficient use of the supply of compressed clean air. In the closed-circuit (circle) system, air is rebreathed after undergoing chemical filtration. Literally, the breathable air flows unidirectionally inside a low-pressure hose arranged in a complete circle. The unidirection flow of air is maintained within the circle by two one-way valves. Other components included within the closed (circle) system include a carbon dioxide-removing filter placed within the circle to remove carbon dioxide from the responder's expired air. A chemical reaction occurs between the expired carbon dioxide and the material within the carbon dioxide filter, often soda lime. This reaction removes carbon dioxide from air within the circle. However, this type of reaction characteristically produces a great quantity of heat that is released into the surrounding environment. Such a heat-producing reaction is known as an exothermic reaction. If such a system were to be employed within a Level A suit (essentially a somewhat insulated encapsulated cocoon) some mechanism of heat removal must be in place, or very shortly intolerable and dangerous levels of temperature would build up within the suit's interior. A full-face facepiece covers the responder's face. The responder's metabolism produces carbon dioxide, which is then exhaled through the facepiece of the respirator into the circle system. Simultaneously, the responder's metabolism also decreases the percentage of oxygen in the air within the circle. To prevent this phenomena from becoming problematic, an oxygen analyzer is placed within the circle to measure the percentage of oxygen within the breathable air. When the percent oxygen within the circle system decreases below a preset level, the oxygen analyzer, which is electronically coupled to a tank of high-pressure 100% oxygen (not air) within the unit, releases a measured quantity of oxygen into the circle system through the second stage (low-pressure) reducing valve. This restores the oxygen removed from the circle by the responder's breathing to acceptable levels. The first stage high-pressure reducing regulator is directly attached to the outlet valve of the oxygen tank. Advantages of the closed-circuit (circle) system include a longer period of usage without refilling the oxygen tank, usually in the neighborhood of 4 hour, as opposed to a maximum usage of 1 hour with an open system employing compressed air. Disadvantages would include the heavy weight of the system, although the open circuit is also heavy, and the exothermic (heat producing) reaction that takes place between carbon dioxide and soda lime or other carbon dioxide-removing substances. Oxygen readily supports combustion which is an

obvious disadvantage, as well as the necessity of having to periodically change the carbon dioxide-filtering material. If changing the carbon dioxide-filtering material is ignored, carbon dioxide levels within the circle system will continue to increase. As levels of carbon dioxide increase initially a cerebral vasodilatory effect (dilatation of blood vessels within the brain, which increases pressure on the brain) will occur, the extent of which will be directly proportional to the level of carbon dioxide. This will be expressed clinically with the development of an increasingly severe headache. At levels of carbon dioxide of approximately 80 mmHg or above (36–40 mmHg is considered normal), carbon dioxide will exert an anesthetic effect on the responder, and he may initially become light headed, dizzy, and may fall into a deep sleep from which arousal is difficult or impossible, depending on the levels of carbon dioxide within the circle system. A chemical reaction occurs within the filtering material when it becomes saturated with carbon dioxide and needs to be changed. The color of this material will undergo a color change, usually from white to blue. One should note that at very cold temperatures the efficiency of the carbon dioxide-filtering material becomes compromised, and residual carbon dioxide may remain within the circle system (NIOSH, 1985). Another disadvantage of a closed (circle) system would include the collection of water vapor from the responder's expired air which initially cools, then condenses to liquid within the hoses of the closed (circle) system. This water would then have to be drained from the system through a one-way valve mechanism.

F. Air-Purifying Respirators

With this respirator type contaminated air at the job site is passed through a filter, and the filter removes the contaminants. The exact composition of an air-purifying filter will be discussed later in this chapter. Numerous restrictions apply to this type of respirator usage. What is important is that the filter employed be able to remove the contaminant and in the concentrations present. In atmospheres containing toxic contaminants in concentrations below the contaminant's IDLH value (as per OSHA charts) either an atmosphere-supplied respirator or an air-purifying respirator may be employed. In atmospheres containing toxic contaminants at or above their IDLH value only atmosphere-supplied respirators may be used, and for HAZMAT (29 CFR 1910.120) and CBRNE (42 CFR 84) operations only atmosphere-supplied respirators of the pressure-demand, SCBA type may be employed.

G. Respirator Nosecup

The respirator nosecup is a small mask, which snuggly covers only the nose and mouth of the responder and is located within the facepiece of a full-face respirator. Air is usually introduced into it by two one-way valves (one on each side) located inside the nosecup. These open to admit air from the inside the respirator's facepiece, but close to prevent air within the nosecup from being exhaled back into the inside of the respirator's facepiece. Some respirators, especially those of British origin, lack the one-way valves, but strategically place the interconnection between inside and outside of the nosecup in a location that will not interfere with proper functioning of the nosecup. The nosecup is directly connected to the exhalation valve of the respirator. Therefore, flow of air would be from the respirator's source of air to the inside of the respirator's full-face facepiece, through a one-way valve into the nosecup, followed by inspiration and expiration by the responder, then through the respirator's expiratory valve into the surrounding atmospheric air. The nosecup (type employing one-way valves) principally accomplishes two goals: (1) it decreases the volume of respirator dead space presented to the responder, thus increasing ventilatory efficiency and (2) it prevents the responder's warm moist expired air from coming into contact with the inside of the eye lens of the full-face respirator. If this latter were to occur the temperature and humidity content of the air on the inside of the eye lens would be significantly greater than that on the outside of the eye lens, with resulting condensation of water vapor to liquid water on the inside of the eye lens, with the resulting obscuring of vision.

H. Respirator Hood

A hood is a garment made of cloth material, which is then externally covered and impregnated with chemical-resistant material, usually of butyl rubber or halogenated butyl rubber. It totally covers the head, has an opening for the facepiece of the respirator that is surrounded by an elasticized band to tightly secure the hood to the respirator facepiece, and additionally covers the shoulders and the upper chest (both front and rear aspects), and is secured by two straps that extend from the outside of the bottom of the rear aspect of the hood, with one strap extending under each axilla (armpit), and subsequently both straps attaching to the outside of the lower margin of the front aspect of the garment.

II. PHYSIOLOGICAL CONSEQUENCES OF PERSONAL PROTECTIVE EQUIPMENT USAGE

A. Heat Stress

The protection provided by the PPE does not come without a price. PPE as a class possesses high thermal insulating properties (they retain heat), coupled with low moisture permeability (they allow only a slight loss of moisture, thus maintaining a high level of humidity within the protective garment). These conditions lead to heat stress. Metabolic work needed to perform a task when wearing PPE is far greater than the metabolic work needed to perform the same task without PPE (Musa, 2002). Since heat production increases as metabolic work increases, a rapid buildup of heat within PPE is typical. Environmental influences on heat regulation within PPE include environmental temperature, humidity, wind velocity (convection), and the way that the PPE is constructed (vapor proof versus nonvapor proof, impermeable versus semipermeable material, one-piece versus two-piece construction, etc.). If the task is performed in a sunny area, then radiant energy from the sun in the form of infrared rays provides additional heat accumulation within the ensemble. Worker issues that contribute to heat stress within PPE would include the worker's state of health, state of acclimatization, state of fitness, level of hydration, and type of medications taken. High blood flow to the skin aids in heat dissipation from the skin. The following medications decrease blood flow to the skin: diuretics by producing fluid loss and dehydration with accompanying constriction of blood vessels, beta blockers by decreasing cardiac output, and alpha agonists by constricting blood vessels. Weight-loss medications have multiple effects, including increasing the rate of metabolism and hence heat production; and anticholinergic drugs decrease or prevent sweating thus decreasing or eliminating evaporative cooling. Both the intensity and duration of the task performed have a great influence on heat stress (SAIC, 2002b).

Consequences of heat stress would include reduced psychological functions including reduction in speed of thought, alertness, and reaction time. Due to the increased temperature and humidity within PPE the rate of sweating dramatically increases. This causes rapid dehydration, with accompanying hypovolemia, reduced vision secondary to condensation of sweat on the transparent part of the facepiece of the protective ensemble (and respirator's eyepieces), and wet and slippery hands. A compensatory mechanism to decrease heat production is to decrease the rate of work; hence, accomplishment of a task takes longer in PPE. As a consequence of heat stress, accidents and injuries are increased when employing PPE. Resting body metabolism will produce heat energy equivalent to that produced by a 100 W light bulb, moderate work increases this to about 250 watts, whereas extreme work, such as running, increases this to approximately 1000 watts. So the key to temperature homeostasis is that heat produced by the body's metabolism must constantly be lost from the body's surface to the outside environment, or the body's temperature will rise (SAIC, 2002a). The body attempts to dissipate heat production by increasing blood flow to the skin and increasing sweating. Consequently, blood flow to the abdominal viscera is reduced. Cardiac output initially increases, but will fall as circulating volume decreases by sweating, and syncopal (fainting) episodes may result.

PPE prevents the dissipation of heat generated by the body to the outside environment by forming a protective semi-insulated cocoon around the individual. The more impervious this cocoon, the greater will be heat and moisture accumulation within the PPE ensemble. When heat production exceeds heat dissipation, body temperature will begin and continue to rise. Dire consequences will follow.

Heat dissipation within PPE is almost nonexistent because of greatly reduced (1) conductive and (2) convective driven heat exchange. However both of these represent minor methods of body heat loss. In a normal environment (no PPE) the majority of the body's heat production is dissipated to the environment by the effect of (3) evaporative cooling that accompanies sweating, that is, liquid water (sweat) is changed to water vapor, with an accompanying decrease in body temperature. However, sweating also produces dehydration. This method of cooling relies on the ability of the surrounding atmosphere to accept water vapor. Hence, the lower the humidity of the surrounding air, the more humidity it will accept, and the greater the degree of evaporative cooling that will occur. Air that is 100% saturated with water vapor will no longer accept additional moisture, and hence evaporative cooling comes to a halt. Air within the PPE very rapidly becomes 100% saturated with water vapor and thus no further evaporation of water (and hence cooling) can occur. As temperature increases within PPE sweating will increase, but this sweating lacks evaporative cooling, and thus sweating in PPE only produces dehydration, without accompanying cooling. The average rate of sweating in PPE is approximately 2 liter per hour, and the maximum rate of oral rehydration is approximately 1.2 L per hour (SAIC, 2002a). Most civilian PPE ensembles do not provide facilities for oral rehydration. Use of PPE ensembles in contaminated areas can produce dangerous degrees of dehydration and accompanying hypovolemia (lack of circulating volume) if strict adherence to work–rest protocols are not followed. One easily sees how large volume dehydration quickly becomes the norm.

So in summary, wearing PPE increases metabolic work. This in turn increases skin temperature, sweating, and skin blood flow. Blood flow to the viscera is reduced. There is an extremely reduced conductive and convective heat loss when wearing PPE (minor factors); as well as essentially no evaporative cooling (major factor). The latter normally constitutes the principal method by which body-generated heat is lost to the environment. When wearing PPE all that sweating accomplishes is the production of rapid and extensive dehydration without accompanying evaporative cooling; consequently heat rapidly builds up within the PPE and hypovolemia ensues (SAIC, 2002a).

B. Pathophysiology of Heat Accumulation

Heat buildup within the protective ensemble provides the physiological basis for (1) painful heat cramps in muscle tissue, (2) heat exhaustion, and (3) heat stroke. If patients experience heat exhaustion they are still capable of sweating and controlling thermoregulation (temperature control of body). This less-severe diagnosis of heat exhaustion is a diagnosis of exclusion, whose symptoms include exhaustion, weakness, headache, fatigue, light-headedness, and dizziness. Signs of heat exhaustion may also include lethargic, depression, irritability, and confusion, elevated pulse rate, and orthostatic hypotension. Heat stroke produces a situation in which the body's ability to sweat has been lost, so the skin is hot and dry; additionally thermoregulation of body temperature (a function of the brain) has been lost; consequently, body temperature rises rapidly to excessive levels. Heat stroke is a true medical emergency, and serious medical consequences, including death, may result if external cooling of the body is not immediately instituted. Signs of heat stroke include delirium, disorientation, combativeness, seizures, collapse (fainting), low blood pressure, and a compensating weak, rapid pulse. Pre- and postdeployment medical monitoring of responders using PPE is absolutely paramount for their safety.

C. Clothing Worn during PPE Usage

The absolute minimum amount of clothing should be worn within PPE, as each layer of clothing not only increases heat retention by the layering effect but also increases metabolic work

(and hence increased energy requirements with accompanying increased heat production) by the hobbling effect.*

In summary, heat stress is created by the interaction of multiple factors including (1) those created by the environment (temperature, air flow, radiant heat, humidity, and PPE), (2) those created by the task (work rate and duration), and (3) those created by the worker (acclimatization, state of hydration, fitness, general health, and medications) (SAIC, 2002b).

D. Physiological Consequences of Respirator Usage

A respirator increases (anatomical) dead space, resistance to breathing, and respiratory work. Respirator usage may double respiratory work, from approximately 3% to 6% of the cardiac output (NIOSH, 1996). Anatomical dead space (the volume of air in the respiratory tree that must be moved before fresh air can be admitted to the alveoli) is usually 1 cc per pound of body weight. This would be 150 cc for a 150 pound individual not wearing a respirator. A full-face respirator may increase this anatomical dead space from 150 cc to approximately 850 cc (OSHA on line course 2220c, unknown year). Tidal volume and hence minute ventilation increase significantly to compensate for this added dead space. However, the increase in tidal volume and minute ventilation is usually not as great as the increase in dead space; consequently alveolar ventilation decreases slightly. Usually respiratory rate decreases slightly with initial respirator usage, although this will increase as the level of exercise increases, and the corresponding need for oxygen increases. Consequently alveolar and blood carbon dioxide levels may rise. Since cerebral blood vessel diameter is directly proportional to blood carbon dioxide concentration within the range of 20–80 mmHg, cerebral blood vessels dilate; this may slightly increase intracranial pressure, and a headache often results.

All respirators increase expiratory resistance to some degree. Air-purifying respirators increase expiratory resistance far less than atmosphere-supplied pressure-demand respirators. The latter maintains a significant positive pressure throughout the entire expiratory cycle. This creates a positive-end expiratory pressure (PEEP) effect on the airway. This in turn keeps more alveoli ventilated (opened) during expiration, which increases the oxygenation of the blood. The bad news associated with this PEEP effect of pressure-demand respirators is that PEEP is well known to decrease cardiac output in susceptible individuals; hence the need to medically screen responders before allowing respirator usage. If responders are also hypovolemic (low circulating volume in the circulatory tree), as may occur with the increased sweating accompanying PPE usage, and undergo inadequate oral rehydration, and the previously mentioned PEEP effect, lower cardiac output may result. This lowered cardiac output at worst could result in syncope (fainting), and at best a decreased endurance at increased levels of exercise. However, several studies have shown that this is not a practical concern in healthy individuals (NIOSH, 1996).

Negative-pressure air-purifying respirators (gas masks) increase inspiratory resistance to a far greater extent than expiratory resistance. With negative-pressure air-purifying respirators, as minute ventilation increases, inspiratory and expiratory resistances increase geometrically thus individuals may decrease their peak inspiratory and expiratory flow rates to decrease the resistance to air flow. Moreover, respirator users must increase their respiratory rate (Hodous, 1989) to meet the demands of increased minute ventilation. The need for increased minute ventilation results from an increased metabolic demand for oxygen as a consequence of the increased metabolic work needed to perform any task when wearing PPE. Negative-pressure air-purifying respirators significantly decrease the maximum voluntary ventilation of an individual and are notorious for tasking individuals with reactive airway disease (RAD), thus precipitating bronchospasm (airway narrowing), with a resultant decrease in laminar airflow and a corresponding increase in turbulent (and much less-efficient)

* Hobbling effect is the interference with joint movement caused by the bulkiness of the multilayered uniform (Musa, 2002).

airflow. Bronchospasm (narrowing of the bronchi) may result in hypoxia (decreased oxygen) and hypercarbia (increased carbon dioxide) in the blood.

The additional weight of a SCBA, approximately 30–35 pounds, significantly increases cardiac work. An increase in respiratory work, due to increased expiratory resistance as described above, will also increase cardiac work by a ripple down effect especially as work loads increase.

In summary, the increased tidal volume, increased inspiratory and expiratory airway resistances (the magnitude of which vary depending on the type of respirator employed), increased anatomical dead space, as well as the initial decrease in respiratory rate, result in decreased alveolar ventilation, increased work of breathing, and decreased endurance at maximum work loads. Fatigue and discomfort increase. Additionally, due to the increased negative and positive thoracic pressures associated with respirator usage, and the increased mathematical difference between these two pressures (NIOSH, 1996), there may be an increased risk to individuals with a history of spontaneous pneumothorax (collapse of the lung) associated with respirator usage (OSHA on line training, unknown year).

Generally negative-pressure air-purifying respirators tend primarily to increase respiratory work that may then later have a ripple down effect to increase cardiac work in individuals with metabolic compromise.

Atmosphere-supplied pressure-demand respirators generally increase cardiac work significantly due to the increased weight of the respiratory equipment. Additionally, increased respiratory work is generated by the increased muscular effort needed to continue breathing against the positive pressure created by the respirator during the entire expiratory cycle. Cardiac problems associated with respirator usage include increased cardiac work with decreased endurance at maximum work loads (OSHA on line training, unknown year), as well as the possibility of arrhythmias due to increased levels of catecholamines that accompany increasing cardiac work.

Interestingly enough, a responder using a negative-pressure air-purifying respirator during a period of very heavy exercise will increase his maximal negative oral pressure during inspiration to about 15–17 cm of water pressure. The maximum peak positive oral pressure generated during very heavy exercise with a respirator in the positive pressure mode (e.g., pressure-demand SCBA) is also about 15–17 cm of water pressure (NIOSH, 1996). The risk of barotraumas with respirator usage is significantly less than that associated with a vigorous cough, which can generate maximum positive pressures of 200 cm of water (NIOSH, 1996). Normal individuals not employing respirators can generate pressures as great as 80–160 cm of negative water pressure during inspiration (NIOSH, 1991). Although the previous set of negative-inspiratory pressures were generated during supermaximal inspiratory effort, normal maximal negative-inspiratory pressures generated in the pleural may be as great as −40 cm of water (NIOSH, 1996).

SCBA tanks contain air with extremely low levels of humidity at room temperature, and this air must be both humidified and warmed by the responder's respirator tree during respiration. This process produces further dehydration while requiring additional energy utilization. This increases the responder's metabolic work load that ultimately decreases physical endurance.

E. Physical Limitations of Personal Protective Equipment Usage

Additional limitations incurred by an individual in PPE include a decrease in manual dexterity and communications difficulties, both in hearing as a result of the noise created by the respirator, and by producing an almost unintelligible quality of speech. Some respirators come equipped with a voicemitter to overcome this difficultly, and although this is an improvement, much is left to be desired. Both reaction and decision times are prolonged in PPE, and errors of omission are more common. Heightened emotions in the form of anxiety, as well as a feeling of claustrophobia and isolation frequently occur. In addition to a decrease in visual fields, the sensation of touch is decreased, and smell and taste are also compromised. It is well documented that accomplishment of an individual task requires more time when wearing PPE (Musa, 2002).

Drying of the eyes due to an increased or diverted airflow is a problem with some respirators (e.g., powered air-purifying respirators and SCBAs) (NIOSH, 1996). Individuals with decreased tear production, or an impaired blinking mechanism, may experience drying of the eyes under such conditions.

Thus PPE has its own unique problems and challenges. Principal among these are rapid heat buildup secondary to increased metabolism, increased sweat production, loss of evaporative cooling, and rapid production of dehydration. All of these lead to the production of a metabolic environment that results in a decreased work endurance at maximal levels of exercise, heat cramps, heat exhaustion, and possibly heat stroke.

III. LEVELS OF CIVILIAN PERSONAL PROTECTIVE EQUIPMENT

A. Criteria Employed for Selection of an Appropriate Level of Personal Protective Equipment

The Code of Federal Regulations 29 CFR 1910.120 outlines four levels of protection established by the U.S. Environmental Protection Agency (EPA) (Table 20.1). OSHA has adopted these levels of PPE and they are recognized as a national standard (Table 20.2) (United States Army, 2003). The selection of which level to be employed is a multifactorial determination. The level of PPE is based on type of agent, its toxicity, and measured concentration, as well as the type of exposure (vapor, liquid, solid, or some combination thereof). When the agent's identity, concentration, or levels of exposure are unknown, Level A is always selected (NJHAZMAT, 2000). Additionally, exposure to any extremely toxic or corrosive gases, liquids, or solids that may cause significant skin damage on exposure require Level A. Downgrading to a lower level of protection occurs as a threat analysis if the situation allows.

B. Level A

Level A protection is a totally encapsulating protective cocoon. The garment's material is totally impermeable to both vapor and liquid. Although the garment has seams and a zipper, both of these

TABLE 20.1
Levels of Civilian Personal Protective Equipment

	Suit Protection		Respiratory Protection		
	Vapor Barrier	Liquid Splash Barrier	Self-Contained Breathing Apparatus	Air-Purifying Respirator (Positive or Negative Pressure)	Comments
Level A					Totally encapsulated protective cocoon, highest degree of protection, highest likelihood of heat and dehydration problems occurring in responders
Direction of air flow from suit to outside atmosphere controlled with one-way valve	Yes	Yes	Yes	No	
Level B	No	Yes	Yes	No	
Level C	No	Yes	No	Yes	Positive- or negative-pressure respirator
Level D	No	No	No	No	

TABLE 20.2
Protection Level Guide

FF = Fit Factor of the Respirator	Respiratory Protection	Body/Skin Protection	Comments
Level A	SCBA pressure-demand respirator FF 10,000	Vapor and Liquid (both immersion and splash)	Only vapor tight level, maximum respiratory and skin protection (both vapor and liquid)
Level B	SCBA pressure-demand respirator FF 10,000	Liquid splash only, not continuous liquid, no vapor protection	Levels B and C have same level of skin protection
Level C	Powered air-purifying respirator: FF 1,000; Negative pressure Air-purifying respirator (gas mask): FF 50	Liquid splash only, not continuous liquid, no vapor protection	Only difference between Levels B and C is the level of respiratory protection
Level D	None	None	Work uniform, example: surgical scrubs and surgical mask, police, firefighters' uniform, etc.

are constructed to also be totally impermeable to vapor (gas) and liquid (NIOJ, 2002). This is the highest level of protection since it completely isolates its occupant from toxic vapor and liquid contamination from the outside. Through the use of one-way valves the suit exhausts expired air and yet maintains an extremely slight positive pressure inside the suit with reference to the surrounding environment. The extent of this positive pressure is determined by the resistance to air flow exerted by the suit's one-way exhaust valves. To provide maximum respiratory protection an atmosphere-supplied (air-supplied) SCBA, usually of the open-circuit variety (i.e., air is not rebreathed by the individual), utilizing a pressure demand-type valve is employed, and this unit is carried within the body of the protective suit.

As previously stated, a pressure-demand respirator maintains a slightly positive pressure within the facepiece of the respirator (with reference to the surrounding atmospheric pressure) in every phase of both inspiration and expiration. Should there ever be a leak in the seal between the operator's face and the respirator's facepiece, the air flow would be from inside of the mask (higher pressure) to the outside of the mask (lower pressure) and should never be vice versa. In the case of a Level A ensemble the outside of the mask would actually be the inside of the Level A suit, which itself maintains a slightly positive pressure compared with the surrounding contaminated atmosphere through the use of a one-way valve mechanism. In the event of a facepiece leak when wearing a Level A ensemble a double set of protective barriers (outward airflow only from the respirator's facepiece to the inside of the Level A suit, and then from the inside of the Level A suit to the contaminated atmospheric air) between the responder's respiratory tract and the surrounding contaminated atmospheric air would be provided. Another advantage of this type of respirator is that the air supply to the responder is totally independent of the surrounding atmospheric contaminated air. Literally this is a ''bring your own air'' type respirator. This independent air supply allows the respirator to be used in oxygen-deficient atmospheres*, as well as in atmospheres containing contaminants at levels that are at or in excess of IDLH. An unofficial definition of IDLH, but one that bears a great degree of truth, is that IDLH is the concentration close to which physiological signs and symptoms begin to appear. Notice that IDLHs express the concentration of vapor, and not a total dose of vapor. The total dose of vapor exposure

* The normal percentage of oxygen in air is 20.9%; <19.5% represent an oxygen-deficient atomosphere per OSHA 1910.134 (d); ~17% oxygen leads to increased minute ventilation and increased heart rate; 14%–16% oxygen leads to rapid fatigue, very poor muscle tone and coordination, and intermittent respirations; 6%–10% oxygen concentration leads to nausea, vomiting, inability to perform, unconsciouness; <6% oxygen leads to spasmodic breathing (Cheyne-Stokes respiration), convulsions, and death within minutes (OSHA online trainning, year unknown).

would be expressed as the concentration of the agent multiplied by the time of exposure in minutes. One should remember that the total dose of the toxic agent, and not the concentration in the atmosphere, affects physiological and pathological outcome.

The ease of inspiration in pressure-demand (positive pressure throughout all of inspiration and expiration) mode allows only a minimal increase in inspiratory work compared with no respirator usage; however, the responder does expire against a positive pressure that would significantly increase respiratory work. This effect is identical to a hospitalized patient on a ventilator in which PEEP has been applied, but this PEEP effect is usually well tolerated by healthy individuals. There is no mobility restriction because of the self-contained air tank, and no exothermic heat is produced, as may occur with a closed-circuit rebreathing device (which has been covered in this chapter) (NIOJ, 2002). Respiratory expiration when wearing Level A may produce a slight breeze within the suit that may have a very slight convective cooling effect. These units are extremely common, as any fire department employs them. However, they must be CBRN certified by NIOSH to be used in response to a CBRN incident. Such equipment certification information is available on the NIOSH Web site. As previously discussed, the slight positive pressure maintained during expiration would foster a slight PEEP effect on expiration. This may help to increase alveolar recruitment (keeping more alveoli open during the respiratory cycle) in the lungs, thus decreasing ventilation/perfusion abnormalities and raising arterial p_a0_2, and consequently increasing blood oxygenation. Disadvantages would include the significant weight of the unit, usually 30–40 pounds, thus carrying this weight would significantly increase cardiac work. These units are also bulky, have a limited air supply (30–60 min), and require regular maintenance by qualified individuals. Failure of any of its many high-pressure components could result in catastrophic explosive traumatic injury.

In summary, Level A is employed with (1) unknown chemicals; or (2) when known chemicals pose a high level of threat to the respiratory system, skin, or eyes; (3) if operations are to be conducted in confined or poorly ventilated areas; or (4) if the chemicals present with known or suspected skin toxicity or carcinogenity (ATSDR, 2000; NIOJ, 2002).

C. Level B

Level B is employed when the maximum level of respiratory protection is needed, but a lower form of skin protection is required. The chemicals have been identified. This lower form of dermal (skin) protection provides protection against liquid splash only. Level B lacks the totally impermeable protective seal that is seen with Level A. Environmental toxic vapors can come into contact with the skin when Level B is worn. Should a potential problem arise if toxic vapor were to come in contact with the skin, Level B should not be worn. This would necessitate an upgrade to Level A. Level B suits provide no skin protection from vapor exposure, nor any protection against continuous contact with liquids. All Level B suits are made of impermeable material.

Level B suits can actually exist in one of the three forms. The first is a totally encapsulating Level B suit made of impermeable material. To outward appearances it looks just like Level A; and just like Level A the suit's material, but not the suit's seams and zippers, is totally impermeable to vapor (gas) and liquid. The suit is therefore designed as splash resistant, that is, resistant to liquid splash, but not liquid proof because of the method of construction of seams and zippers. Unlike Level A zippers and seams, those in Level B suits would allow penetration of vapor and liquid. This latter design significantly reduces the cost of suit production. Since it is totally encapsulating, the SCBA is worn within the suit, and the main purpose of this type of Level B suit is to protect the expensive SCBA and other equipment from splash contamination by toxic liquid. The second type of Level B suit is a one-piece type coverall with hood. While the suit's material is impermeable to vapor and liquid, the suit's seams and zippers are not, thus allowing the penetration of toxic vapor and liquid. A large open area in the front of the hood allows the SCBA mask to snuggly fit over the face. Clearly the space created between the suit and the facepiece of the respirator could be penetrated by vapors and liquids, unless measures are taken to prevent this. The SCBA is worn

outside the suit on the responder's back. The respirator's facepiece is then taped to the suit (to decrease, but not eliminate, agent penetration) with chemical-resistant tape for which agent permeation data exist or, alternatively with duct tape for which no agent permeation data exist. In the open-faced suits, as opposed to the totally encapsulating suits, poor fit often exists below the respirator's facepiece in the area of the chin. Frequently, this area is not covered by the protective suit, thus exposing the area of the face immediately below the respirator's facepiece directly to toxic agent exposure. Devices such as a ''hood'' (previously defined) and a respirator ''second skin'' have been designed to deal with this problem. The latter is a device exteriorly mounted on the facepiece of the respirator made of chemical-resistant material (especially vesicant-resistant material) such as butyl rubber, halogenated butyl rubber, or EPDM (ethylene propylene diene monomer). It is larger in circumference than the facepiece of the respirator, and is particularly elongated in the area below the respirator's facepiece to provide protection to the chin and neck. A photograph illustrating this lack of protective coverage of the chin area is included in the photographic section of this chapter. A third type of Level B suit is also made in the coverall configuration as discussed above, but is manufactured in two pieces (jacket and trousers) instead of one.

D. Level C

Level C employs an air-purifying respirator instead of an atmosphere (air)-supplied respirator. Even though the level of respiratory protection has been downgraded from Level B, the degree of skin protection offered in Level C is the same as Level B, namely liquid splash protection only. There is no skin protection against vapor contact or continuous contact with liquid. The respirator may be either a powered air-purifying respirator (regarded by OSHA as a positive-pressure respirator)*, or a negative-pressure air-purifying respirator. The former is often referred to as a PAPPER, whereas the latter is referred to as a gas mask. Two design types of Level C suits exist, the designation being determined by the type of material used in the suit's construction, that is, impermeable or semipermeable material. The first type is identical to the coverall suit (impermeable material in either a one- or two-piece design) described for Level B. The second presentation of the Level C suit ensemble is usually a two-piece (although one-piece suits do exist) semipermeable activated carbon-lined suit. The permeable, or more correctly, semipermeable suit is actually constructed in two layers. As an example, the previously fielded military version of this suit was known as Battle Dress Overgarment (BDO). The BDO has been replaced by the Joint Service Lightweight Integrated Suit Technology (JSLIST) ensemble. The JSLIST suit provides two layers of protection. The outer layer is constructed of a 50% nylon per 50% cotton poplin rip-stop material that has been chemically treated with a heavy-duty water repellent. The inner layer consists of a nonwoven front laminated to activated carbon spheres bonded to a tri-cotton knit back. The JSLIST overgarment incorporates an integrated hood on the jacket. This suit provides users with 45 days of wear and 120 days of service life. Unlike its predecessor (BDO), the JSLIST can be laundered up to six times. The JSLIST protective ensemble provides users with 24 h of protection (United States Army, 2003). It is claimed that unlike the BDO the activated carbon will not transfer from the suit to the responder's clothing (USARIEM, unknown year). Civilian versions would be structurally identical to these chemical-protective suits, although the coloring on the outside of the suit may be in various colors and patterns. The newer versions of the civilian suits followed the military lead and are now equipped with a built-in hood. Semipermeable suits would allow the outward passage of certain entities to a limited extent, such as body moisture and heat. At the same time, the suit's outer layer would repel certain atmospheric components such as rain, dirt, etc., and the inner layer of activated carbon would adsorb materials such as toxic agent. Any suit containing activated carbon should not be exposed to becoming saturated with water or liquid, as this degrades the suit, compromises the activated

* The pressure inside the facepiece is positive in pressure compared with that of the ambient air. OSHA regards powered air-purifying respirators as examples of positive-pressure respirators (OSHA online training, year unknown).

carbon, and may allow for passage of toxic material through the protective carbon material and into the suit. Indeed the military does use this type of suit in decontamination procedures employing liquid decontaminating materials. However, before conducting decontamination exercises the wearer dons a TAP (toxicological agent protective) apron, which is a totally impermeable butyl rubber apron with long sleeves that covers any area of the suit that might become exposed to liquid.

1. Activated Carbon

Activated carbon (sometimes referenced as activated charcoal) is a specially formulated carbon-based product. Materials used include various carbon-based products including coconut shells, various woods, coal, etc. These materials may be pretreated with acids, and then subjected to combustion at varying degrees of time, temperature, and additionally with pressurized steam. This process expands the porous nature of these materials, and tremendously increases the surface area for adsorption of toxic materials onto the surface of the activated carbon. Adsorptive surface areas of 500–1400 m^2 per gram are common (Pope, 1996). In a typical chemical warfare filter in excess of 200 gram of activated carbon fill is used. As an example, using 950 m^2 (the average of 500 and 1400 m^2) and multiplying this by 200 gram (the minimum weight of activated carbon in the filter) the surface adsorptive area of the activated carbon in the filter would be at least 190,000 m^2. A football field measures 360 by 160 feet, or 57,600 $feet^2$ (Journey North, 2002). Converting square feet to square meters yields 5351.215 $meters^2$ (Online conversion, 2006), and dividing 190,000 by 5351.215 equals 35.5. Thus the minimal activated carbon adsorptive area of an average chemical warfare filter is greater than 35 times the size of a regulation football field. During the manufacturing process, activated carbon is also impregnated with salts of various inorganic metals. The exact composition of the impregnated metals varies according to the type of toxic materials that the activated carbon is designed to filter.

2. Example of the Construction of an Air-Purifying Respirator Filter

The current military filter, designated C2A1, which is designed for all chemical, biological, and radiological warfare agents and some TICs, contains ASZM triethylenediamine (TEDA) Carbon. This activated carbon is impregnated with the salts of silver, copper, zinc, molybdenum, as well as triethylene diamine. Generally speaking, removal of chemical contaminants by activated carbon is based on two things: (1) the vapor pressure of the toxic contaminant, and (2) its reaction chemistry. Activated carbon has difficulty in removing compounds with low molecular weight (Pope, 1996), as exemplified by carbon monoxide and ammonia, etc. Briefly, substances with vapor pressures below 10 mmHg are adsorbed onto the surface of the activated carbon, where they are usually held there by electrostatic forces and then slowly undergo degradation by hydrolysis. These would include blister and nerve agents. The impregnated salts of inorganic metals react chemically with toxic gases having higher vapor pressures, including chlorine, phosgene, cyanide, cyanogen chloride, as well as other acidic gases. The most difficult toxic materials for the filter to remove will be the contaminants that will be the limiting factor in the serviceable life of the filter. These agents will destroy the protective filter barrier the quickest, and they are the ''blood'' or cyanide agents, that is, cyanogen chloride and hydrogen cyanide. The exact nature of these chemical reactions is beyond the scope of this chapter. The interested reader is referred to an excellent paper by Robert W. Morrison (unknown year). The C2A1's total filtering capacity for the most difficult of the nerve agents to filter (sarin) is 300,000 mg-min per m^3. Sarin is the most difficult nerve agent to be filtered by the activated carbon because it has the highest vapor pressure of all the nerve agents. Sulfur mustard was used as the vesicant representative, again the total filtering capacity was also 300,000 mg-min per m^3. Phosgene was the representative of the lung damaging agent category, and the total filtering capacity was 120,000 mg-min per m^3, whereas cyanide represented the blood agents and the total filtering capacity was only 80,000 mg-min per m^3. Chlorine, phosgene, the cyanides (hydrogen cyanide and cyanogen chloride), and other acidic gaseous are removed by reaction chemistry of

their molecules with the impregnated salts of inorganic metals, rather than by adsorption onto the surface of the activated carbon (Morrison, unknown year). This activated carbon, impregnated with the salts of copper, silver, zinc, molybdenum, and TEDA, is contained in the second compartment of the filter (measuring from the filter's inlet to the filter's outlet). The accepted abbreviation for this carbon is ASZM-TEDA. An older type of carbon known as ASC contains the salts of copper, silver, and chromium (in its hexavalent form). Hexavalent chromium has been implicated in causing cancer, and is therefore considered a hazardous material, and must be disposed of by employing a hazardous material waste disposal protocol. This type of carbon is contained in the older C2 and M17 military filters, since replaced by the C2A1 filter. The first compartment (measured from the filter's inlet to the filter's outlet) of a CWA filter contains a P-100 HEPA filter* that removes particulate (solid) material, including chemicals in the micro pulverized state (riot control agents), biologicals (bacteria, viruses, toxins, etc.), and alpha and beta radiological particles. A third compartment (closest to the outlet of the filter) contains another, much smaller P-100 filter whose purpose is to remove any activated carbon that may have become dislodged, thus preventing this from entering the facepiece of the respirator. Activated carbon, impregnated with the salts of inorganic metals, is contained in the filters of all air-purifying respirators, and also the inner lining of semipermeable protective suits. It is also present in selected glove and boot liners that are specially designed to adsorb toxic chemical agents. The CBRN filter (male connection) is screwed into the facepiece of the respirator (female connection) using a standardized 40 mm NATO (North Atlantic Treaty Organization) thread. One needs to be sure that a gasket constructed of EPDM or butyl rubber (free of defects and in good working order) is present at the interface of the facepiece with the filter as this gasket actually forms the gas-tight connection that prevents the ingress of any unfiltered atmospheric air into the facepiece of the respirator.

Conditions of Level C usage: For the safety of the responder, several conditions must be met before Level C may be employed. The first of these conditions is that the identity of the toxic agent is known, its concentration is also known, and that concentration is below IDLH. IDLHs of some common CWAs and TICs, both in ppm and mg/m^3, and molecular weights may be found in Table 20.3. A second condition of Level C usage is that is cannot be used in an oxygen-deficient atmosphere, that is, one in which oxygen by volume is $<19.5\%$. A third condition of Level C usage is that the filters on the respirator must be capable of filtering the toxic agent in the concentration present; that is, a condition of Level C usage is that either IDLH or maximum use concentration (MUC), whichever is the smaller number, be used as the limiting factor of respirator usage (see the next paragraph). Additionally, the toxic material must have good warning properties (irritating odor, tearing, choking, coughing, etc.) in case of respirator failure.

An assigned protection factor (APF) is the level of protection that a respirator is expected to deliver when appropriately maintained and used within the confines of a respiratory protection program. Assigned protection factors are assigned by OSHA (Janssen, 1999; Runnion, 2003). Actually most modern respirators that have been properly maintained can deliver protection factors far in excess of these assigned protection factors. MUC is the maximum total amount of the contaminant in question for which a respirator can protect an individual. This is mathematically calculated by multiplying the APF of the respirator by the required OSHA permissible exposure limit (PEL), short-term exposure limit (STEL), or ceiling limit (CEL). Should the MUC of the respirator (APF multiplied by the PEL) exceed the IDLH concentration for the contaminant, then the IDLH concentration becomes the limiting factor for use of the respirator. If the APF multiplied by the PEL is less than IDLH, then this quotient (MUC) becomes the limiting factor of respirator usage (NIOSH,

* HEPA filters are of three efficiencies 95%, 99%, 99.97% (referred to as 100); and N = not resistant to oil, R = resistant to oil, P = oil proof, so a P-100 HEPA filter is at least 99.97% or greater efficient and oil proof (Woods, 1999). HEPA filter efficiency may be derived by measuring penetration by dioctyl phthalate, a molecule that in nature exists in 0.2–0.3 μ size. It has been found that this size particle is the most difficult size particle for a HEPA filter to remove (partially attributed to OSHA online training, year unknown).

TABLE 20.3
IDLH of Various CWAs and TICs

	Tabun GA	Sarin GB	Soman GD	VX	Mustard HD	Lewisite L	Cyanide AC	Cyanogen Chloride CK	Phosgene CG	Chlorine Cl_2	Ammonia
IDLH in ppm	0.03[c]	0.03[c]	0.008	0.002	0.0004	None stated	50	None stated	2	10	300
IDLH in mg/m^3	0.2	0.2	0.07	0.02	0.003	0.003[b]	60	None stated	10	30	230
Molecular weights[a]	162.13	140.1	182.178	267.38	159.08	207.35	27.03	61.48	98.92	70.91	17.03[d]

[a] United States Army (2005).
[b] Foust (1999).
[c] Manning (2002).
[d] Wikipedia (2006).

2005). More information on this topic is contained in 29 CFR 1910.134, which may be viewed on the Internet at the following address: http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_id = 12716&p_table = STANDARDS.

An excellent NIOSH web page for one-stop shopping on respirators is http://www.cdc.gov/niosh/npptl/topics/respirators/ (NIOSH, 2006).

Actual FFs of an individual respirator on an individual responder can be determined with a quantitative measuring device such as TSI's Port-A-Count®. This numerical value then can be stored in a computer program with the responder's name, respirator number, and date of test.

3. Powered Air-Purifying Respirators

The powered air-purifying respirator (PAPR or PAPPER) employed in Level C has many advantages including (1) minimal mobility restrictions, (2) small size, (3) easy maintenance because of few parts, (4) minimal cardiovascular effect, (5) easy storage in small spaces, (6) lightweight (only a few pounds without the blower, which can be worn on the belt), (7) cheaper cost per unit than atmosphere (air)-supplied respirators, (8) far less complicated design than atmosphere (air)-supplied respirators (since they lack a high-pressure tank, hoses, and high-pressure reduction valves), and (9) relatively simple to use. They characteristically filter at least 4–6 cubic feet per minute (cfm) of air (113–170 L per min) (3M Instruction Manuals, 2004) (Scott Safety C420 Brochure, 2005), and depending on the filters and blowers used, rates as high as 9 cfm (254.85 L per min) are obtainable (Minnesota Mining and Manufacturing (3M) Technical Assistance, 2006 Personal communication via telephone 800-243-4630, accessed 10 April 2006). Regulations emphasize that blowers of PAPRs employing a tight mask like facepiece must be capable of producing at least 4 cfm air flow, whereas those of loose-fitting hood-like facepieces must be capable of producing at least 6 cfm. The greatly increased volume of flow of filtered air created within the facepiece of the respirator by the blower would create an outward flow (from the respirator's facepiece to the surrounding contaminated atmosphere) of air, should a faceseal leak occur. This outward flow of filtered air would significantly decrease the possibility of inhalation of any significant amount of unfiltered toxic atmospheric air, especially in the hooded variety of respirators since no faceseal exists within many models with this type of respirator. Hence, all PAPRs (both tight-fitting facepiece and hood variations) are considered positive-pressure respirators by OSHA once the manufacturer has proven this by laboratory testing.

Those individuals with RAD as exemplified by asthma, as well as many forms of chronic obstructive pulmonary disease (COPD), may benefit greatly from a PAPR, when employed in an escape-only respirator format. Significantly, increased airway resistance during inspiration, such as that which is generated when employing a negative-pressure respirator, has long been recognized as a precipitating factor of a RAD/asthmatic attack. Individuals with moderate to advanced COPD are respiratory cripples, with significantly to greatly decreased respiratory muscle strength. Since the respirator's blower, and not the individual escapee's respiratory muscles, generates the force necessary to move the contaminated environmental air through the respirator's filters, inspiratory resistance to breathing for the individual escapee is for all practical purposes normal.

Disadvantages of a PAPR would include the inability of the respirator to be used in atmospheres which restrict the usage of any air-purifying respirator, and increased dead space (characteristic of any respirator).

By convention, both inspiratory and expiratory resistances of respirators are determined at minute ventilations of 85 L per min.

Other disadvantages of air-purifying respirators (both powered and negative pressure) include limited filter capacity and the inability to use them with chemicals that do not have warning properties (SAIC, 2002b). Often they cannot be used to filter compounds with low molecular weights (Pope, 1996), or whose vapor pressure is >10 mmHg, unless the filter canister is specially designed to remove specific contaminants (Morrison, unknown year), usually by employing reac-

tion chemistry. Lesson learned: always check to make sure the filter you wish to employ is capable of filtering the toxic agents in the quantities present.

In some cases, the thickness of filters used with PAPRs is made significantly thicker than those used with nonpowered (negative-pressure) air-purifying respirators. Although the added thickness equates to more respiratory protection through additional filtering, blower failure in such a case would result in inspiratory resistances far greater than that encountered with negative-pressure respirators (because of the increased filter thickness). Another disadvantage of PAPRs would include crimping or kinking of the low-pressure hose that joins the facepiece of the respirator and the filter/blower unit. Additionally, the blower is noisy, requires periodic maintenance, and runs on battery power. Care must be taken to be sure the batteries are always maximally charged and in working order. The noise created by the blower would negate the use of most PAPRs in a stealth operation.

4. Hooded Powered Air-Purifying Respirators

There is a subset of PAPRs that do not form a tight seal with the face. These are known as hood-type PAPRs. A large, loosely fitting hood usually made of a butyl rubber-coated material covers the head, shoulders, and upper chest.

Two different subsets of these hooded respirators exist. In one subset, there is a stretchable butyl rubber dam (or other suitable material) that surrounds the neck, similar to the stretchable rubber dam on a dry suit used for diving. This dam prevents the ingress of unfiltered atmospheric air. Since this forms a tight seal with the neck, a theoretical disadvantage of this type of hood is that the tight seal is delivered at about the level of the carotid body, and could cause bilateral stimulation of the carotid body, leading to bradycardia (abnormally slow heart rate, less than 60 beats per min), and syncope (fainting) in a sensitive individual. At this time, I am unaware of any such reports in the literature. Of note is that all OSHA escape-only hood-type respirators (which are usually negative-pressure air-purifying respirators, although a positive-pressure PAPR hood known commercially as the SCape CBRN[30] has recently been introduced) employ this stretchable neck dam.

A second type of hooded respirator employs a duplication of the hood (two hoods) that covers the chest below the level of the facepiece. There is no stretchable rubber neck dam in this type of respirator, hence no pressure on the neck. The first hood is tucked inside the protective suit garment, whereas the second hood is draped over the chest on the outside of the protective suit. Although this type of hood has no neck seal, it relies on the volume and flow pattern of filtered air from the blower to prevent inhalation of any unfiltered air. Although this type of hooded respirator has an expiratory outlet valve, the large volume of air flow created by the blower (>6 feet^3 per min) forces a small amount of the filtered air into the inside of the protective suit, and in the event of blower failure the air within the suit would act identical to the reserve bag in an anesthesia circle system, and provide a reservoir of questionably filtered air (remember Level C suits do not have to be vapor impermeable), which at the very worst would have levels of atmospheric contaminants similar to those in the environment. Since Level C can only be employed if the atmospheric contaminant is below IDLH values, this is the greatest level of contaminant the responder could be exposed to in the event of respirator failure. Moreover, IDLH values are purposefully constructed to provide a great margin of safety in the event of respirator failure before significant physiological manifestations of toxic vapor inhalation would be present, allowing time for a safe escape from the toxic environment.

An additional advantage of this type of hooded respirator is that as long as the blower is operating a small percentage of filtered air is blown into the protective suit. This air is then exhausted to the outside atmosphere; consequently there is a small flow of filtered air whose movement is always from the (1) hood to the (2) inside of the Level C suit and then to the (3) outside of the suit. This flow pattern and velocity of filtered air decreases the potential for ingress of the toxic atmospheric air into the suit.

Moreover, this type of powered respirator creates a rather rapid current of air within the facepiece of the hood, and to a much lesser degree the protective suit. This gives a cooling effect (convective and perhaps evaporative) to both the head and possibly the body. In the absence of a

nosecup the responder's warm, moist expired air could condense on the inside of the viewing lens of the hood, thus interfering with vision, as well as causing the condensed water to drip down into the inside of the protective garment. The high volume of filtered air created by the blower has significant velocity and flow, which helps prevent any significant amount of the responder's expired warm and moisture-laden air from reaching the inside of the facepiece's viewing lens. This decreases the possibility of formation of condensation on the inside of the lens. Hooded-type respirators (PAPR and PAPR escape hoods) traditionally lack a nosecup. In the absence of a nosecup some responders will treat the inside of the viewing lens with antifogging material.

Yet another significant advantage of this type of PAPR is that it is exempt from OSHA's fit-testing requirements. Please note that even though this respirator does not require fit testing, all responders who are required to wear any respirator as a responder, including this one, must undergo a medical evaluation to determine the employee's ability to safely use a respirator. Escape-only respirators are exempt from the medical evaluation requirement. Additional advantages including special eye lens adaptors are not needed, as the responder's regular eyeglasses may be worn. The responder does not have to be clean shaven, since there is no need to provide a tight seal between the respirator and the user's face; consequently, both subsets of hooded respirators are ideal for bearded individuals, or those with facial deformities or injuries that would prevent a traditional respirator from forming a gas-tight seal with the responder's face. These are also ideal for the responder with claustrophobia, providing a distinct psychological advantage as the average responder has less apprehension about donning this type of respirator than a traditional respirator employing a facepiece with a tight faceseal. Its field of vision is the best of any type of respirator, preventing the responder from having to turn his head to view parts of his surrounding. These respirators are well tolerated by individuals who are less-than-perfect physical specimens of a well-conditioned body. Overwhelmingly, the hooded PAPR has been selected for hospital decontamination teams requiring Level C equipment.

5. Negative-Pressure Air-Purifying Respirators

Negative-pressure full-faced air-purifying respirators are best illustrated by the traditional military gas mask. Please note that the military itself does not refer to these respirators as gas masks; this term has evolved from slang terminology. Indeed, the formal military designation of these respirators is field-protective masks. The name says it all: it is a protective mask for use in the field, not within buildings. Following a civilian lead the military has subsequently adopted respiratory standards that would prevent this type of respirator from being employed within a toxic atmosphere containing contaminants in levels equal to or in excess of IDLH except on the battlefield. Extensive data collected by the military clearly show that when a proper gas-tight faceseal between the respirator and the soldier is present, and the respirator has been well maintained, including use with a currently serviceable chemical warfare filter, this type of respirator is fully capable of providing respiratory protection for the soldier against several LCt_{50} doses of CWAs (SBCCOM, 2003). This is the respirator that is issued to military personnel for use in combat. Despite these data, civilian authorities (OSHA) will not permit this type of respirator to be used in concentrations that equal or exceed IDLH levels. Two arguments are advanced: (1) that military personnel have far better and repetitive training in the use of field-protective masks and (2) the military, unlike its civilian counterparts, is willing to accept a certain level of casualties in battle. Nonetheless, this does seem like a double standard.

Negative-pressure full-faced air-purifying respirators (gas masks) have advantages that include lightweight, small size, being the least expensive type respirator, capable of being easily maintained, providing only a minimal increase in cardiac work, and with minimal mobility restrictions. However, they possess significant disadvantages that include exclusion from use in oxygen-deficient atmospheres ($<19.5\%$ by volume), or any other atmosphere which would preclude the usage of an air-purifying respirator including contaminants at or above IDLH. Although these respirators cause little direct increase in cardiac work, they can indirectly contribute to increased cardiac work because of their direct effect on increasing respiratory work.

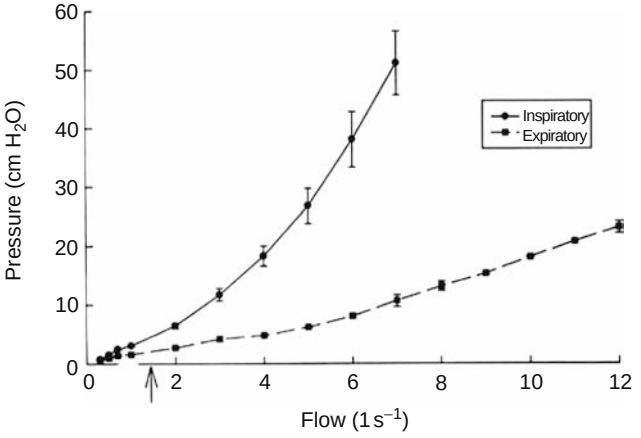


FIGURE 20.1 Pressure–flow relationships of a U.S. Army M40 field mask. (From Musa, S.R., Banderet, L.E., Cadarette, B, *Textbook of Military Medicine: Medical Aspects of Harsh Environments, Volume 2*, K.B. Pandolf and R.E. Burr, eds, Office of the Surgeon General, Department of the Army, United States of America, Falls Church, VA, 2002.)

Most notably, these respirators significantly increase both inspiratory and expiratory resistances; however, the effect on inspiratory resistance is far greater than the effect on expiratory resistance. Moreover, both of these resistances increase in a geometrical (not arithmetical) fashion with increasing rates of minute ventilation. Please see the accompanying graph (Figure 20.1) of inspiratory and expiratory resistances (Musa, 2002). These respirators specifically significantly decrease maximum voluntary ventilation, peak inspiratory and peak expiratory flow, and forced inspiratory flow 50%. The geometric increase in respiratory resistance with increasing minute ventilation also contributes to a geometrically increased respiratory workload (Musa, 2002). Significantly increased inspiratory and expiratory resistances achieved with increasing airflow rates resulted in decreased submaximal oxygen uptake, minute ventilation (V_E), and physical endurance when work levels above 135 watts were achieved (Musa, 2002). The decrease in maximum voluntary ventilation that resulted in significant decreases in submaximal oxygen uptakes also impacted negatively on oxygen availability to the tissues, and thus decreases exercise tolerance and endurance. There is a significant increase in dead space (as is true of any respirator), thus decreasing alveolar ventilation, with accompanying increases in both alveolar and arterial carbon dioxide partial pressure and tension. This decrease in alveolar ventilation is compensated for by increasing the inspired (tidal) volume (Musa, 2002). Individuals sensitive to increased carbon dioxide tensions will increase their respiratory work in an attempt to normalize carbon dioxide tension (Musa, 2002). There is a distinct increase in bronchospasm during use of negative-pressure respirators, and this is especially well documented in individuals with RAD.

6. Respirator Facepiece Construction

Another factor when considering respirator selection is the material that was used to construct the facepiece. Certain materials, such as butyl rubber, especially butyl rubber that has been impregnated with the halogenated salts of chlorine or bromine, are especially resistant to permeation by the vesicant agents. Although all chemical agents or compounds (warfare and otherwise) will eventually permeate through the facepiece of respirators and other chemical-protective equipment, the real question is one of permeation time. Is this permeation time expressed in seconds, minutes, hours, days, weeks, months, years, decades, centuries, or millenniums? It should be noted that generally the permeation time of facepiece material (or any chemical-protective material) increases as the

thickness of the material increases, and this increasing permeation time generally follows more of a geometric, rather than an arithmetic pattern. Of all CWA classes (lung damaging agents, cyanides, vesicant, nerve agents, incapacitating agents, and riot control agents), the vesicant group of CWAs are the quickest to permeate chemical-protective material, including facepieces. Additionally, respirators built with silicone facepieces have particularly poor resistance to permeation by vesicants (Gander, 1997), and require a second-skin vesicant permeation barrier. This second-skin barrier is often made of EPDM, although butyl rubber (particularly those impregnated with chlorine or bromine salts, i.e., halogenated) is an excellent alternative.

The U.S. Army/Marines M40 and M40A1 mask, and the FR-M40 (First Responder M40) are excellent examples of a respirator employing a silicone facepiece. Silicone definitely has its strengths. These include: (1) an excellent ability to contour (or mold) to the face thus decreasing the possibility of leaks, (2) resilience in returning to its original shape after distortion, thus preventing leaks, and (3) possessing minimal allergenic properties. This is helpful to those individuals who are sensitive or allergic to latex and other rubber products. As a general rule of thumb, any facepiece material will resist permeation (within the time frame of field usage) by lung-damaging agents (choking agents), cyanides (blood agents), and nerve agents. The real test of permeation resistance of a respirator's facepiece is when it is tested against the vesicants. These include mustard (sulfur and nitrogen), Lewisite, and phosgene oxime. Realistically, the chance of encountering sulfur mustard (''the king of battlefield gases'') is greater than the other vesicants because of the ease of its synthesis. Mustard is a simple straight-chained molecule (Campbell, L. (2003), personal communications via telephone, accessed 18 November 2003), and its molecular shape allows it to easily permeate certain materials. By contrast, nerve agents tend to be larger and branched molecules (Sidell et al., 1997). The key to the selection of respirator facepiece material is to have permeation data against the chemical agents to be encountered.

An extremely important point about permeation of chemical warfare (or any chemical agent) through chemical-protective materials should be made at this point. Although permeation data of CWAs through types of protective material are readily available from other sources, these data are based on only the CWA and the chemical-protective material in the equation. If the CWA is now mixed with a solvent, permeation data for the CWA through the protective material may drastically change according to the nature of the solvent. Common solvents may include petroleum products, such as gasoline and oil, and other hydrocarbons. As an example, petroleum products applied to butyl rubber drastically reduce the permeation time of CWAs through it. One should exercise great care to prevent petroleum or petroleum distillate products from coming in contact with chemical-protective equipment made of butyl rubber, halogenated butyl rubber, nitrile, etc. Should such contact occur it is imperative that the butyl rubber, nitrile, or other protective products be immediately discarded and replaced with new pieces of similar protective products not exposed to petroleum products. Such contact would include storing personal protective equipment products in buildings where they could be exposed to liquid petroleum products or petroleum vapors or fumes. As an example, in a clinical test employing 1% sarin (GB) mixed in a solvent of 99% saline, permeation through butyl rubber glove material took greater than 6 h. However, by simply changing the solvent from 99% saline to 99% hexane or 99% chloroform and keeping all other test factors the same, the permeation time was reduced from greater than 6 h to 5 min. A similar test under the same conditions employing nitrile glove material reduced the permeation time from greater than 2 h to 5 min (Battelle Medical Research and Evaluation Facility, January 1999, Table 10). The take-home message is that permeation data must be based on not only the CWAs and type of protective material, but also on any solvent that may be involved. Additional factors that would influence these data are total dose of CWAs, thickness of the protective material, temperature, humidity, and any breeze (that would cause evaporation of the CWAs) that would be blowing under test conditions, etc. Clearly data from one set of permeation tests are not necessarily applicable to another set of permeation tests involving the same chemical agents and protective material unless all other variables are exactly the same, a highly unlikely occurrence.

Finally, materials commonly employed in the construction of respirators would include natural rubber, butyl rubber, chlorobutyl rubber, bromobutyl rubber, neoprene, and silicone.

7. Protective Suit Construction

Let us now consider other issues related to PPE. The first of these is the construction methods of protective suits. Multiple varieties of materials are used in the construction of protective suits. These materials are employed to produce either a unilayer or a multilayered laminated-type garment. All suits are made from either impermeable or semipermeable (usually referred to as permeable) material. Level A or B suits are constructed of impermeable material, whereas Level C suits may be constructed of either impermeable or semipermeable material. Usually, permeation testing on materials used in the construction of protective suits is evaluated for a total of 480 min (8 h) duration. No civilian responder will stay in PPE for longer than 8 h; hence, permeation testing is stopped at that point. If the chemical agent has not permeated the suit in that time frame the suit is considered impermeable to that test agent, but it may be totally permeable to another chemical agent. As pointed out earlier, solvents may drastically alter permeation data. Learning point for emphasis: know the permeation data of your respirator, protective suit, hood, gloves, and boots against the chemical warfare (or TICs) that you are likely to encounter; as well as other incidental chemicals, which are commonly encountered in the field, such as petroleum products (gasoline, etc.). Additionally, suits may be classified as being one- or two-piece construction. Suits may be classified according to one or all of the previously mentioned criteria.

Level C suits may be made of either semipermeable (generally referred to as permeable) or impermeable material, and may be made as a one- or two-piece ensemble (jacket and trousers). This two-piece construction is commonly seen in semipermeable activated carbon-impregnated suits. One should note that the elbows and knees in permeable suits are usually reinforced with the internal addition of butyl rubber at the sites of maximum stress, such as elbows and knees. Let us briefly consider the difference between impermeable and permeable (semipermeable) suits. The following is a summary of a SBCCOM report (SBCCOM, June 2001). Impermeable suits are generally less expensive than permeable suits, they are for one-time use only, and most impermeable suits models are available with hoods and attached booties. Due to their one-piece construction with impermeable material, heat retention within these suits is a much greater problem than with permeable suits. However, impermeable suits can be exposed to water or other liquids, whereas exposure of permeable suits to these same fluids will begin degradation of the protective carbon products. Impermeable suits are preferred for decontamination procedures, but permeable suits can be used without degradation if a butyl rubber apron is worn over the suit and covers all areas of the suit, including arms, which might become exposed to water or any liquid. Impermeable suits are smaller and more compact for storage purposes, and generally come in bright colors. In addition, the material from which impermeable suits are constructed is stiff and makes rather loud noises on movement; hence, impermeable suits are usually unsuitable for sheath operations.

Although permeable suits may be reused after washing according to the manufacturer's instructions, these suits should never be reused after exposure to a toxic substance, as some of the toxic substance will have been adsorbed by the protective activated carbon. If this toxic material does not undergo hydrolysis (chemical decomposition by combining with water usually resulting in less-toxic or nontoxic materials) when adsorbed on the activated carbon of the suit, it may well desorb from the suit at a later time. Due to the permeable nature of the suit's components, coupled with the usual two-piece construction, heat is dissipated much more quickly than with a one-piece impermeable suit. However, this two-piece construction of some models potentially leaves body areas that might be exposed to toxic atmospheric pollution during movement by the individual. Careful attention should be given to such measures as tightening the securing string located at the bottom of the jacket. Additionally the hook-and-pile closures (also known by their proprietary name of Velco®), whose upper halves are located at the bottom of the jacket, should be firmly attached to

their accompanying halves located at the top of the trousers. Tightening the hook-and-pile closures located at the base of the arms and legs will also decrease the likelihood of such a problem occurring. Taping the joint between the jacket and trousers, suit and gloves, pants and boots, and hood and respirator, or any interface between two chemical protective ensemble components, with chemical resistance tape is another method to decrease ingress of unfiltered vapor into the suit's interior. Such ingress of unfiltered atmospheric air into the interior of the suit through these opened joints during movement has been referred to as the bellows effect (McIntosh, R. (October 2002), personal communication).

Generally, Level C suits do not have attached gloves and booties, although many models do come with attached hoods. Although permeable suits are produced in one-piece coverall-type construction, they are indeed rare. Characteristically, both impermeable and permeable Level C suits, as well as the coverall type of Level B suits, leave a gap in protection at the level of the chin just below the respirator. Pictures of this problem abound in the literature (SBCCOM, June 2001). Some of the newer suits have a flap on the lower part of the suit's hood that will swing across the chin directly below the respirator and attach by hook-and-pile to the base of the suit's hood on the opposite side. Some respirators have an enlarged EPDM second skin that extends below the respirator to cover this exposed area of the chin and neck, and tucks into the suit at the level of the upper chest, the FR-M40 respirator being a prime example. A common practice with suits that have a built-in hood is to take chemical resistance tape (several small pieces) and tape the hood to the respirator. When taping always turn an end of the tape in upon itself (such that adhesive is in contact with adhesive), this will leave a tear tab (dog ear) to later easily remove the tape. This has been illustrated in a picture included in the photographic section at the end of this chapter. Duct tape, lacking permeation data, has also been used for this purpose. Another method of protecting this area is by wearing an external respirator hood made of fabric that has been coated with a chemical protective coating, usually of butyl rubber. These hoods directly adhere to the respirator (usually with an elastic band), and cover the head, shoulders, and upper chest. Tests have shown that hoods significantly decrease chemical agent exposure to the neck, chin, and face. When employing such a hood a good idea is to place the hood, minus respirator, over your head and look up at a light. Should there be any break in the butyl rubber protective barrier one will be able to observe the light. Should this occur, one should replace the hood. The older practice of using duct tape to patch these defects should be discouraged.

Level C impermeable suits usually are manufactured as one-piece ensembles, whereas semipermeable suits usually come as two-piece ensembles. One-piece suits usually have fewer areas for ingress of toxic vapor contaminants. Impermeable suits characteristically are lighter in weight than semipermeable suits.

The most vulnerable areas of the skin are (1) the chin directly below the respirator and (2) the scrotum. The latter is explained by the scrotum's skin being thin, warm, moist, and covered. Skin meeting the previously mentioned criteria is highly susceptible to attack by chemical agents. WWI chemical causality care mustard data clearly prove this point.

A summary of effectiveness of commercially available personal protective suits, including permeation breakthrough times for sarin and mustard, is available from several sources (Ellison, 2000).

E. Level D

OSHA and EPA Level D is the normal work uniform, without respirator or any protective suit, although a surgical mask, gown, and gloves (frequently of latex) may be worn. This would be the type of PPE traditionally worn by hospital laboratory workers.

F. Boots

Boots are usually made of vinyl or suitable rubber (usually butyl) material that resists chemical agent permeation. Vinyl boots, when contaminated by liquid chemical agent, will desorb vapor over

a prolonged period of time, and this should be considered when unmasking (CCCD, July 2000). Traction with these boots can be a problem. The practice of wearing boots several sizes larger than shoe size creates traction problems, and suggests an increase in the metabolic work of walking (Musa, 2002), probably through the hobbling effect.

G. Gloves

At least two sets of gloves should be worn with the chemical protective ensemble, the inner usually cotton (although nitrile and latex gloves have been used) glove and the outer chemical protective glove. An intermediate layer glove (forming three layers of gloves) may be added at the individual's discretion. Actually, the practice of three glove layers is becoming common. Usually these outer gloves are made of butyl rubber or halogenated butyl rubber, a substance highly resistance to permeation by chemical agents, although recently gloves made of nitrile and Viton have been employed. However, note petroleum products easily permeate butyl rubber. On the other hand Viton has excellent permeation resistance to oil and gasoline products, hydraulic fluids, and hydrocarbon solvents, whereas nitrile has very good permeation resistance to oil and gasoline, superior resistance to petroleum-based hydraulic fluids, and good resistance to hydrocarbon solvents (DuPont, 2006). Any contact of butyl rubber with petroleum products (gasoline, oil, etc.) will rapidly degrade the protective effect of this material, and they should be immediately discarded. Nor should butyl rubber be used for insulation against electrical shock. Butyl rubber gloves usually come in three thicknesses, 0.007, 0.014, and 0.025 in. You may hear these referred to as 7, 14, and 25 mil gloves. Testing suggest that the 14 and 25 mil gloves can be used up to 24 h in a contaminated environment before requiring decontamination. However, the 7 mil gloves, used for operations requiring good tactile properties (medical types, typing and computer usage), must be decontaminated and inspected within 6 hours (CCCD, July 2000).

H. Toxicological Protective Aprons

The TAP is a totally impermeable, heavy, and hot, butyl rubber apron with long sleeves that covers any area of the suit that might become exposed to liquid. It should be worn over any semipermeable suit if the suit is to be used during decontamination procedures employing liquids.

I. Cooling Vests

Lightweight cooling vests for wear when in PPE are becoming more popular. Several manufactures have produced these. Careful balance of cooling effect versus weight of the vest is of utmost concern.

J. Vision-Assisting Devices

Vision-assisting devices include (1) traditional eyeglasses (spectacles), (2) metal and plastic eye lens respirator optical inserts, and (3) contact lenses. Traditional eyeglasses with earpieces cannot be used with tight-fitting respirators since the earpieces would break the faceseal of the respirator. However, traditional eyeglasses can be employed with hood-type Level C respirators (both powered and negative-pressure air-purifying respirators; the negative-pressure Level C group being represented by the escape-only type respirators). Optical inserts are constructed of either metal frames or plastic frames that accept prescription lenses. They look like traditional eyeglasses (spectacles) except they lack earpieces, hence they will not break the faceseal of the respirator. They are made to be of either one- or two-piece construction. One-piece construction is usually made of metal or heavy duty plastic, and they have prongs or spikes (of plastic or metal) at the sides of the insert which snuggly fit into premanufactured depressions in the inside of the facepiece of the respirator. Two-piece optical inserts are made of either metal or plastic. The first piece fits within the inside surface of the respirator usually just above the nose where it attaches to the inside of the respirator with a glue-like material. Thus it becomes permanently attached to the respirator at this point. The other side of this piece contains a

hook-and-pile pad to accept and hold the second piece. The second piece, containing the prescription lens frame, attaches to the first piece with hook-and-pile. The newer types of these two-piece optical inserts are made of plastic. Older metal two-piece optical inserts (e.g., M17 and M40 and older M40A1 military masks) have a metal circular-like frame that fits directly against and around the inside perimeter of both eyepieces of the respirator. At the lateral (outside) side of each of the respirator eyepieces a small metal spring attaches directly to the prescription lens frame, thus the prescription lens frame is suspended within the respirator and may be subjected to movement or bounce as the responder moves. Additionally, spectacles possessing a very thin elastic or rubber head strap in place of earpieces are available. The thickness of this head strap is very thin and does not break the respirator's faceseal. Contact lens wear were usually not recommended for use with a respirator, although little documented evidence exists to support this point of view (NIOSH, 1996). It was felt that contact lenses could slip and change their position due to the possibility of pressure on the outside corners of the eyes from a full-face respirator, or a speck of dirt or dust might get under them when they are worn, and only respirator removal and adjustment of the lenses (unacceptable in an atmospheric contaminated environment) would rectify this situation (United States Nuclear Regulatory Commission, unknown year). Older fears had included the possibility of a small amount of toxic vapor becoming trapped between the lens and the eye, resulting in absorption of toxic material into the eye. OSHA studied this problem with a research project conducted by the Lawrence Livermore National Laboratories, who concluded that contact lenses were perfectly acceptable to wear with a full-facepiece respirator (DaRoza and Weaver, 1985). As a result of this study and other papers, OSHA published an enforcement procedure authorizing the use of rigid gas-permeable and soft contact lenses in all workplaces and with all types of respirators (American College of Occupational and Environmental Medicine, 2003). The United States Department of Homeland Security, Center for Domestic Preparedness in Anniston, Alabama, allows the use of contact lenses meeting the previously mentioned requirements in their Toxic Agent Training Facility (the only such facility available to civilian responders in the United States) where students are actually exposed to very high-purity sarin (GB) and VX in concentrations below IDLH (United States Department of Homeland Security (2005), p. 7).

K. Miscellaneous Equipment

1. Head Harness

Military respirators usually have a six point head harness; two straps each on the forehead, the temporal region, and the chin. These straps meet at a central location at the posterior of the skull, and here a small pad is located which serves as the terminal of these straps, as well as providing a degree of cushioning effect for the posterior of the skull. Civilian respirators may have either a six point or a five point head harness. The latter employs only one centrally located forehead strap. Usually these straps are made of elastic material, although some straps may be made of flexible chemical-resistant rubber. An alternate to this arrangement is a skull cap-type head harness. The six much shorter straps of the harness end not in a small pad at the posterior of the skull, but at a large skull cap; much like that worn by a medieval monk, or a large prayer cap covering a large part of the posterior head. This is illustrated in the photographic section of this chapter. Some responders feel these are more comfortable and slip less than traditional head straps.

2. Outserts

These polycarbonate lenses attach to the outside of the respirator's eye lens usually by elastic chemical-resistant rubber rings attached to the periphery of these devices. Clear outserts protect the eye lens of the respirator from damage and scratching from blowing sand, dirt, or loose objects. If outserts become scratched or damaged they are easily changed. In addition, all outsert types help reduce fogging of the respirator's eye lens in cold or moist weather. This is accomplished by the

outserts reducing the degree of temperature change between the outside and the inside of the eye lens of the respirator's facepiece; that is, the outserts act as a temperature buffer zone by increasing the distance between the cooler outside air and the warmer air within the respirator's facepiece. Their action is identical to that of the double glass layer in insulated windows. Tinted or smoked colored outserts, in addition to providing protection as described above, also provide filtering of the sun's ray, identical to sunglasses. Laser outserts are designed to filter out specific laser light wavelengths (e.g., ruby, 694 nm wavelength and neodymium, 1064 nm wavelength). Ballistic outserts strongly resist damage from fragmentation; that is, they are ballistic resistant, not ballistic proof.

3. Reversed Faceseal

A relatively narrow strip of faceplate material is attached completely around the inside circumference of the respirator's facepiece close to the periphery of the facepiece material. This is seen only in the newer (fourth generation) respirators. The lateral aspect of this strip of material is fused into the inside of respirator's facepiece while the medial unattached edge runs toward the center of the facepiece. This material, therefore, extends from the periphery toward the central area of the respirator's facepiece, and is therefore reversed from central to peripheral orientation of the respirator's facepiece material. This strip makes direct contact with the responder's face, and is compressed firmly against it by pressure generated with the tightening of the respirator's head straps. The water seal (created from the responder's perspiration) that forms between the responder's face and the respirator facepiece is what forms the vapor-proof seal of the respirator. Since this reversed seal runs much more parallel to the responder's face in the forward area of the respirator's facepiece, this strip greatly increases the area of direct contact between the respirator and the responder's face, thus greatly increasing the area of the respirator's vapor-proof water seal. Consequently, this reversed seal greatly decreases the likelihood that the water faceseal between the respirator and the responder's face will break during times of stress; that is, facial movements such as talking, chewing, and swallowing, or during bouncing of the respirator's facepiece, which may be created by body movement.

4. Voicemitter

The voicemitter is a device placed in the front or side of the facepiece of the respirator to increase the quality and volume of the responder's speech. Respirators tremendously decrease the quality and the volume of the spoken word, and other responders often find their speech unintelligible. This device contains a vapor-proof diaphragm that aids in the transmission of a higher-quality speech pattern with greater volume, although this is far from perfect. Voicemitters on the side of the respirator improve the quality and the volume of speech transmitted when using a telephone.

5. Voice Amplifiers

These battery-powered devices attach externally to the outside of the front voicemitter of the respirator (either by a thread-like mechanism or external clips) and electronically amplify the volume of the responder's speech. Consequently, these devices are used in crowd control or where multiple responders must be addressed at once; however, they do increase the weight on the front of the respirator's facepiece and this could possibly aid in breaking the vapor seal of the respirator.

In summary, clearly all normal bodily functions such as vision, eating, drinking, speaking, eliminating body waste, and recognizing individuals are all compromised when wearing PPE.

In January 2005, OSHA published *OSHA Best Practices for Hospital-Based First Receivers of Victims from Mass Casualty Incidents Involving the Release of Hazardous Substances*. This publication, a must-have item for hospitals, is available at: http://www.osha.gov/dts/osta/bestpractices/firstreceivers_hospital.pdf.

Most importantly, remember that employing PPE is a team sport. When using PPE, either in training or in a true toxic environment, one should never be without a buddy also dressed in the same level of PPE. Both responders should go everywhere together, never losing sight of each other. This will benefit everyone's safety.

IV. MISCELLANEOUS TOPICS RELATED TO PERSONAL PROTECTIVE EQUIPMENT

A. Skin Exposure Reduction Paste Against Chemical Warfare Agents

A new material, currently available only to the military, is known as Skin Exposure Reduction Paste Against Chemical Warfare Agents (SERPACWA). This is an FDA-approved Teflon®-based paste that is applied to any area of the skin where gaps in the protective suit ensemble may occur, care being taken to keep it away from the eyes and mucous membranes. This material will significantly increase the time required for skin permeation by CWAs, while decreasing the amount of total agent permeated. However, it should be noted that it will not totally stop CWA permeation through the skin. An instructional diagram included with the paste shows the areas of skin to which SERPACWA should be applied. Generally speaking, these areas include all areas where there is an overlap of two pieces of protective clothing, that is, top half of protective suit over bottom half of protective suit, sleeves of suit and gloves, trousers and boots, etc.

Although SERPACWA forms a barrier paste, it does not detoxify (chemically neutralize) CWAs. A second generation of this paste, now being developed, will accomplish this goal. This material cannot be exposed to fire or burnt as toxic fumes from the ignition of Teflon® would result. Combustion of Teflon® produces perfluroisobutylene (PFIB), which is a lung-damaging agent that is 10 times more potent than phosgene. Should skin treated with SERPACWA (e.g., fingers) come into contact with material that will later be burnt (e.g., a cigarette), small amounts of SERPACWA could be transferred to the surface of that material. If such a transfer of SERPACWA occurred, subsequent combustion of this material (i.e., cigarette) would release toxic fumes of PFIB as previously stated.

Incidentally, SERPACWA superbly protects skin against the irritating effects of urushiol (the active ingredient in both poison ivy and poison oak), and this was proven in tests conducted by the military employing human beings. Since it is unethical to conduct tests that expose human beings to CWAs, the active ingredient of these previously mentioned materials was substituted. Although currently available only to the military, who owns the patent rights, commercial development of this product (for use against poison ivy and poison oak) may occur at a later time in an effort to recoup developmental costs. Lukey et al. (2007) provide additional coverage of SERPACWA in chapter 21.

B. Alternate (Nontraditional) Forms of Personal Protective Equipment for a Large-Scale Chemical Event

Should a major terrorist CWA release incident occur in a large public area such as a mall, many victims would be dead or seriously injured by the time the HAZMAT unit arrives and suits up. Under normal HAZMAT conditions, a reasonable response time would be 45 min, and usually these teams are composed of fewer than one dozen individuals. Under chemical warfare conditions, this would equate to not enough responders arriving too late. Planners have looked for other possible options for availability of responders. One such option is the use of firefighters in their usual turnout gear with very specific limitations. This option was studied by the U.S. Army Soldier and Biological Chemical Command's (SBCCOM) Domestic Preparedness Chemical Team at Aberdeen Proving Ground, Maryland. Consequently, a set of guidelines for usage of firefighters in turnout gear was formulated. This unique and important report is entitled *Guidelines for Incident Commander's Use of Firefighter Protective Ensemble (FFPE) with Self-Contained Breathing Apparatus (SCBA) for Rescue Operations During a Terrorist Chemical Agent Incident*, Final report, August 1999. This 65 page report should be read in its entirety, but briefly two general guidelines (as stated on the cover

of the report) are included here. ''Standard turnout gear with SCBA provides a first responder with sufficient protection from nerve agent vapor [not liquid] hazards inside interior or downwind areas of the hot zone to allow 30 min rescue time for known live victims.'' Note the words ''known live victims.'' Since these people are still alive within the confines of a nerve agent-contaminated environment without employing any PPE, this would imply that the concentration of vapor nerve agent would be on the low side, or that the effects of any liquid nerve agent had not had time to occur. Either situation requires immediate response. The second guideline states ''Self-taped turnout gear with SCBA provides sufficient protection in an unknown nerve agent environment for a 3 min reconnaissance to search for living victims (or a 2 min reconnaissance if HD [sulfur mustard] is suspected)'' (Domestic Preparedness Chemical Team, August 1999). In this scenario, the status of the victims would initially be unknown. This report contains many conditions of operation, and again it is emphasized that the report must be read in its entirety.

C. Wet Bulb Globe Temperature Index

The most disruptive element of providing care for chemical casualties when wearing PPE is caused by heat stress and subsequent dehydration of the responder. To decrease heat stress, published tables provide work–rest cycles based on temperature being measured by the Wet Bulb Globe Temperature Index (FM 3-11.4, June 2004). Careful adherence to these tables will decrease heat-related casualties among responders. This index considers the effects of temperature, humidity, radiant energy, and wind. It produces a temperature based on integration of the effects of the above four factors (Stafford County Public Schools, unknown year).

D. Conversion of Parts Per Million or Parts Per Billion to Milligrams Per Cubic Meter

An absolutely annoying feature of dealing with chemical compounds is that their concentration may be expressed either in (1) ppm and ppb or (2) mg/m^3 (milligrams per cubic meter). Tables expressing concentration data in ppm or ppb cannot be interchanged with tables expressing the same data in mg/m^3. Luckily, there is a formula for converting one form of measurement into the other. The conversion formula is

$$mg/m^3 = (\text{ppm})\ (\text{g.m.w.})\ \text{divided by}\ (R)(T) \tag{20.1}$$

The key to this formula is as follows: ppm is parts per million (not ppb, be careful of this). In addition, remember that 1 ppm = 1000 ppb and 1 ppb = 0.001 ppm. The term g.m.w. is gram molecular weight (molecular weight of 1 g of the substance expressed in daltons), which is obtained from a chart of molecular weights of substances. Table 20.3 provides molecular weights of some CWAs and toxic industrial compounds. Material safety data sheets (MSDS) are another good source for providing molecular weights of toxic compounds. The term R = a constant, and at sea level this constant is equal to 0.08205. The expression T is temperature in absolute degrees (Kelvin) (Ellison, 2000). Kelvin temperature is centigrade temperature + 273, so 25°C = 298 K. Additionally, degrees Fahrenheit = 1.8 times degrees Centigrade + 32. Consequently, 77°F is equal to 25°C. If one computes the denominator of the fraction (i.e., R multiplied by T) using a temperature of 77°F, which is equal to 25°C, which is also equal to 298°K, then the value of the fraction's denominator is 24.45. Hence, at temperatures at or close to 77°F one may substitute 24.45 for RT in the denominator of the formula, realizing that the further one moves away from 77°F in either direction, the greater the error will become. Luckily, this error is small in the range of temperatures normally encountered in the environment.

E. Respirator Storage

Certain guidelines should be used in the storage of respirators. Respirators should be stored in a clean, dry, cool place, which is free of contaminants (liquids or vapors). Remember how petroleum or petroleum distillate vapors (as well as many other chemical liquids and vapors) can damage many types of protective material, and drastically decrease permeation times of chemical agents through

them. Pay careful attention not to store respirators or filters near petroleum or petroleum products or any chemical liquids or vapors. Storage temperatures should never exceed 120°F. Manufacturer's instruction manual should always be read in their entirety. They should be stored in a storage bag or container away from flames, direct heat, or sunlight. A face form (an internal skeleton-like device usually made of plastic and molded to conform exactly to the inside contours of the respirator) should be used for long-term storage, and no weight, which might distort the respirator during long-term storage should be placed on the storage container. Keep filters sealed or in sealed containers until just before use. Do not use the respirator storage container as a pillow for sleeping. This has been a problem in the military, and such action may break or distort the respirator.

F. Shelf and Service Lives of Personal Protective Equipment

Remember that every component of the personal-protective ensemble (respirator, suit, gloves, boots, hoods, TAP aprons, etc.) has a shelf life; that is, how long the manufacturer is willing to stand behind their product to perform its job as the manufacturer has described. The military makes a further distinction between the terms ''shelf life'' and ''service life.'' Shelf life may be defined as the period of time that a piece of chemical-protective material is expected to retain all of its protective properties if it is left unopened in its own factory (often vacuum sealed) protective packaging. As an example, JSLIST chemical-protective suits are assigned a lot number and each lot number is assigned a shelf life of 5 years. Throughout the 5 year shelf life lots from different times of manufacture are tested at random. If a lot fails testing, suits in that lot will be determined to be no longer serviceable. Such unserviceable suits may only be used in training exercises, where they will be in a nonhazardous environment and thus not be exposed to toxic substances; otherwise they must be discarded. At the end of 5 years if testing continues to prove the lot serviceable, then the shelf life of that individual lot may be extended beyond 5 years. Service life refers to the period of time that chemical-protective equipment will satisfactorily perform its protective role once it has been removed from its factory-sealed protective packaging. One might call service life ''out of the bag'' protective life. Military field manuals have charts that include (1) service life, (2) wear time once contaminated with toxic agent, and (3) if the equipment or garment is capable of being laundered or decontaminated (United States Army, 2003). Instruction manuals, supply bulletins, and technical orders should always be consulted for shelf and service lives. Should the instruction manual not provide you with sufficient information on these lives, then direct written communication with the manufacturer would be appropriate. Filters for air-purifying respirators traditionally have two distinct and different shelf lives. The first is the expiration date of the filter when it is kept unopened within the manufacturer's vacuum-sealed protective storage container. The second, which the military would call service life, would occur once it has been removed from its factory-sealed storage container and attached to the respirator. Useful service life of a filter once removed from its vacuum-sealed protective storage container is highly dependent on multiple factors including, but not limited to (1) the hours of use of the filter, (2) the minute ventilation (volume of each breath multiplied by the number of breaths per minute) of the responder when employing the filter, (3) the concentration and time of exposure to the chemical agent, (4) the nature of the chemical agent itself as some are more destructive to filtering material than others, and (5) the temperature and humidity of the environment in which the filter was stored open and later used by a responder. As an example, the cyanide or blood agents (hydrogen cyanide and cyanogen chloride) have the shortest breakthrough time when tested against the standard military C2A1 nuclear/biological/chemical (NBC) filters of any class of CWAs; hence the cyanide or blood agents dictate the service life of these filters. The army has charts, published in field manuals, of expected service life for chemical warfare filters removed from their vacuum-sealed storage containers but not exposed to CWAs. The same filter would perform satisfactorily for the longest period of time in an arctic (cold with low-humidity) environment, would perform satisfactorily for a moderate period of time in a temperate environment, and would perform satisfactorily for the shortest period of time in a tropic (hot and

humid) environment (US Army, 1994). As humidity increases, the useful life of activated carbon material, as found in respirator filters, semipermeable suits, gloves, and booties, decreases. Water vapor in the form of humidity condenses and adsorbs onto the activated carbon, thus occupying binding sites that would otherwise be occupied by toxic compounds; hence the adsorptive surface of the activated carbon for binding toxic compounds is decreased. Respirator filters should never be used if they have exceeded the unopened shelf life date (except for training purposes in a nonhazardous environment), have been submerged in water or any sort of liquid, stored open in any area where chemical vapors are present, stored open near petroleum or chemical products, if the canister has been cracked or dented, or if loose particles can be heard moving when the filter is gently turned from side to side. Care should be exercised not to drop filters as this may dislodge or displace protective filtering material and allow for the ingress of unfiltered toxic atmospheric air. Consult local laws regarding the deposal of filters, as in some jurisdictions they may be considered hazardous waste.

V. GOVERNMENTAL OVERSIGHT AND REGULATIONS

A. Respirator Protection Program for Workers

An all-encompassing respiratory protection program for workers is mandated by OSHA, a division of the United States Department of Labor. The exact OSHA standard is addressed in 29 CFR 1910.134(c). This program must include any person who will be subjected to respirator usage. An excellent online review, written by OSHA, of this program may be found at the internet address http://respiration.elearning.dol.gov/. OSHA's complete respiratory protection standards (regulations) are covered in 29 CFR 1910.134, which is available on the internet address http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_id = 12716&p_table = STANDARDS.

A summary of this material is given below. It is strongly suggested that the above standard be reviewed in its entirety.

Every aspect of the program must be supported by written documentation. Every aspect of the following topics must be addressed in detail. At a very minimum, these documents should address the following subjects: (1) respirator selection, (2) medical evaluation for respirator usage, (3) fit testing of respirators, (4) proper use of respirators, (5) maintenance, inspection, and care of respirators, (6) the quality of breathing air employed, (7) training requirements, and (8) a program evaluation. This written document must be updated as changes in the workplace require.

This program requires that an employer provide an employee working in an environment with a respiratory hazard all of the following: (1) a respirator, (2) training in use and maintenance of the respirator, and (3) medical evaluations as deemed necessary at no cost to the employee.

These documents must be available to all workers and OSHA inspectors on request. The program must include a qualified program administrator and requirements for such a person are covered by these regulations (OSHA on line course 2220b, unknown year).

Record keeping is an important part of this program. The record-keeping aspects of this program are covered in 29 CFR 1910.134(m). Although these requirements are far too complex to be dealt with in this review, an example of these requirements would include that the medical evaluation questionnaire be considered a medical record, and hence maintained in accordance with 29 CFR 1910.1020(d)(1)(i) ''Employee Medical Records.'' Under this standard, this questionnaire must be kept for the duration of the worker's employement plus 30 years. This OSHA manadatory medical evaluation questionnaire is found in Appendix C to Sec. 1910.134, which is available on the internet at http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table = STANDARDS&p_id = 9783.

B. Fit Testing of Respirators

Although some respirators may be fit tested employing qualitative methods that rely on the responder's senses to detect substances such as isoamyl acetate (banana oil), saccharin (sweetener

aerosol test), bitrex (bitter aerosol test), stannic chloride, or irritant smoke (such as CS tear gas), federal regulations require that respirators used for HAZMAT and CBRN operations must be quantitatively tested. This may be done by employing either a (1) generated aerosol, (2) ambient aerosol, or (3) controlled negative-pressure technology.

A device frequently and easily employed for such quantitative measurement relies on generated aerosol methodology. This device internally generates large super micron alcohol droplets from submicron aerosol particles, some as small as 0.02 μ (TSI, 2006, p. 47). These super micron alcohol droplets are manufactured by the aerosol generator from the surrounding nontoxic ambient air. This is accomplished by the addition of highly purified isopropyl alcohol (in the neighborhood of 99% pure). Initially these manufactured large aerosol particles are distributed to the atmosphere immediately surrounding the respirator's facepiece via an open-ended tube. This tube is connected directly to the body of the aerosol generator. Later this same tube will be used to measure the atmospheric concentration of the particles that immediately surround the outside of the respirator's facepiece. A second tube is connected to the inside of the respirator, often coupled to the respirator's drinking tube, or lacking this, a modified respirator facepiece to which a sampling port has been added. This tube connects directly to the aerosol generator's aerosol-counting component. Inside the facepiece, air should be sampled between the nose and the mouth. The concentration of aerosol particles on the outside is designated C_o, and the concentration of aerosol particles on the inside is designated C_i. The ratio of C_o/C_i (C_o divided by C_i) is known as the FF. An example of such a device is the TSI Port-A-C ount® (OSHA on line course 2220d, unknown year).

OSHA regulation 29 CFR 1910.134(f) requires that any time a change has been made in the model, type, or size of the respirator, or any change in the employee status that may affect respirator faceseal has occurred, for example, facial trauma, a fit testing must be done. Interestingly, this test may be done with the employee's own respirator, or an identical surrogate respirator (i.e., exactly the same manufacturer, model, and size). Additionally, fit testing must be conducted and documented annually.

OSHA regulation 29 CFR 1910.134(f)(7) requires that the FF of a tight-fitting full-facepiece respirator be $\geq$500. FFs far in excess of this are common with modern respirators, many of them are capable of generating FF of 10,000 or more. The FF may change as the responder performs different functions or activities. Certain activities are more prone than others to break the faceseal of the respirator and allow the ingress of contaminated atmospheric air. Therefore, a protocol for testing FF should include multiple different activities with which the responder will become involved. An example of the OSHA fit-testing protocol (29 CFR 1910.134(f)(5) and 29 CFR 1910.134 Appendix A) would include measurements obtained during all of the following activities: (1) normal breathing, (2) deep breathing, (3) moving the head from side to side, (4) moving the head up and down, (5) talking, (6) grimacing, (7) bending down and touching toes, and finally (8) normal breathing. Since FFs can vary over time and the nature of activity, an average calculation for each of the above-mentioned activities is obtained. With each activity, an atmospheric sample is first obtained, immediately followed by a sample obtained from within the respirator, and then another atmospheric sample. An average value is then automatically calculated by the sampling device for each of the previously mentioned activities. Responders should pay careful attention to the results of each of these activities and remember which ones are more likely to create a break in the faceseal of the respirator, and consequently exercise caution when performing those activities. The average measured FF for each of the tested activities for every responder are easily stored in a computer using the manufacturer's readily available (downloadable from the manufacturer's Internet address) computer software.

C. Federal Chemical, Biological, Radiological, or Nuclear Standards for Personal Protective Equipment

New Federal CBRN standards have taken much of the guess work out of the CBRN equipment selection process, thus making the responder's life much simpler. If the piece of PPE equipment is NIOSH CBRN approved it has been tested against the classical CWAs, the biological agents, and

the radiological agents. But in addition to CWAs it has been tested against multiple TICs, each tested industrial chemical represented a group of TICs, and the tested material has satisfactorily protected the responder against those agents. Inclusion of chemicals into a particular group is by possessing certain chemical characteristics. All chemicals within a group have similarities that make them behave in a similar manner, although some TICs within each group permeate protective material faster and easier than other chemicals within that same group. The most difficult chemical to protect against (easiest chemical to permeate protective equipment) in each group was selected as the representative of the group. The idea being that if you can protect against the worst offender in each group of chemicals, you can protect against all the other chemicals within the group. This provides reliable permeation data while holding down the costs of permeation testing. Included in these groups are approximately 140 TICs in approximately 11 groups. In one classification format, a TIC is defined as a compound which has an LD_{50} of less than 100,000 mg min/m^3 in any mammalian species, and is produced in quantities exceeding 30 tons per year at one production facility (NIOJ, 2002). These are subsequently broken down into high-, medium-, and low-risk TICs. Another classification format produces a risk level for each TIC (high, medium, or low) by integrating its IDLH (concentration in ppm), vapor pressure, the number of continents in which it is produced, and the number of producers (Sun and Ong, 2005). Again be cautioned that these tests included the TIC and the protective material only, they did not include any additional solvents.

The new CBRN standards established by the federal government currently require that only repirators which are CBRN certified by NIOSH and display the NIOSH CBRN certification may be used in CBRN rescue operations. At the time of this writing NIOSH CBRN regulatory certification for other PPE equipment (suits, gloves, boots, etc.) does not exit. For any agency, public or private, to use respirators not certified as NIOSH CBRN approved in such rescue operations would be opening up an entirely new area of liability and litigation should physiological, psychological, or medical harm befall the rescuer immediately following the event or in the future. Manufactures of PPE equipment must submit multiple pieces of identical equipment to NIOSH for testing, along with a substantial testing fee, when seeking NIOSH CBRN respirator approval. This approval testing is supervised and conducted by The National Personal Protective Technology Laboratory located in Pittsburgh, Pennsylvania. This organization maintains its own website at the following internet address: http://www.cdc.gov/niosh/npptl/ default.html. This site includes, but is not limited to, access to 42 CFR Part 84 (Federal Respiratory Regulations), a listing of CBRN-approved respirators, standard testing procedures, the CBRN standards for equipment certification, an interactive training site, a respirator selection logic site, and respiratory user notices (including changing standards, revocation of approval, etc.), and guidance documents. This site represents one-stop shopping and an absolute must internet address for anyone seriously interested in CBRN PPE standards and certified equipment.

D. Code of Federal Regulation and Other Governmental Regulations

In addition to federal regulations, additional state, county, and local regulations may also apply. In no way should the above discussion on governmental regulation be considered inclusive and the reader is referred to other sources, particularly those found in the Code of Federal Regulations available on the Internet. These include 29 CFR 1910.120 ''Hazardous Waste Operations and Emergency Response'' (HAZWOPER). Respiratory Standards are found in 29 CFR 1910.134, and 42 CFR 84 is the location of the new NIOSH CBRN standards, and a listing of NIOSH-approved CBRN equipment.

PHOTOGRAPHIC ACKNOWLEDGMENTS

Special acknowledgment and thanks are extended to all the individuals who either provided me with the photographs, or helped me produce the photographs used in this chapter. The most important acknowledgment is to my wife Kathleen (Kathy), who for many months on end thought I had married our computer instead of her, and who temporarily became a ''chapters of the book'' widow.

PHOTOS

PHOTO 20.1 Level A. The suit's impermeable material is formed into flexible booties at foot level, which are continuous with the main body of the garment. This maintains the vapor-proof integrity of the suit. The responder then places the booties into the chemical-protective hard boots shown in the photographs. A band of the suit's impermeable material mounted exterior to the suit then overhangs and surrounds the hard boots worn by the responder.

PHOTO 20.2 Level A: Side and rear view, notice that the entire self-contained breathing apparatus (SCBA) is contained within the vapor- and liquid-proof protective suit. The suit material, zippers, and seams are made of vapor-impermeable material and construction. Level A is the only level to maintain vapor-proof integrity. (Photos 20.1 and 20.2 are courtesy of the Center for Domestic Preparedness, Department of Homeland Security, Anniston, Alabama. These images are the work of an employee of the Center for Domestic Preparedness, taken or made during the course of official duties. As a work of the U.S. Federal Government, the images are in the public domain.)

(a) (b) (c)

PHOTO 20.3 (a and b) Level B: Notice the taping of the gloves and boots to the outside of the suit. In addition note how some of the impermeable suit material overhangs the boots and is taped to the boots using duct tape (tape with known chemical agent permeation data would be preferable) with a ''tear off'' tab. Several layers of gloves are usually employed. (Author's personal photos with him serving as the responder, taken at Lakehurst, N.J. Naval Air Station by Sergeant Daniel Mitten of New Jersey Task Force 1 and the New Jersey State Police.) **(c)** Level B: Another picture illustrating the support mechanism of the SCBA allowing it to ride on the hips. Notice how the tank is mounted with a tank shut-off valve and the first stage of the regulator (high-pressure reducing stage) is mounted under the tank. (Author's personal photos with him serving as the responder, taken at Lakehurst, N.J. Naval Air Station by Sergeant Daniel Mitten of New Jersey Task Force 1 of the New Jersey State Police.)

(a) (b)

PHOTO 20.4 (a and b) Level C: One-piece impermeable material suit with tight-fitting powered air-purifying respirator, that is, PAPR. (Author's personal photos with him serving as the responder, taken at Lakehurst, N.J. Naval Air Station. Thanks to Len Dotson, photographer and member of New Jersey Task Force 1 who spent a good deal of time serving as photographer and photographic advisor, and Sergeant Daniel Mitten of New Jersey Task Force 1 of the New Jersey State Police.)

PHOTO 20.5 Level C: Close-up of facepiece of a tight-fitting powered air-purifying respirator. Federal regulations require that flow be 4 cubic feet/min or greater. Transparent facepiece of respirator is not well visualized in this photograph. (Author's personal photos with him serving as the responder, taken at Lakehurst, N.J. Naval Air Station. Thanks Len Dotson, photographer and member of New Jersey Task Force 1 who spent a good deal of time serving as photographer and photographic advisor, and Sergeant Daniel Mitten of New Jersey Task Force 1 of the New Jersey State Police.)

PHOTO 20.6 (a and b) Level C: Powered air-purifying respirator of the loose-fitting hood-type variety with a one-piece impermeable material suit. This model is very popular with hospital decontamination teams, as beards, eyeglasses, and facial deformities are well tolerated. Some models contain a stretchable neck dam seal; others do not, relying instead on duplication of the hood placed both interiorly and exteriorly to the protective suit. (Author's personal photos with him serving as the responder, taken at the Washington Township Division of the Kennedy Health System of New Jersey by Kimberly Cavallaro of the Emergency Management Department.)

(a)

(b)

(c)

PHOTO 20.7 Level C: (a) This loose-fitting hood-type powered air-purifying respirator creates the least amount of claustrophobia, as well as the least amount of spatial disorientation of all respirator types. By federal regulation loose-fitting hood-type respirators must generate a flow of 6 cubic feet/min or greater. (b and c) Two types of blower and filter units for PAPRs. (Author's personal photos with him serving as the responder, taken at the Washington Township Division of the Kennedy Health System of New Jersey by Kimberly Cavallaro of the Emergency Management Department.)

PHOTO 20.8 Level C: Negative-pressure air-purifying respirator (M40A1 military respirator) with one-piece impermeable suit. Observe taping of zipper and facepiece. (Author's personal photo with him serving as the responder, taken at the Washington Township Division of the Kennedy Health System of New Jersey by Kimberly Cavallaro of the Emergency Management Department.)

(a) (b)

PHOTO 20.9 (a and b) Level C: Negative-pressure air-purifying respirator (M40A1 military respirator) with one-piece impermeable suit. Respirator equipped with external clip-on smoked colored eye lens outserts. (Author's personal photos with him serving as the responder, taken at the Washington Township Division of the Kennedy Health System of New Jersey by Kimberly Cavallaro of the Emergency Management Department.)

PHOTO 20.10 Level C: Negative-Pressure Air-Purifying Respirator (FRM40) with two-piece semipermeable activated carbon-lined suit. Notice the built-in hood and Velcro closures at the wrist, waist, legs, and crotch (permanently attached to the rear of jacket, pulls under the crotch of the suit with an uplifting effect on trousers, and attached by Velcro to the front of jacket). An elasticized stretchable band surrounds the face area of the hood making very close contact with the respirator. There is an adjustable string closure tie built into the lowest part of the jacket, as well as Velcro lining both along the inside bottom of the jacket and the outside top of the trousers. The yellowish color of the suits is due to the picture being taken through protective glass at the toxic live agent training chamber in Anniston, Alabama. All of these measures are taken to decrease the amount of contaminated air that could possibly enter the suit through gaps occurring at locations where different components of protective equipment meet (gloves to suit, etc.). This effect occurs during movement and is known as the bellow's effect.

PHOTO 20.11 This photograph is another picture of the exact same suit type as Photo 20.10 (without the color distortion from protective glass) taken in an uncontaminated training environment. The responders in photo 20.10 are actually in an environment contaminated with sarin (GB) and VX at levels below IDLH values in the live agent chamber at the Center for Domestic Preparedness, Department of Homeland Security in Anniston, Alabama. This is a truly unique (only facility for civilians in United States) and extremely worthwhile training event. (Photos 20.10 and 20.11 are courtesy of the Center for Domestic Preparedness, Department of Homeland Security, Anniston, Alabama. This image is the work of an employee of the Center for Domestic Preparedness, taken or made during the course of official duties. As a work of the U.S. Federal Government, the images are in the public domain.)

PHOTO 20.12 Level C: Two-piece semipermeable activated carbon suit with M40A1 (military) respirator. A separate hood made of a butyl rubber-impregnated garment covering the head, shoulders, and chest is being employed. An elastic band holds the facial opening of the hood in close contact with the respirator; a second elastic band at the neck level decreases the possibility of ingress of contaminated air. Straps under both arms secure the hood's position in place extending from back to front of the responder (attached by clips, older models with hook-and-pile). This picture shows both the front and rear of the hood. (From the website of the United States Military Academy at West Point, N.Y., Corps of Cadets, Class of 2008, Cadet Basic Training 2004, photo 87. (www.usma.edu) U.S. Army Photo. This image is the work of an employee of the United States Military Academy, taken or made during the course of official duties. As a work of the U.S. Federal Government, the image is in the public domain.)

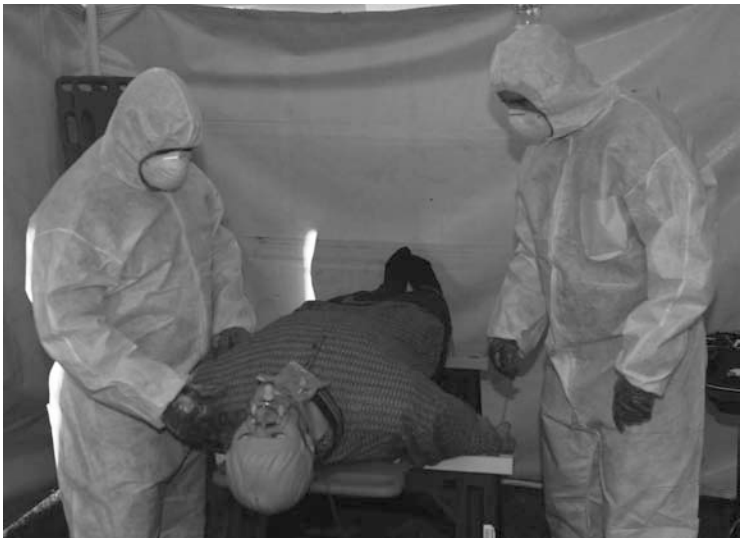


PHOTO 20.13 Level D: Work clothes and street clothes. This level offers no chemical and minimal biological protection. Other examples would include surgical scrubs and mask, policeman and firefighter's ensembles. (Courtesy of the Center for Domestic Preparedness, Department of Homeland Security, Anniston, Alabama. This image is the work of an employee of the Center for Domestic Preparedness, taken or made during the course of official duties. As a work of the U.S. Federal Government, the images are in the public domain.)

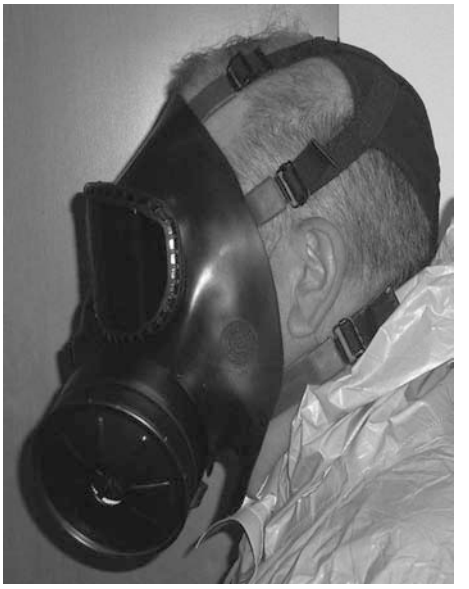
PHOTO 20.14 What's wrong with this picture? Note that the zipper is not fully closed; however, even if it were, skin would still be exposed. A perfect example of the usefulness of a respirator hood. A buddy could place duct tape around the face for sealing the suit to the respirator. The newer type second skin of the M40A1 could also help eliminate this problem.

This picture shows a defect in the chin area that is common with all Level B non-encapsulating suits as well as all Level C suits. For suits with an incorporated hood this defect is remedied by adding a chin closure flap directly below the face at the time of the suits construction. This flap's closure is secured with hook and pile (velco®). Additionally the hood is taped to the respirator with chemical permeation tape. On suits lacking an incorporated hood, a separate hood (with or without a respirator facepiece "second skin" as is appropriate) is employed. This hood is taped to the respirator with chemical permeation tape in a manner identical to that employed with an incorporated hood. See photographs 20.12 (hood), and photographs 20.15 (c and d) (second skin). (Author's personal photo with him serving as the responder, taken at Lakehurst, N. J. Naval Air Station by Sergeant Daniel Mitten of New Jersey Task Force 1 of the New Jersey State Police.)

(a)

(b)

(c)



(d)

PHOTO 20.15 (a, b, c, and d) In these four photographs, the upper two (a and b) show facial coverage by the M40A1 military field protective mask, which is identical to the coverage offered by the FR-M40 (the civilian First Responder M40 respirator). The lower two photos (c and d) show the addition of the newer version of a vesicant-resistant ''second skin,'' and clearly illustrates the additional facial-protective coverage given to the forehead, both sides of face, and the elongated coverage of the chin and neck area. Comparison of the second skin coverage area should be made to photo 20.14 showing this frequently unprotected skin covering the chin and neck. (Author's personal photos with him serving as the responder, taken by his daughter Barbara Ross.)

(a)

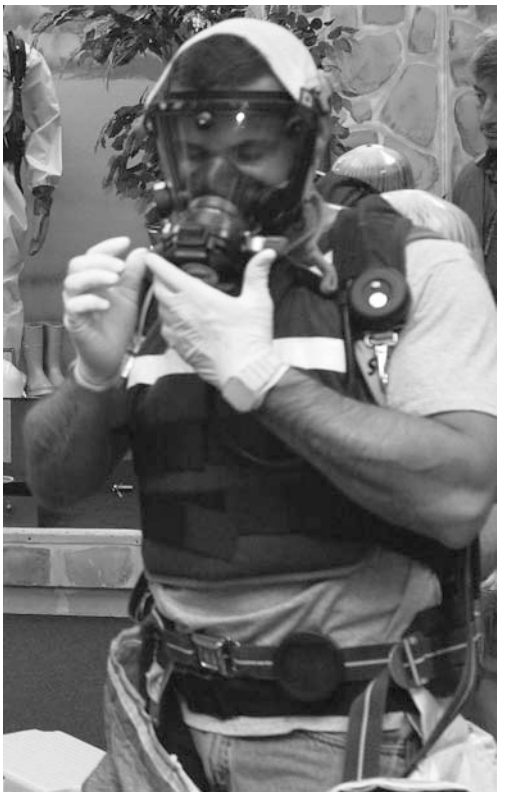
(b)

PHOTO 20.16 (a, b, and c) TAP Apron: TAP = Toxicological Apron Protective. This is a thick, hot, somewhat heavy, one-piece, butyl rubber apron worn over a two-piece semipermeable activated carbon suit during decontamination procedures involving water and other liquids (often involving activated chlorine compounds). These pictures show the front, side, and rear of this apron (with opening for donning and heat dissipation). Photograph 20.16(c) shows a close-up of the apron's sleeves covering the responder's butyl rubber gloves. Note the apron's attached closure straps. (Author's personal photos taken when attending the *Field Management of Chemical and Biological Casualty Course* given by the Chemical Casualty Care Division of the United States Army Medical Research Institute of Chemical Defense at Aberdeen Proving Ground, Maryland.)

(*continued*)

(c)

PHOTO 20.16 (a, b, and c) (Continued)



(a)

(b)

PHOTO 20.17 (a, b, and c) These three photographs illustrate the front, side, and rear of a cooling vest. In the far left photograph, also note the air gauge on the responder's left arm just to the right (facing responder) of the cooling vest. (Courtesy of the Center for Domestic Preparedness, Department of Homeland Security, Anniston, Alabama. This image is the work of an employee of the Center for Domestic Preparedness, taken or made during the course of official duties. As a work of the U.S. Federal Government, the images are in the public domain.)

(*continued*)

(c)

PHOTO 20.17 (a, b, and c) (Continued)

(a)

(b)

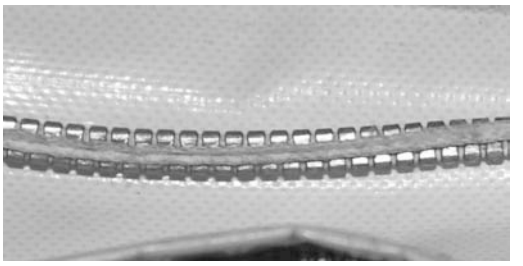
PHOTO 20.18 (a and b) Chemical protective outer gloves. Multiple layers of gloves (two or three layers) are worn. Butyl rubber and halogenated butyl rubber gloves have excellent permeation resistance to the classical CWAs, but extremely poor permeation resistance to petroleum and petroleum products (gasoline, etc.). ((a) Courtesy of the Center for Domestic Preparedness, Department of Homeland Security, Anniston, Alabama. This image is the work of an employee of the Center for Domestic Preparedness, taken or made during the course of official duties. As a work of the U.S. Federal Government, the images are in the public domain. (b) Author's personal photos taken at Lakehurst, N.J. Naval Air Station. Thanks to Len Dotson, photographer and member of New Jersey Task Force 1 who spent a good deal of time serving as photographer and photographic advisor, and Sergeant Daniel Mitten of New Jersey Task Force 1 of the New Jersey State Police.)

(a)

(b)

PHOTO 20.19 (a and b) Boots are made of material that is highly resistant to permeation by chemical warfare agents, and in sufficient thickness as to decrease the chance of accidental perforation. Note the hook and button fasteners on the boots in the left picture, and the ridges in the soles of the boots which create good traction in the right picture. The size of the boot is molded into the sole of the boots in the picture on the right, although this is not easily seen in the picture. ((a) Courtesy of the Center for Domestic Preparedness, Department of Homeland Security, Anniston, Alabama. This image is the work of an employee of the Center for Domestic Preparedness, taken or made during the course of official duties. As a work of the U.S. Federal Government, the images are in the public domain. (b) Author's personal photos taken at Lakehurst, N.J. Naval Air Station. Thanks to Len Dotson, photographer and member of New Jersey Task Force 1 who spent a good deal of time serving as photographer and photographic advisor, and Sergeant Daniel Mitten of New Jersey Task Force 1 of the New Jersey State Police.)

(a)



(b)

(c)

(d)

PHOTO 20.20 (a, b, c, and d) The vapor-proof zipper of the Level A suit shown in two views, from a distance and up close. The flap that overlays this zipper is secured by Velcro (c), and then ideally tapped with chemically resistance tape, or in this case, duck tape (d). Since the suit is vapor proof, air exhaled from the SCBA would remain within the suit and expand the suit; at some point, the increased pressure would rupture the suit. This is prevented by one-way valves that allow gas (expired air) to exit the suit but prevents any outside atmosphere from entering the suit. Pictures of one valve from both outside and inside the suit (Photos 20.21 and 20.22) are provided. (Author's personal photos taken at Lakehurst, N.J. Naval Air Station. Thanks to Len Dotson, photographer and member of New Jersey Task Force 1 who spent a good deal of time serving as photographer and photographic advisor, and Sergeant Daniel Mitten of New Jersey Task Force 1 of the New Jersey State Police.)

PHOTO 20.21 Outside of Level A vapor-proof suit: Both the outside protective ''tent'' made of impermeable suit material and the outside rigid housing of the one-way air valve (air flow direction from inside to outside of suit) are seen in this photograph. A flapper disc made of chemically impermeable material (often halogenated butyl rubber or EDPM rubber) is within this housing. (Author's personal photos taken at Lakehurst, N.J. Naval Air Station. Thanks to Len Dotson, photographer and member of New Jersey Task Force 1 who spent a good deal of time serving as photographer and photographic advisor, and Sergeant Daniel Mitten of New Jersey Task Force 1 of the New Jersey State Police.)

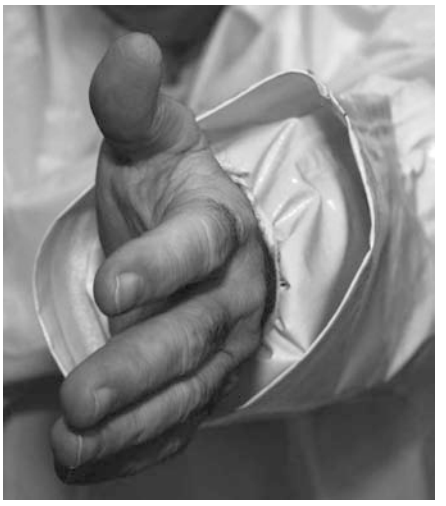
PHOTO 20.22 Inside of Level A vapor-proof suit: One can see the one-way air escape valve flapper disc that opens to the outside atmosphere when pressure within the suit exceeds atmospheric pressure. As soon as the pressure is equalized the disc valve closes. Sticking of this air escape valves mechanism would cause air expired from the SCBA to be retained within the suit and increase pressure within the suit, buildup of pressure could rupture the suit. One should always be sure these valves are functioning properly before donning the suit. (Author's personal photos taken at Lakehurst, N.J. Naval Air Station. Thanks to Len Dotson, photographer and member of New Jersey Task Force 1 who spent a good deal of time serving as photographer and photographic advisor, and Sergeant Daniel Mitten of New Jersey Task Force 1 of the New Jersey State Police.)

(a)

(b)

(c)

PHOTO 20.23 (a, b, and c) Escape-Only Masks: These negative-pressure air-purifying respirators are designed for escape only. All have a stretchable tight-sealing neck dam constructed of chemically resistant material to maintain a vapor seal from the surrounding atmosphere. They are stored very compactly in small, lightweight, vapor-tight storage containers, usually either a chemical-protected bag or a can with a self-tearing tab to remove the lid. The object is to have these respirators immediately available to the user through convenient packaging. Allowable escape time varies from manufacturer to manufacturer. One would be wise to consult the NIOSH Web site to see any additional data that may be available on these respirators. Photographs courtesy of: (a): ''NBC Escape Hood,'' www.firstlinetech.com, point of contact: Amit Kapoor, President; (b): ''MSA Response Hood,'' www.msanet.com, point of contact: Steve Grasha, Mine Safety Appliance (MSA), Manager, North America Creativity Center; (c): ''North Safety Products ER2000,'' www.northsafety.com, point of contact: David Barricelli, Jr., North Safety Products Director, Marketing Communications. Recently an ''escape-only'' hooded respirator that employs a battery-powered blower activated by removal of the unit from its storage container has been introduced by ILC Dover. This powered air-purifying hood is considered a positive-pressure escape-only respirator by OSHA and NIOSH. Like its negative-pressure counterparts it employs a butyl rubber dam to form the neck seal, unlike its negative-pressure counterparts, it does not employ a nosecup, but relies on the volume of filtered air from the blower to dilute any of the escapee's warm moist expired air from reaching the inside of the facepiece and condensing on it. Visit http://www.ilcdover.com, select ''personal protection'' on ILC Dover's home page and visit ''SCapeCBRN[30].'' A video of the donning procedure is also available for your viewing.



(a)

(b)

(c)

PHOTO 20.24 (a) Elastic on an inner band of suit material clings to the arm, while an external overlap of suit material will cover the outer layer of gloves. (b and c) The suit's booty fits inside the boot while the suit's overlap material will cover the outside of the boot and may be taped to the boot. (Author's personal photos with him serving as the responder, taken at Lakehurst, N.J. Naval Air Station. Thanks to Len Dotson, photographer and member of New Jersey Task Force 1 who spent a good deal of time serving as photographer and photographic advisor, and Sergeant Daniel Mitten of New Jersey Task Force 1 of the New Jersey State Police.)

(a)

(b)

(c)

PHOTO 20.25 (a, b, and c) Note how these Level A gloves screw and lock onto the body of the Level A suit. A chemical-resistant gasket material is on the inside of the glove and the outside of the suit at the point of jointing to create a vapor-tight suit. (Author's personal photos with him serving as the responder, taken at Lakehurst, N.J. Naval Air Station. Thanks to Len Dotson, photographer and member of New Jersey Task Force 1 who spent a good deal of time serving as photographer and photographic advisor, and Sergeant Daniel Mitten of New Jersey Task Force 1 of the New Jersey State Police.)

PHOTO 20.26 Exterior of Level A suit. The exterior ''tents'' covering the one-way exhaust valves are visualized just above the waist on the responder to the left, and on the top rear of the hood of the responder on the right in this photograph. (Courtesy of the Center for Domestic Preparedness, Department of Homeland Security, Anniston, Alabama. This image is the work of an employee of the center for Domestic Preparedness, taken or made during the course of official duties. As a work of the U.S. Federal Government the image is in the public domain.)

PHOTO 20.27 Duct tape around the suit's leg, notice the ''dog's ear'' of tape on tape (L) to make removal easier. (Author's personal photos with him serving as the responder, taken at Lakehurst, N.J. Naval Air Station. Thanks to Len Dotson, photographer and member of New Jersey Task Force 1 who spent a good deal of time serving as photographer and photographic advisor, and Sergeant Daniel Mitten of New Jersey Task Force 1 of the New Jersey State Police.)

(a) (b)

PHOTO 20.28 (a) Kappler Chem-Tape has been permeation tested against chemical warfare agents with published results and (b) Duck tape that has not undergone permeation testing against CWA, but has been widely used. (Photos 20.28 (a) and (b) are author's personal photos taken at Lakehurst, N.J. Naval Air Station. Thanks to Len Dotson, photographer and member of New Jersey Task Force 1 who spent a good deal of time serving as photographer and photographic advisor, and Sergeant Daniel Mitten of New Jersey Task Force 1 of the New Jersey State Police.)

PHOTO 20.29 Inside view of M40A1 military negative-pressure air-purifying respirator (gas mask). *Note*: The inside surface of the respirator would be in direct contact with the responder's face, and thus establish the vapor-proof water seal (derived from the responder's perspiration) between the respirator and the responder's face. When the respirator is worn by the responder, the free edge of the ''reversed seal'' would fold forward into the body of the facepiece of the respirator as the head straps are tightened by the responder. (Author's personal photograph taken by him.)

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21 Chemical Warfare Agent Decontamination from Skin

Brian J. Lukey, Harry F. Slife Jr., Edward D. Clarkson, Charles G. Hurst, and Ernest H. Braue Jr.

CONTENTS

I. CHEMICAL AGENT ISSUES: BATTLEFIELD/TERRORISM

This chapter focuses upon decontaminating skin exposed to chemical warfare agent (CWA). Although the protective equipment chapter discusses effective barriers to protect an individual, there is a high probability that the items may not be worn at the time of an attack. Consequently, the next barrier, the skin, becomes critical, but has a limited time before it allows an agent to penetrate. Rapid decontamination is of utmost importance, before the skin barrier is compromised. To best understand the process, this chapter will discuss military and civilian issues concerning skin exposure, the characteristics of the skin itself, medical protective measures, and evaluation of skin decontaminants.

In battle, the warfighter will be exposed to a variety of environment factors that may cover up a potential chemical warfare incident. These include smoke, rain, gunpowder, diesel exhaust, oil leaks, and fog. Because the first step in the skin decontamination process is to recognize that a CWA exposure occurred, the warfighter must quickly differentiate these environmental variables from chemical agents. The detection chapter in this book emphasizes the need to rapidly detect the chemical agent. This urgency is underscored here because the quicker a CWA is decontaminated from the skin, the lesser it will penetrate, and lesser the damage that will be caused by the agent. However, detectors may take several minutes to identify an agent and may not be monitoring during a particular attack. Consequently, the warfighter should also rely on other indicators to determine the need to decontaminate.

Skin decontamination deals primarily with liquid exposure. However, agent vapor from off-gassing of clothing or materials can be a concern, particularly with sulfur mustard. Warfighters first

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need to realize liquid is on their skin. That recognition may not be obvious at times of high stress and activity. One's sweat could mask a foreign liquid on the skin. Even if a foreign substance is recognized on the skin, it must be identified as a threat great enough to warrant stopping their current operations to decontaminate. With so many stressors on the battlefield, one could easily downplay the liquid as some harmless fluid that perhaps leaked from a military vehicle. Hostile fire may appear a much greater threat at that moment. However, CWAs are just as lethal and should be immediately addressed. Forensic epidemiology and analysis will not be immediately available. Being conscious of the threat and observant of one's surroundings is the most valuable detection system available.

If the situation allows, and the threat of agent use by an adversary is probable, all foreign materials should be treated as harmful agents and decontaminated. Unfortunately, there will be a first-use scenario where victims will be caught unaware and potentially suffer high casualty rates, thereby raising the level of awareness and caution in subsequent encounters. Once the warfighter believes a liquid is a CWA, he or she should remove the agent as soon as possible. The critical element is time. The longer the agent remains on the skin, the greater the penetration and consequently, the greater the threat to the individual. Fortunately, warfighters are taught to recognize CWAs and to individually decontaminate their skin with issued decontamination kits. Additionally, they are taught to decontaminate each other.

With regard to National defense, a terrorist attack with a CWA could create chaos. Most civilians are not taught to recognize such an attack and do not carry a military issued decontamination kit. Nevertheless, the same concepts of toxicity apply; the longer the agent remains on the skin, the greater the potential toxicity to that person. Quick recognition of the situation and the possible agent employed, or at least family of agents, can result in saving most victims. Training of our first responder personnel and emergency room staff is essential to a good outcome if a chemical agent is ever employed.

Again, the first rule of decontamination is removal. The lack of access to sophisticated decontamination solutions or materials should not prevent quick intervention using any materials that are available to first remove gross contamination by scraping off and using absorbing materials. The exposed area should then be flushed with copious amounts of liquid, using anything available (water, soda, etc.)

The characteristics of the skin itself are important to best understand the skin as a barrier and to best develop barrier creams that enhance protection against CWAs and decontamination methods to best remove the agent from the skin.

II. SKIN CHARACTERISTICS

Skin might at first glance appear a simple structure, but this would be a naive assumption. Nearly 2 m^2 of skin covers the human body and is a complex three-layered organ responsible for numerous critical functions. The primary function of the skin is to prevent exogenous materials such as chemicals, microbes, ultraviolet radiation, and foreign matter from getting into the body while retaining body fluids and containing the vital organs of the body. Additionally, the skin serves roles in the immune system, heat regulation, physical protection, and psychosocial make up.

A. Structure

Three major layers or distinct divisions comprise human skin—epidermis, dermis, and subcutaneous fat. Each layer can be further subdivided.

Epidermis. The outer most layer of the skin is the epidermis. It is made up of an outer layer of dead cells providing protection to the underlying layers. The cells of the epidermis are called keratinocytes. As keratinocytes go through maturation they lose their nucleus and become ''keratinized'' to form the dead protective layer, the stratum corneum. The barrier function is provided by

this upper most layer of dead, cornified (keratinized) cells. The cornified or horny layer ranges from a few cells up to the very thick areas on the soles and palms. The cells are organized in a stacked geometric fashion surrounded by lipids produced in the epidermis. These lipids are a unique mixture of oil-like compounds enriched in cholesterol that are adapted to protect the host against water loss. Additionally, varying degrees of skin oil, called sebum, from sebaceous glands are on the surface. Both sources of oil play important protective roles. The bottom layer of the epidermis, the basal cell layer, is normally one to two cells deep. This is the self-replicating layer which replaces the epidermis approximately every 30 days and averages about 100 μm in thickness. The epidermis as a whole and specifically the stratum corneum serve not only to protect from excessive water loss, but also against ingress of water, microbes, and toxic substances.

The complex biochemistry and anatomical structure of the epidermis is devoted, in large part, to producing this dead protective stratum corneum. Abnormal keratin production leads to diseases like Psoriasis and Ichthyosis and can be accompanied by profound defects in the skin's barrier function capability. The skin's protective function is also dependent on the integrity of the epidermis and its attachment to the dermis, which is maintained by elaborate intercellular adhesion structures, desmosomes, and hemidesmosomes. Diseases which alter the cellular connectivity both cell to cell and epidermis to dermis result in diseases such as Pemphigus Vulgaris, Bullous Pemphigoid, and the various forms of Epidermolysis Bulosa and is thought to be the mechanism of action of vesicating chemical agents that cause fluid filled blisters on the skin resembling the symptoms of these diseases.

Dermal–Epidermal Junction (DEJ). The DEJ is a specialized attachment area forming an extensive interface between the epidermis and the dermis. The major function of the DEJ is to keep the epidermis and dermis firmly attached. Sulfur mustard, Bullous Pemphigoid, and forms of Epidermolysis Bulosa form vesicles (blisters) at this junction.

Dermis. The dermis consists primarily of connective tissues: collagen, elastin, and ground substance, which provides protection against trauma by adding strength and flexibility to the skin. Within the dermis are blood vessels, lymphatics, nerves, and the epithelial organs: hair follicles, sebaceous glands, and sweat glands (apocrine and eccrine).

Subcutaneous Fat. The subcutaneous (SQ) fat serves to maintain heat, store energy, and act as a shock absorber protecting structures within the body. The basic SQ unit is the microlobule which is composed of fat cells, adipocytes.

B. Permeability, Breakdown of Protection: Hydration/Dehydration, Abrasion, Penetration (Absorption) Rates, Toxicity, Inflammation/Disease

The rate of absorption of materials through the skin is limited by the stratum corneum layer. This is due to tight cell–cell adhesion and the presence of intracellular lipids that are hydrophobic and repel most aqueous substances. Unfortunately, toxic substances or disease can rapidly breakdown the skin's protective barrier. Because the skin is hydrophobic, lipid-based toxic compounds and mixtures may rapidly penetrate epithelial tissue. Thin skin, containing little or no stratum corneum, also allows for rapid penetration of toxins. No stratum corneum, such as mucosa (eyes, mouth, and sinuses) permits very rapid penetration approaching that of an intramuscular injection.

Due to protein–water binding, human skin has the capacity to absorb five to six times its weight in water. The extent of skin hydration is directly related to the penetration rate of toxic materials. Controversy exists about whether hair follicles contribute to increased penetration, but some studies do show increased absorption through hairy skin. Inflammation in the skin leads to vasodilatation and increased blood flow and subsequently increased penetration of toxic material. Aging contributes to decreased lipid barrier protection and decreased intercellular cohesion and increased penetration of toxic material.

III. BARRIER PROTECTION—BARRIER CREAMS

Topically applied protectants, as a means of delaying absorption of toxic substances through the skin, have been of interest to the military since World War I (WWI). The purpose of these materials, as with protective suits, was minimizing exposure and prolonging the effective window for decontamination. Applying a topical protectant to vulnerable skin surfaces prior to entry into a chemical combat arena was proposed as a protective measure against percutaneous CWA toxicity soon after the use of sulfur mustard by Germany at Ypres, Belgium in 1917 (Papirmeister et al., 1991). In the summer of 1917, the U.S. Army began examining various soaps and ointments for their protective qualities. Although several simple formulations were found to be effective in reducing skin irritation produced by agents such as hydrogen sulfide, no product was made available to the warfighter before the end of the war (Papirmeister et al., 1991). Research in the area of protective ointments continued after the war, but this effort did not produce a fielded product before the beginning of World War II (WWII).

During WWII, a concentrated effort to develop ointments for protection against sulfur mustard took place at the Chemical Warfare Service, Edgewood Arsenal, Maryland. The Army produced the M-5 protective ointment, which was manufactured in 1943 and 1944. However, because of limited effectiveness, odor, and other cosmetic characteristics, the M-5 ointment was no longer issued to soldiers by the mid-1950s.

Between 1950 and the early 1980s, the focus on research shifted to medical countermeasures and away from protective creams. In the early 1980s, a limited research effort returned to development of a protective ointment and produced two nonactive barrier skin cream formulations based on a blend of perfluorinated polymers. The two formulations, developed at the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD), Aberdeen Proving Ground, Maryland, were transferred to advanced development in October 1990 (McCreary, 1997). After extensive testing, the most efficacious formulation was selected and progressed through development with an Investigational New Drug (IND) filed with the Food and Drug Administration (FDA) in 1994 and approval of a New Drug Application (NDA) in 2000.

This new product was called Skin Exposure Reduction Paste Against Chemical Warfare Agents (SERPACWA). SERPACWA consisted of fine particles of polytetrafluoroethylene (PTFE) solid dispersed in a fluorinated polyether. The excellent barrier properties of this polymer blend were related to the low solubility of most materials in it. SERPACWA is now a standard issue item to U.S. forces when there is a threat of CWA use.

SERPACWA is an antipenetrant barrier cream for use by service members to protect against the toxic effects of CWAs, for example, blister (vesicant) and nerve agents and percutaneously active biological agents. It does not neutralize CWAs. When used in conjunction with mission-oriented protective posture (MOPP) gear, SERPACWA will prevent, or significantly reduce, the absorption of liquid agent through the skin. It is less effective against vapor hazards. It is used as an adjunct to MOPP, not as a substitute. Interestingly, the effective barrier of SERPACWA has also been found to protect against poison ivy and poison oak.

The SERPACWA is used at the direction of the Commander. Each person is issued six packets of SERPACWA. The entire contents of the packet are to be used in one application. When in a high-threat area, with the additional heat stress of wearing MOPP, SERPACWA should be applied every 8 h. This translates to the soldier's basic load being sufficient for 2 days. SERPACWA is not water soluble, and so it cannot be washed off by water or removed by sweat without brushing and scrubbing. However, it will physically wear off with time. Abrasion of SERPACWA by clothing or other contacts, such as sand or dirt, will reduce the wear time. The SERPACWA needs to be reapplied when the coating is generally embedded with particulate matter (dirt or sand), or the sites are decontaminated. Minimally, the 8 h rule applies. Insect repellents on the skin, such as DEET, decrease the effectiveness of the SERPACWA. If DEET is wiped off using a dry towel, gauze, or piece of cloth before application, then SERPACWA can still provide significant protection.

The effectiveness of SERPACWA is dependent on the thickness and integrity of the SERPACWA layer and the length of time between application and agent exposure (wear time). Under normal conditions, SERPACWA is effective when spread over the skin as a thin layer (0.1 mm thick or 0.01 mL/cm^2). One packet of SERPACWA contains 1.35 fluid ounces (about 2.7 weight ounces or 84 g). This amount of SERPACWA produces a smooth coating on the skin which has a barely visible cream color and is slightly detectable by touch. The first priority for skin application should be given to covering those areas adjacent to the closures of the individual protective ensemble (neck, wrists, and lower legs around the top of the boots). If the situation permits, SERPACWA should also be applied to the creases and crack of the buttocks and around the waist. It could be applied to the armpits and groin area but this is less important because SERPACWA does not provide significant protection against agent vapor. It is not applied to open wounds. It should never be applied to the entire body, because it serves as a barrier to sweat and could potentially add to the heat stress of the body, especially if wearing MOPP.

The use of SERPACWA makes decontamination easier for those areas that are protected by the barrier because CWA is more easily removed from the SERPACWA layer than from the skin. Service members should still perform skin decontamination immediately after chemical contamination, as the effectiveness of SERPACWA decreases with time.

The SERPACWA has no vapors, and so it does not register a false alarm with the automatic vapor detectors, such as the Improved Chemical Agent Monitor (ICAM). It also does not register with systems that detect chemical liquid, such as M8 or M9 paper. The SERPACWA paste on the surface of M8 or M9 paper will prevent CWA absorption by the paper and render it ineffective.

The SERPACWA increases the protection provided by the standard protective suits used by the U.S. warfighters and extends the window for decontamination. Although SERPACWA offers several advantages, it is not the ideal barrier cream and has limitations. The SERPACWA acts as a passive protective barrier and does not neutralize chemical agents into less toxic compounds. Evaluation of SERPACWA in animals using a saturated vapor cup model did not demonstrate any efficacy against challenge by sulfur mustard vapor or soman vapor. In fact, against a minimum sulfur mustard vapor challenge (saturated vapor cup for a short time) SERPACWA actually produced a significantly worse lesion (261% increase) over nontreated positive control animals (Braue, 2007). VX has such a low vapor pressure that it is generally not considered a vapor threat.

To overcome the limitations of SERPACWA, the USAMRICD began a program to develop an improved SERPACWA in 1994. This program, known as active Topical Skin Protectant (aTSP), was designed to address the two main limitations of SERPACWA—nonreactivity and inability to protect against an agent vapor threat. Its goal was to develop a formulation that would act as both a protective barrier and an active, destructive matrix to detoxify CWAs. The types of molecules that potentially could be used to neutralize or detoxify CWAs were known. The compounds fell into three general classes: oxidizers, reducers, and nucleophiles. An important limitation, however, was that the final formulation could not irritate the skin. This restriction eliminated many of the most reactive species. The aprotic nonpolar environment of SERPACWA provided a unique but challenging medium for active moieties to neutralize CWA. It was necessary for the improved SERPACWA to provide increased protection without degrading a warfighter's performance (Braue, 1999).

Using the two components of SERPACWA, perfluorinated-polyether oil and polytetrafluoroethylene solid, as a base cream, USAMRICD scientists evaluated over 150 different active components. Classes of compounds tested included organic polymers, enzymes, hybrid organic–inorganic materials, polyoxometalates, inorganic composites, inorganic oxides, metal alloys, and small organic molecules. These compounds were incorporated into the base cream to produce over 500 candidate formulations (patents by Braue et al., 2002a, 2002b, 2002c, 2002d, 2002e; Hill et al., 2004 and Hobson et al., 2002a, 2002b, 2002c, 2002d).

The candidate formulations were evaluated for efficacy in both in vitro and in vivo models. Using a decision tree network (DTN) approach, two candidate formulations were downselected for

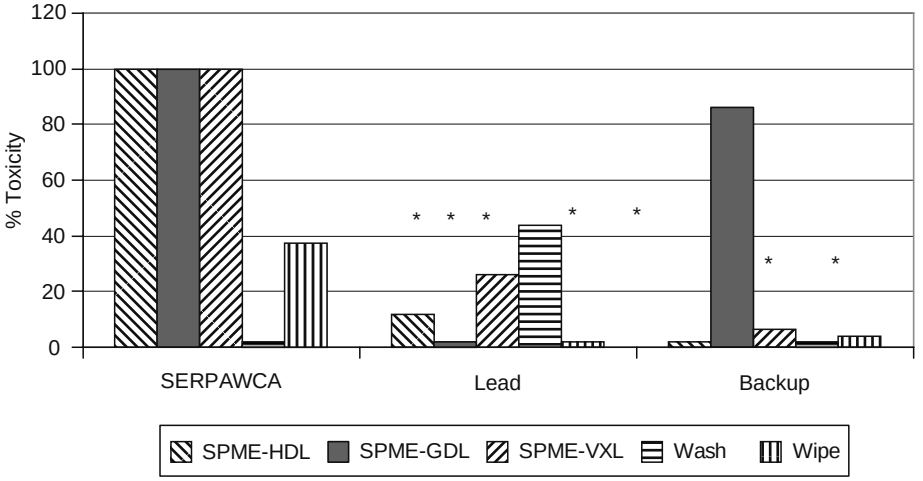

FIGURE 21.1 Critical DTN in vitro tests comparing SERPACWA efficacy with the lead aTSP formulation and the backup aTSP formulation. % Toxicity is a normalized efficacy scale where 100 means no destruction of agent in the solid phase microextraction (SPME) headspace tests and easy removal in the wash and wipe tests. * indicates significant ($p < 0.05$) improvement compared with SERPACWA. "L" with agent name indicates liquid, "V" indicates vapor. The lead formulation containing organic polymers provided significantly improved protection in every model except for the wash test. The backup formulation, containing S-330, provided improved protection for HD and VX and equivalent protection for GD in the neutralization tests. It also provided improved protection in the wipe test and equivalent protection in the wash test.

transition into advanced development. The lead formulation was a mixture of organic polymers, surfactants, and the base cream of perfluorinated-polyether oil and polytetrafluoroethylene solid. The backup formulation contained the S-330 (Sigma-Aldrich cat # S706485; CAS # 19103-02-7) and the base cream of perfluorinated-polyether oil and polytetrafluoroethylene solid. The results summarized in Figures 21.1 and 21.2 clearly demonstrated the improvement in efficacy provided by the lead and backup formulations over the original SERPACWA (Braue, 2006).

The improved SERPACWA was transitioned to advanced development in 2004. However, limited resources with higher priority products in the queue prohibited the U.S. Army Medical Research and Materiel Command to continue development of these improved formulations at that time. It is unclear at the time of this writing whether these new and improved formulations will be finalized into a usable product. If the decision is made to continue advanced development, it is likely to take 7–10 years to obtain the FDA's approval and bring this product to market.

IV. DECONTAMINATION

Characteristics of an Optimal Decontaminant. In battle, the warfighter will not likely have time to identify the specific agent on his skin, but will expect the decontaminant to universally remove all chemical and biological warfare agents. This decontaminant should obviously not facilitate the penetration of the agent into the skin but instead wick any penetrated agent out of the skin.

The most effective decontaminant is only effective if we can get it into the hands of the user. We must be conscious of fiscal and manufacturing constraints. Part of a comprehensive evaluation of decontamination solutions must include the entire life cycle of cost of research and development; manufacturing, fielding, maintenance, and disposal. An optimal candidate would move through the acquisition process quickly and preferably be already commercially available for another use and easily approved by the FDA for this intended use.

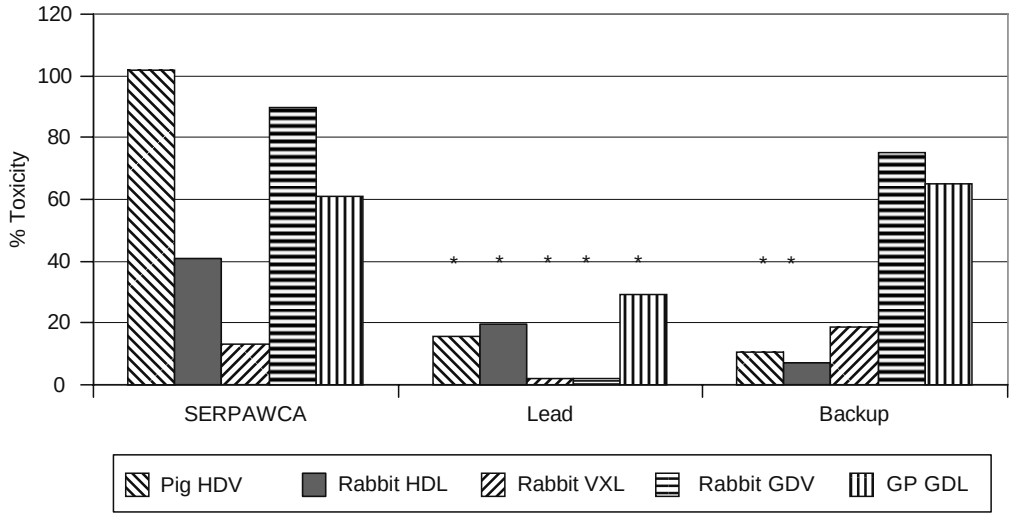

FIGURE 21.2 Critical DTN in vivo tests comparing SERPACWA efficacy with the lead aTSP formulation and the backup aTSP formulation. % Toxicity is a normalized efficacy scale where 100 is no protection and 0 is complete protection. * indicates significant ($p < 0.05$) improvement compared with SERPACWA. "L" with agent name indicates liquid, "V" indicates vapor. The lead formulation containing organic polymers provided significantly improved protection in every model. The backup formulation, containing S-330, provided improved protection for HD and equivalent protection for VX and GD.

The manufacturer must be able to scale-up production to make sufficient quantities for all users and do so under good manufacturing practice such that all lots are equally efficacious. The resulting product should have a long shelf life and be stable under extreme temperatures to support arctic and desert operations. Also, a single product deliverable from several devices for both mass casualty and individual use would be a logistical plus. One must recognize, however, that decontamination falls into two general categories. The first is immediate lifesaving decontamination, which is performed at the time of exposure by the individual or buddy. This is generally limited to a small area on the skin and requires relatively small amounts of decontamination material. The second category is patient decontamination that not only protects casualties but also protects emergency responders, medical treatment personal, and the treatment facility. Patient decontamination includes removal of clothing and washing the entire body.

Also, the warfighter's needs must be considered. Complicated decontamination procedures are unacceptable, because they increase the probability of error during the stressful exposure scenario and require excessive training. The warfighter must be able to employ the device quickly to minimize transdermal penetration. The individual device should be lightweight, not have an offensive odor, residue, or form dust that irritates the eyes or lungs. The product must be medically safe, in that it is nonirritating and nonallergenic. Ideally, the product would also be environmentally safe to use by itself and render the chemical/biological agent environmentally safe.

Though an ideal decontaminant may be difficult to attain, many alternatives exist that are readily available and meet many basic requirements. van Hooidonk et al. (1983) evaluated a variety of household products as decontaminants for VX, soman, and sulfur mustard. His paper provides an excellent comparison of these readily available decontaminants to include flour, soapy water, talcum powder, and tissue paper. We have selected several other candidate decontaminants that are under investigation, in advanced development, or currently fielded. These include the currently fielded U.S. Department of Defense skin decontaminants (M291 Skin Decontamination Kit [SDK], 0.5% hypochlorite, and 1% soapy water), Reactive Skin Decontaminant Lotion (RSDL), Diphoterine, Sandia Laboratory's Decontamination Foam, and the Decontamination Sponge.

Both rabbits and guinea pigs have been used to evaluate the efficacy of decontamination products by the U.S. Department of Defense. In these models, the hair is clipped and the agent is applied to the clipped area. After a 2 min delay, the decontamination product is used on the exposed skin area. A limited number of experiments have been done at shorter and longer times. The animals are generally under sedation during the exposure and decontamination procedures. Efficacy is expressed as a protective ratio that is defined as the LD_{50} of treated animals divided by the LD_{50} of untreated control animals. No decontamination has a protective ratio of 1.

M291 Skin Decontamination Kit (SDK). The M291 SDK was first issued to U.S. forces in 1989 and remains the primary kit that soldiers use to remove CWA from skin. The kit consists of a wallet-like carrying pouch containing six separate decontaminating pads, enough to perform three decontamination procedures. Each pad has a loop that fits over the fingers so that the user can easily pat it over contaminated skin. The pads are designed to absorb and neutralize liquid CWAs and are particularly useful when water is limited.

The M291 pads are nonwoven, fiber-fill, laminated, and impregnated with the decontaminating compound, Ambergard XE-555 resin, which is a black, free-flowing powder. This powder is a carbonaceous adsorbent that can remove agent from the skin and two ion exchange resins that neutralize the agent. Each pad provides the soldier with a single-step, nontoxic, nonirritating decontamination application, which is safe to use on intact skin. However, the pads should not be used on wounds, in eyes, or on abraded skin (Hurst, 1997).

To date, the M291 SDK has not been used to decontaminate humans exposed to chemical agent. Extensive animal data suggest that the kit will provide significant protection to exposed soldiers. The resin itself was first tested for efficacy in rabbits with shaved backs that were exposed to soman, thickened soman, and VX. Two minutes after exposure, the animals were treated with the resin. The protective ratios were observed to be 2.0, 10.4, and 22.8, respectively (Joiner et al., 1988).

In another study, clipped rabbits were exposed to sulfur mustard, lewisite (L), thickened soman, and VX and were decontaminated between 30 s and 5 min postexposure. For the vesicants (sulfur mustard and L), lesion areas were compared. Decontaminating for 1 min resulted in the lesion areas being reduced 21-fold for sulfur mustard and 22-fold for L, relative to no decontamination. For the nerve agents (soman and VX), the inhibition of blood acetylcholinesterase (AChE) was used as a marker. Using a 2 min decontamination time, the dose of nerve agent required to produce 50% inhibition of AChE was increased 1.8-fold for thickened soman and 18-fold for VX (Hobson et al., 1993).

Recent studies in the clipped, haired guinea pig model, however, demonstrated that the M291 SDK was only marginally effective against GD, GF, and not effective against VX, VR, and other agents (Braue, 2006; Hanssen et al., 2006).

The activated carbon leaves a black residue on the skin of the user making the M291 unpopular with soldiers. It is not expected that use of the M291 would be influenced by this fact if the probability of exposure was high.

RSDL. In 1995, the Canadian Defense Research Establishment at Ottawa and Suffield developed and patented a liquid skin decontaminant for CWA exposure named RSDL. RSDL is a formulation of potassium 2,3-butanedione monoxime (as free oxime and potassium salt) in a solvent of polyethylene glycol monomethylether (MPEG) of 550 nominal molecular weight with 10% w/w water (pH 10.6) (Sabourin et al., 2001; Bide et al., 2005; Material Data Safety Sheet, 2006).

RSDL is currently manufactured by E-Z-EM, Inc (Lake Success, NY) and was selected to gradually replace the M291 kit for U.S. military. The Milestone C decision was made on March 16, 2007. RSDL is currently fielded by Australia, Belgium, Canada, Ireland, the Netherlands, New Zealand, Slovenia, and Sweden. This lotion has the benefit of being a wet decontamination system, which is generally more effective than dry systems.

The effectiveness of RSDL at blocking damage caused by nerve and blistering agents was tested in hairless guinea pigs by van Hooidonk et al. (1996). Measurements were taken for lesion areas, necrosis scores, Draize scores, and agent adducts in blood or skin. The RSDL was compared in

similar tests with three other decontaminants—Dutch Powder (HuidOntsmettingsPoeder), Fuller's earth pad, and the M291 SDK. The RSDL ranked the most efficient in decontaminating L, thickened L, VX, and thickened sulfur mustard. However, it ranked last in decontaminating sulfur mustard (van Hooidonk et al., 1996).

Further testing of RSDL in haired guinea pigs has been done at the USAMRICD. Braue (2006) and Hanssen et al. (2006) demonstrated that RSDL is superior to the M291 kit, 0.5% hypochlorite solution, and soapy water against a broad spectrum of agents, including soman, cyclosarin, VX, and VR. Braue showed that RSDL provided protective ratios of 16, 26, 66, and 140 for soman, cyclosarin, VX, and VR, respectively.

The FDA, under 510(k) file 023969, has approved RSDL has as a medical device (FDA 2003a) and has reported that RSDL neutralizes the effects of many agents, including VX, G series agents, sulfur mustard, and T-2 fungal toxin (FDA, 2003b).

One drawback that may limit the soldiers desire to use RSDL in the field, is that RSDL leaves an oily residue on the skin that makes one's hands slippery and makes handling weapons more difficult.

Diphoterine. Diphoterine is a French decontamination product manufactured by Prevor Laboratory (Vlamondois, France) (Material Data and Safety Sheet, 2007). Unlike the other decontaminating systems discussed, Diphoterine was also designed to be used in decontaminating the eyes. It is supplied as an odorless, colorless, sterile solution in water (pH 7.4). It is classified as a slight dermal irritant (Jones et al., 1987) but a nonirritant in the eye as tested in the rabbit model (Clouzeau and Read 1990).

Diphoterine is a polyvalent, hypertonic, amphoteric, chelating liquid. The liquid nature rinses off surface contaminants. Its hypertonicity reduces the penetration of chemicals into the tissue by wicking the chemical agent out of tissue. The amphoteric nature neutralizes acids and bases. Also, Diphoterine can chelate radionuclides such as strontium, cobalt, cesium, or uranium and anions, such as oxalate, but doesn't chelate calcium or magnesium, which would cause biological damage to cells.

Most of the published material on the testing of Diphoterine is in French, but an excellent review article was written by Hall et al. (2002). In this review article, they described the work of Gerard et al. (2000) in which Diphoterine's effectiveness in decontaminating an alkali agent in rabbit eyes was studied. Gerard et al. (2000) exposed the eye to filter paper soaked with concentrated sodium hydroxide for 1 min and after a delay between 1 and 30 min, irrigated for 3 min with running water, an isotonic tears solution, or Diphoterine. Although all lavage solutions did not protect against the destruction of the corneal epithelial surface, Diphoterine reduced the amount of stromal edema compared with water. Also, the endothelial cells were completely destroyed with water lavage, partially destroyed with the isotonic tear solution, and slightly destroyed with Diphoterine.

Several case studies describe the effectiveness of Diphoterine in treating patients accidentally exposed to chemicals. Simon (2000) compared water and Diphoterine rinsing of 375 chemical splashes during a 7 year period at a chemical factory in France. This retrospective analysis identified 205 cases where water was used as the rinsing agent, resulting in 68 cases (33%) with no after effects, whereas 170 cases that used Diphoterine resulted in 88 cases (52%) with no after effects. Also, Hall et al. (2002) translated an unpublished report from Konard et al. which described 45 occupational accidents at Bergheim, Germany, involving sodium hydroxide or other strong bases. The finding was a significant reduction in lost work time when Diphoterine was used relative to water. Also, Diphoterine-treated patients did not require further significant medical treatment, whereas the water-treated patients did.

Gerasimo et al. (2000) compared the ability of soapy water, physiological saline, and Diphoterine to decontaminate sulfur mustard. They exposed human skin obtained from elective abdominoplasty to C14 labeled sulfur mustard in vitro for 5 min. They added the lavage to the test tube and removed the skin after 3 min, 10 min, or 3 successive 10 min washes. In each case, Diphoterine significantly removed more sulfur mustard than the other two treatments. For the 3 successive washes, Diphoterine removed 50% of the applied agent compared to 37% for soapy water and 32% for physiological saline.

Diphoterine was tested in soldiers exposed to ortho-chlorobenzylidene malononitrile (CS) ''tear gas.'' Five French Gendarmes entered a standard training CS exposure chamber. Four developed skin–eye exposure symptoms and were then decontaminated with Diphoterine. One Gendarme used Diphoterine as a pretreatment and then entered the chamber. Those who entered the CS chamber without prior application of Diphoterine developed the expected effects of excessive lacrimation, eye irritation, and blepharospasm. After CS exposure and Diphoterine decontamination, these effects rapidly resolved and all four were fully operational. The single Gendarme who used Diphoterine as a pretreatment developed milder symptoms and remained fully operational on exiting the chamber (Viala et al., 2005). Testing conducted at the Battelle Memorial Institute (Columbus, OH) showed that Diphoterine was not effective (equivalent to no decontamination) against HD in the clipped rabbit model. HD was delivered as a 1 μL liquid droplet followed by Diphoterine application 2 min after challenge (Braue, 2007).

Sandia Foam. In the late 1990s, Sandia National Laboratories (Albuquerque, New Mexico) sought to develop a decontaminating solution that used ''off the shelf'' technology and products. The basic concept was to use common household products already approved and marketed in the United States for easier FDA approval as a medical product.

The first product developed was Mass Decontaminating Foam-100, licensed for manufacture to two companies: Modec Inc. (Denver, Colorado) and Envirofoam Technologies Inc. (Draper, Utah). Modec marketed the product as Modec Decontamination Foam (MDF-100), stored in two separate containers. The first contains a solution of 6.6%-*N*,*N*,*N*,*N*′,*N*′-Pentamethyl,-*N*′-Tallow Alkyl 1,3-Propanamine Diammonium; 2.6%-*n*-Tallow-Pentamethyl Propane Quaternary Ammonium Compounds; Benzyl-C12-18 Alkyl Dimethyl; 1%-Isopropyl Alcohol, and the other a solution of 8% hydrogen peroxide. When the two solutions are mixed and sprayed, they generate foam, which settles into a liquid in under 30 min.

The ability of MDF-100 foam to protect against nerve agent exposure was tested by cutaneously exposing clipped haired guinea pigs to varying doses of VX or soman and 1 min later removing the agents by wiping the side of each animal four times with gauze soaked in MDF-100. Animals were observed over 24 h and then euthanized. MDF-100 did an excellent job of protecting against soman and VX exposure. In untreated animals, the LD_{50} for cutaneous exposure to neat soman is 11.3 mg/kg (Clarkson et al., 2004). However, in guinea pigs decontaminated with MDF-100, the LD_{50} was 400 mg/kg, a 35-fold protective ratio. MDF-100 provides an even higher protective ratio against VX. In untreated guinea pigs, the LD_{50} for cutaneous VX exposure (using 90% methylene chloride as a carrier solvent) is 0.14 mg/kg. However, in animals decontaminated with MDF-100, the LD_{50} is 10.1 mg/kg, a 72-fold protective ratio.

MDF-100 was advertised as being equally effective against biological agents. In response to the terrorist anthrax letters of 2001, MDF-100 was considered by the Environmental Protection Agency (EPA) as a decontaminant for facilities. Use of MDF-100 was cancelled by the EPA in March 2002 because of concerns that it did not neutralize anthrax spores with sufficient speed.

To counter this concern, Sandia labs developed another foam product that was faster acting and less corrosive, DF-200. DF-200 has a lower concentration of peroxide and surfactants and contains an activator to increase the speed with which it breaks down agents.

As a mortuary affairs research project, Capacio et al. (2003) studied DF-200's effectiveness in neutralizing CWAs on dead animal skin with minimal cosmetic damage to the skin. Guinea pigs were exposed to 113 mg/kg of either soman or sulfur mustard. In the case of sulfur mustard exposure, animals were euthanized, and 30 min after exposure animals were placed separately in double polyethylene bags. DF-200 was applied to the shaved skin and the bags closed. The bags were fitted with sampling access ports that could be resealed following headspace sampling. For soman, vapor concentrations were monitored every 30 min up until 6 h, then again at 24 h. For sulfur mustard additional samples were taken at 48 and 72 h.

The studies conducted with soman showed that vapor concentrations dropped below dangerous levels after 6 h in 33% of the containment bags. At 24 h, 100% of the containment bags had

undetectable soman vapor levels. The studies conducted with sulfur mustard showed high levels of vapor in the containment bags over the 72 h tested, with sulfur mustard vapor ranging 1–10 times higher than acceptable safety levels. In contrast to the soman experiments, where concentrations in the decontaminated bags decreased relatively quickly by 6 h, the concentrations of sulfur mustard increased from 0.5 to 1.5 h then decreased relatively slowly over time. The bags containing foam had lower levels of sulfur mustard vapor than the control bags at all points measured.

These experiments indicate that despite causing no cosmetic damage, DF-200 will have limited use in decontaminating human remains exposed to CWAs because it is of limited effectiveness against sulfur mustard.

Polyurethane Sponges. The use of a synthetic sponge, incorporating scavenging enzymes and detoxifying chemicals, has been pursued in an attempt to develop a more effective, self contained, easy to use, field-deployable self-use decontamination device. Sponges afford the dual advantage of physically removing CWAs from skin while providing a means of neutralizing the agent and preventing cross-contamination.

Munnecke (1979) first showed that immobilized enzymes could sequester pesticide organophosphates and Havens and Rase (1993) developed a reusable immobilized enzyme sponge for detoxification of organophosphate pesticide spills. Gordon et al. (1999) developed a polyurethane sponge and demonstrated its efficacy at physically removing agent from the skin. Versions of the sponge have copolymerized soluble cholinesterase enzymes and oximes in the polyurethane matrix to stoichiometrically interact with, sequester, and detoxify organophosphate agents. Other versions of these sponges are now being developed to provide similar detoxifying effects on vesicating agents. Active moieties include polyamines, which provide reactive nucleophiles for sulfur mustard alkylating agents, and tetraglyme to extract the CWAs from skin and wounds.

The synthesis process for the sponges is well established, can be accomplished at room temperature, and the incorporated enzymes have been shown to be stable for over 3 years in a dry state and over 3 months if wet. A large-scale deployment of these sponges is currently limited by the amount of available enzyme. Use of a high-specificity enzyme is desirable but not a necessity because so much enzyme can be integrated into the polyurethane polymer surface area.

To assess efficacy of the sponge, protective ratios were calculated and the amount of CWA removed by the sponge and decontaminated over time was measured. Detoxification of the organophosphate or sulfur mustard was quantified using specific enzymatic assays for each agent. The rate of detoxification was greatest for sulfur mustard whereas VX and soman were detoxified by the sponge mixture at similar rates (Gordon et al., 2006). When compared with the M291 SDK and several commercial formulations, the sponges provided markedly increased protection to neat soman and VX, as well as reduced methylene blue uptake, edema, and histopathology scoring indexes in sulfur mustard exposed animals. The sponge was slightly more efficacious than RSDL for soman and equivalent for VX, but a more effective detoxifier for both VX and soman (Gordon et al., 2006).

Hypochlorite/Soap and Water. In WWI, the Allies used bleaching powder (calcium hypochlorite) as the primary decontaminant. This material proved effective at neutralizing sulfur mustard on the ground. Almost 2000 tons of bleaching powder were sent to the Allied Expeditionary Force during the war (Smart, 1997). Another form of hypochlorite used in the war was Dakin's solution. Dakin's solution consists of sodium hypochlorite (0.4%–0.5%) and boric acid (4%) and was used as a successful field antiseptic. Unfortunately, the solution was unstable and had to be made as needed (Vedder, 1925). Because of its oxidative chlorination properties, it was identified as an acceptable way to decontaminate CWAs. Dakin's solution acts as a solvent on dead cells and hastens the separation of dead from living tissue. This originally was thought to detoxify agent in the skin, but later work showed this was not the case (Smart, 1997).

Current U.S. doctrine calls for using 0.5% sodium or calcium hypochlorite solution for decontamination of skin and a 5% solution for equipment. However, even 0.5% hypochlorite is

contraindicated for the eye, as it may cause corneal injuries. It should also not be used in open wounds (Hurst, 1997).

The effectiveness of dilute bleach as a decontaminant was tested in multiple studies. Dolzine and Logan (1991) conducted an in vitro test in which they determined the percentage of agent that was detoxified after a 5 min exposure to various concentrations of bleach. They found that 67% of sulfur mustard, 69% of GF, and 85% of sarin were destroyed with 0.5% bleach. Undiluted bleach resulted in 79% of sulfur mustard, 91% of GF, and 99% of sarin being destroyed. Undiluted bleach is contraindicated as a decontaminant on skin not only because of its limited effectiveness against sulfur mustard, leaving in excess of 20% of the agent still active after 5 min of decontamination, but it is also toxic to the skin and may create more damage. Zvirbis and Kondritzer (1953) noted that rabbits receiving 20 mm^3 of sarin/kg had no convulsions or deaths without decontamination but had 100% incidence of convulsions and 75% incidence of lethality when decontaminated with 5% bleach. The bleach itself may destroy the protective barrier of skin and allow sarin to pass through the skin. Because of this, U.S. Army doctrine calls for diluting bleach to 0.5% hypochlorite which produces no appreciable irritation to human skin (Hobson and Snider, 1992).

van Hooidonk et al. (1983) conducted in vitro penetration studies using a diffusion cell with guinea pig abdominal skin. They found that diluted bleach (concentration not defined) removed 96% and 97% of VX and soman, respectively. However, they also found that tissue paper wetted with water removed 92% of VX and 91% of soman. In the in vivo experiment, use of dilute bleach resulted in a 100% survival rate when VX (0.25 mg) or soman (5.0 mg) was dermally administered to guinea pigs. However, soapy water and wet tissue paper each produced the same 100% survival rate for the same amount of agent decontaminated. They concluded that physical removal of the chemical agent from the skin quickly is more important than which decontaminant is used.

Wormser et al. (2002) compared the effectiveness of 0.5% hypochlorite with water in decontaminating sulfur mustard on guinea pigs. They found the skin sulfur mustard content was reduced 64% and 68% by water and 0.5% hypochlorite, respectively, indicating that both are equally effective in removing sulfur mustard. However, they found the gauze pads soaked with the decontaminant contained microgram quantities of sulfur mustard when water was used, but no detectable sulfur mustard levels when 0.5% hypochlorite was used. Thus, the most significant neutralizing effect of 0.5% hypochlorite appears to be its ability to destroy the agent removed from the skin and render the material safe.

Decontamination of conventional wounds in a contaminated environment continues to be a major concern. Researchers have looked at the effect of bleach decontamination on damaged skin exposed to CWAs. Gold et al. (1994) evaluated the effects of water or diluted bleach (0.5%) as a wound decontaminant 2 min after hairless guinea pig was exposed to sulfur mustard. The study found that 0.5% hypochlorite and even water soaking for 5 min in a wound contaminated with sulfur mustard (20 mg/kg) cause greater necrosis than when no decontamination was carried out. This does not mean that the wound should not be decontaminated but rather that bleach soaking in the wound is not the route to decontaminant.

Hobson and Snider (1992) evaluated the effectiveness of hypochlorite solutions in decontaminating rabbit intact skin and wounds exposed to VX or sulfur mustard. When the intact skin was decontaminated with bleach at 5% or 0.5% hypochlorite concentrations 1 min after sulfur mustard exposure, lesion areas were reduced by 4.6- and 4.3-fold, respectively. For VX-contaminated intact skin, 5% and 0.5% sodium hypochlorite increased the median lethal dose of VX by 19- and 16-fold, respectively. The results indicate that 0.5% bleach is as effective as 5% in decontaminating sulfur mustard and VX on intact skin. However, when VX was applied to a wound site, the 0.5% bleach was not effective in increasing survival rate, whereas 5% bleach increased the median lethal dose 2-fold.

Recent studies in the clipped haired guinea pig model using 0.5% bleach and a 2 min decontamination delay demonstrated protective ratios for soman, cyclosarin, VX, and VR of 2.6, 6.8, 17, and 60, respectively (Braue, 2006; Hanssen et al., 2006).

Because soap and water (or water alone) are almost ubiquitous in military and civilian environments, uses of these materials as decontaminants are almost guaranteed after a chemical warfare attack. Many emergency response plans drafted after the 1995 Tokyo sarin attack call for the use of hypochlorite in conjunction with soap and water (Volans, 1996). Wetter et al. (2001) did a survey of 224 hospitals in the northwestern United States and found that 72% of their emergency departments had equipment and plans to use water as their method of mass decontamination. Both fresh water and sea water have the capacity to remove chemical agents not only through physical removal but also via slow hydrolysis. However, the generally low solubility and slow rate of diffusion of CWAs in water significantly limits the agent hydrolysis rate (Chang and Ciegler, 1986; Hurst, 1997).

The predominant effect of water and soap solutions is the physical removal or dilution of agents; however, slow hydrolysis does occur, particularly with alkaline soaps. In the absence of hypochlorite solutions or other proven decontaminants, soap and water is a reasonable option to remove CWAs (van Hooidonk et al., 1983; Hurst, 1997).

Recent studies in the clipped haired guinea pig model using 1% soapy water and a 2 min decontamination delay demonstrated protective ratios for soman, cyclosarin, VX, and VR of 2.2, 6.0, 16, and 27, respectively (Braue, 2006; Hanssen et al., 2006).

V. SUMMARY

Several decontaminants are readily available for CWA exposure. The true measure of effectiveness requires that all products be tested identically, to include soapy water as van Hooidonk et al. (1983) did so well for common household products. The most comprehensive data available are the studies conducted at the USAMRICD (Braue, 2006; Hanssen et al., 2006) which directly compared the efficacy of M291 SDK, RSDL, 0.5% bleach, and 1% soapy water challenged with soman, cyclosarin, VX, and VR in the clipped haired guinea pig model. The most important guiding principle of decontamination is rapid removal of the threat agents from the skin. This principle supersedes seeking out sophisticated decontaminants immediately after CWA exposure. The immediate use of soapy water would offer far better results than the best possible decontaminant that was delayed more than 30 min in its use. Future evaluation of potential decontaminants should consider all of the discussed factors to determine the best product.

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22 Chemical Warfare, Chemical Terrorism, and Traumatic Stress Responses: An Assessment of Psychological Impact

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CONTENTS

Portions of this article have appeared in press previously.

I. INTRODUCTION—THE CHEMICAL WARFARE AGENTS

Chemical warfare agents (CWAs) have been used on the battlefields by armed forces as well as against civilians by terrorists and armed forces during the twentieth and twenty-first centuries. This chapter addresses several issues encountered by medical planners when confronted by the use, or threat of the use, of chemical weapons. It discusses the types of CWAs, their principal target organs and physiological effects, and also considers their use in twentieth century warfare and in terrorism. Importantly, it contrasts the military combat and civilian terrorism scenarios. Calling upon a review of the disaster literature and military training and simulations, we summarize the additional physiologic and psychological, both acute and chronic, consequences of the use and threat of use of CWAs in both military and civilian settings. In addition to potential psychological effects of CWA exposure, the threat of chemical attack may evoke fear, helplessness, and horror. We review the methodologies and findings from these diverse studies, attempting to apply them to the fortunately, infrequent circumstance of chemical terrorism. We argue that there is a good basis from these historical studies to support planning the response to chemical terrorism. Such planning must include early involvement of mental health experts in assessment and treatment of psychological health. The early involvement of these experts will mitigate the early as well as the delayed psychological effects.

The twentieth century saw the introduction of CWAs on the battlefield by countries with advanced chemical industrial capabilities and, near its conclusion, by countries with less advanced production means and by terrorist organizations. The principal classes of CWAs are nerve, blister, blood, and choking agents. The simplistic military chemical nomenclature adopted over 50 years ago captures the principal target organ of each class of agent—central nervous system (CNS), skin or epithelial tissues, airways, gastrointestinal tract (GI), the oxygen carrying capability of blood, and the pulmonary system, respectively (Headquarters Department of the Army, 1966). These CWAs were designed to be rapidly lethal (nerve, blood) or incapacitating (blister, choking) at relatively low concentrations. The so-called ''G'' nerve agents, which include tabun (GA), sarin (GB), soman (GD), or cyclosarin (GF) were developed just prior to World War II (WWII). Another prominent nerve agent is VX, which was weaponized by both Cold War superpowers. Nerve agents have thus far had limited use on the battlefield, and they were used recently in terrorist attacks. The most prominent example of a blister agent is sulfur mustard (HD), which was used widely in World War I (WWI) and in the Iran–Iraq War. The most well-known and widely employed and studied blood and choking agents are cyanide and phosgene, respectively. Both chemicals are traditional CWAs, having been used in WWI and the (Second Sino-Japanese War), and ''toxic'' industrial chemicals (TICs), that is, chemicals produced in large amounts for commerce. The combination of toxicity plus availability leads to concern about their use in chemical terrorism. The rapid and toxic action of these agents requires prompt medical response. The major classes of CWAs, their principal physiologic effects, and historic morbidity are represented in Table 22.1.

II. USE OF CWAs IN THE TWENTIETH CENTURY

A. Exposure of Military Personnel during War

Unfortunately, CWAs have been used, or are thought to have been used, in a number of major conflicts in the twentieth century. Instances of CWA use include (1) extensive use in WWI, (2) use by Italy against Ethiopia (Abyssinia), (3) use in the Sino-Japanese War, (4) relatively well-studied use in the Iran–Iraq conflict, and (5) the Matsumoto/Tokyo terrorist incidents. The military effectiveness of these weapons is questionable; however, their psychological value appears significant (Spies, 1986). We briefly discuss the information derived from each of the above instances.

In WWI, HD was the most significant cause of CWA casualties (80%). It produced 125,000 casualties for the United Kingdom alone, and for the United States, approximately 28,000

TABLE 22.1
Morbidity and Mortality of Four Classes of Chemical Warfare Agents and Their Principal Targets (Estimates Derived from U.S. Published Sources)

Class of CW Agent	Historic Lethality/ Morbidity	Principal Target Tissue	Physiological Effects
Choking (e.g., phosgene)[a]	1%/6,834	Deep lung compartment, such as pulmonary capillary	Pulmonary edema, hypoxia
Blood (e.g., cyanide)[b]	Unknown	Cellular respiratory enzymes	Depression of cortical function, unconsciousness, convulsions
Blister (e.g., mustard gas)[c]	2%/27,771 4%/45,000	Skin, airway, eyes, GI tract, bone marrow	Loss of function due to skin, lung, ocular lesions; slow recovery
Nerve (e.g., sarin)[d]	About 1%/ 1,200	CNS, neuromuscular junction; cholinergic synapse	Vomiting, diarrhea, miosis, nausea, weakness, loss of consciousness, convulsions

[a] WWI figures for the United States are estimates because phosgene was often mixed with chlorine; however, a total of 6,834 injured (average hospitalization was 49 days; casualties have been directly attributed to phosgene with 66 fatalities).

[b] No data from wartime use; however, wartime experiences suggest difficulty in achieving militarily effective concentrations unless confined to closed spaces.

[c] WWI, 2% with 27,711 U.S. soldiers injured; Iran–Iraq War, 4% with 45,000 estimated injured. Its use resulted in similar hospitalization duration to WWI.

[d] No data from wartime use; however, sarin was used by terrorists twice in Japan, in 1994 and 1995.

casualties, of which 2% died. The average hospitalization of U.S. casualties was 42 days (Sidell et al., 1997). For each U.S. soldier exposed to HD, two sought medical care without evidence of exposure. A significant instance was during the operation of the 3rd Division at Aisne-Marne, which occurred in the first few months of American forces' action in the War. The division reported 500 casualties over an 8 day period with such symptoms as fatigue, chest pain, dyspnea, coughing, throat pain (ranging from tingling to burning), and ill-defined eye symptoms. Nevertheless, there was no clinical evidence of gas inhalation or burning found in any of those soldiers who were examined within the medical system (Medical History, 1959). The 3rd Division's experience is often used to highlight the psychological impact of these types of weapons on individuals near or even distant from the point of exposure. Overall, the findings in WWI indicated that functional nervous disease was responsible for at least 10% of the evacuations of soldiers during an attack. The majority of these evacuees were reported as cases of hysteria (also called gas neurosis). Additionally, cases of anxiety neurosis were also seen. However, the majority of anxiety cases did not arise during battle, but rather were of slow onset and seen after long continued duty (Marlowe, 2001). Symptoms of disease often continued after the actual illness was resolved, and gas neurosis continued to reproduce the initial symptoms, long since cleared, of exposure to toxic agents in use on the battlefield. Stokes and Banderet (1997) reported that as American forces acquired more combat experience and better training during WWI, these cases of gas neurosis became increasingly rare.

During the Italian–Abyssinian conflict (1933–1935), the extensive use of HD seems to have had a significant effect on the performance of the Abyssinian forces. The New York Times reported "*[HD] burned their shoulders and feet, blinded them, and burned the mouths of their pack animals . . .*" Historians conclude that "*gas seemed to have impaired Abyssinian morale*" and contributed to the speed of the Italian victory (Spies, 1986). It is important to note that military historians emphasized that the effect on morale of both soldiers and the civilian population was significant, as many thousands of civilians traveled with the Abyssinian forces. We do not have good reports on the

extent of civilian casualties or their psychiatric outcomes. Fifty years later, the pernicious nature of mustard was reinforced in the mid-1980s in the Iran–Iraq War, where it produced an estimated additional 45,000 chemical casualties (Carus, 1988). Significant skin, eye, and pulmonary lesions were seen and required extensive hospitalization, often up to 10 weeks (Willems, 1989; see Table 22.1). Reports of neuropsychiatric effects, such as severe apathy, impaired concentration, and diminished libido have appeared in the clinical literature (Balali-Mood and Navaeian, 1986). Recently, there have been a number of studies published on long-term, persistent, adverse health effects of victims of sulfur mustard exposure from that conflict (Balali-Mood and Hefazi, 2006; Hashemian et al., 2006).

B. Exposure of Military Personnel during Training

During WWII, extensive testing of HD was undertaken (by the U.S. Army) for the purpose of evaluating the effectiveness of decontamination and protective equipment. Volunteers in such tests were found to have increased posttraumatic stress disorder (PTSD; 17% prevalence and 33% subdiagnostic PTSD prevalence) when evaluated 50 years later (Schnurr et al., 1996). The number of exposures to the gas was predictive of lifetime PTSD. No acute exposures to HD or immediate problems were reported. McDonough and Romano, in Chapter 4 of this book, discuss reports of the human volunteer experiments done in the United Kingdom over a 50 year period. They reported that there was no evidence that exposure of volunteers to low doses of nerve agents resulted in any adverse medical sequelae, to include psychiatric symptoms.

C. Exposure of Civilians during Terrorist Attacks

Chemical terrorism of civilian populations was first observed at the end of the twentieth century. These included the use of HD against the Kurds and Iranian civilians during the Iran–Iraq War (Hashemian et al., 2006), and of sarin in Tokyo and Matsumoto, Japan, by terrorists (Yanagisawa et al., 2006). The terrorists' use of sarin in two separate instances in Japan is the best studied (of the two events) and has yielded some important new insights into the problem of preparing for CWA terrorism. On 20 March 1995, using a primitive method of dispersal, sarin was released on Tokyo subways by terrorists and resulted in 5500 people seeking medical care; approximately 1500 had defined symptoms of exposure, and 12 died. Less well known is the fact that on June 27, 1994, sarin was released in Matsumoto, Japan, with estimated exposure of 471 people and 7 deaths (see Table 22.1). Acute symptoms typical of sarin were described in both attacks and consisted of miosis (constriction of the pupils), increased tracheobronchial secretions, bronchial constriction, laryngospasm, increased sweating, urinary and fecal incontinence, muscle fasciculations, tremor, and even convulsions/seizures of CNS origin. Unexposed individuals had a variety of symptoms, primarily stress-related responses, and in some cases, these symptoms persisted.

For example, chronic health effects from this acute exposure, including CNS or behavioral changes that were inextricably linked to PTSD, were observed (Yokoyama et al., 1998). In Iran, a recent study found higher prevalence of chronic psychological effects, including PTSD, among civilians exposed to both conventional warfare and chemical attacks than from war alone. This study is discussed later in the text (Section V.C).

Thus, many of the lessons we have learned in the twentieth century were derived from military history, research, training exercises, and analyses (Stokes and Banderet, 1997) and more recently by studies of exposed civilians in Japan (Yanagisawa et al., 2006) and Iran (Hashemian et al., 2006). These studies have yielded valuable insights into the development, nature, and potentially long-term consequences of the psychological reaction to presence or fear of presence of CWA. We will consider the lessons of these experiences, along with discussions of the sociopsychological and neuropsychological impact of natural disasters, warfare, chemical exposures of civilian populations, and terrorist attacks. Despite similarities in the physiological and psychological responses of

TABLE 22.2
Chemical Warfare and Chemical Terrorism: Critical Differences

	Military	Civilian
Population level of training	Trained, protected troops	Generally untrained, unprotected
Population age, gender	Ages 18–45, predominantly male[a]	Ages 0–75, both genders
Reported health	Generally good health, fitness	Wide range of health status
Availability of medical protection	Medical protection available, perhaps pretreated	Postexposure treatment only
Psychological effects	2:1, limited misuse of equipment and antidotes	5:1, some misuse of equipment and antidotes[b]
Leadership	Military command and control	Law enforcement and public safety
Response	Retaliate with overwhelming force[c]	Forensic, judicial response[d]

[a] In January 2003, the proportion of males in the active U.S. forces was 85%.
[b] Experiences from Persian Gulf War, discussed below in text.
[c] This is discussed in Howorth (2000).
[d] Medical response is always under review. See the chapter by Moore and Saunders-Price for a description of latest preparedness and response structure in the U.S. Federal system.

exposed subjects, it should be emphasized that there are significant differences between chemical warfare directed against military forces and chemical terrorism directed against civilian populations that may impact on medical response systems and overall operational responses. These differences and the parameters of response are shown in Table 22.2.

III. TERRORISM, NATURAL DISASTER, AND WARFARE: PSYCHOLOGICAL OUTCOMES

A. Terrorism and Its Outcomes

Terrorist acts involving the use of CWAs evoke all of the outcomes of terrorist or catastrophic events, the most prominent of which is PTSD. We observed this following the events of Matsumoto and Tokyo, and we can learn about this generalized response from many experiences. In the United States, the most prominent, recent examples of terrorism would be the World Trade Center and the Oklahoma City bombing. The most complete longitudinal study appears to have been done in France, which demonstrated a clear relationship between degree of injury and degree of PTSD and a high instance of PTSD in terrorist incidents (Abenhaim et al., 1992). Jehel et al. (2001) reported that after a terrorist bombing attack in Paris in 1996, 41% of victims met PTSD criterion 6 months after the attack, and 34% continued to have PTSD 18 months after the attack.

Smithson and Levy (2000) reported some 139 cases where disaffected political or religiously motivated groups were in some fashion connected to chemical or biological ''agents'' between 1975 and August 2000 in the United States. On the other hand, in her comprehensive review of terrorism in the United States, Smithson concluded that to that point, the major terrorist incidents in the United States have not involved chemical warfare or biological warfare agents. As mentioned above, the most noteworthy examples are the Oklahoma City and World Trade Center attacks.

On April 19, 1995, a terrorist bomb destroyed significant portions of the Alfred P. Murrah Federal Building in Oklahoma City. Before the September 11, 2001 attacks on the Twin Towers of the World Trade Center in New York, the Oklahoma City bombing was the most violent act of terrorism committed in the United States in terms of the number of dead and wounded—167 and 684, respectively. The intense public interest in the event may have resulted in serious efforts to

track the affected populations over several years. For the purposes of this report, the most important papers are those of North et al. (1999, 2002a, 2002b). Their studies reported that 6 months following the catastrophe, nearly one in three met criteria for PTSD. They also indicated that depression, generalized anxiety disorder, and alcohol-use disorder were increased. Pre-existing behavioral disturbance was predictive of developing a disorder related to the catastrophe. These findings are all the more remarkable in that the most severely injured bombing victims were not included in the study. It has been reported several times that injury severity is linked to onset of PTSD (Abenhaim et al., 1992; APA Online, 2006). Acute stress disorder (ASD) was not addressed in this group, perhaps because most of North and his colleagues' cohort demonstrated persistent symptoms.

The medical aftereffects of the September 11, 2001 terrorist attacks on the World Trade Center have received a great deal of media attention. To a lesser extent, the psychological sequelae to this terrorist incident have also been captured and reported (Galea et al., 2002, 2003). The death toll of 3000 was accompanied by thousands of other affected individuals, by no means all in the New York area, who sought health support. Published studies indicate there is evidence of considerable psychological trauma, both in the New York area (Galea et al., 2002; Susser et al., 2002) and elsewhere across the country (Schuster et al., 2001). It is of interest to note that symptoms of stress were experienced by those not directly affected by the incident. Clearly, the attack created a widespread sense of grief and vulnerability in a great number of individuals. Galea and his colleagues analyzed telephone interviews with over 1000 residents of Manhattan, all living below 110th Street, a distance of about 6 miles from the World Trade Center. The interviews took place between 30 and 60 days after the attack. A significant proportion of Galea's respondents showed symptoms of PTSD (7.5%) or depression (9.7%), rates approximately twice the baseline rates for Manhattan. Additionally, in areas closest to the World Trade Center, the rate of PTSD was closer to 20%. There were indications of increased use of cigarettes, alcohol, or marijuana following the terrorist attack. Similar anxiety and mood changes were observed in an Israeli community, even in individuals who were not directly affected by terror attacks (Strous et al., 2003). Susser et al. (2002) stressed the consistency of the findings of the Galea studies with those of North and colleagues in their follow up of victims of the Oklahoma City bombing. Susser et al. (2002) evaluated the data coming from studies of the World Trade Center attack, the Oklahoma City bombing, and general disaster literature to develop estimates of the total psychological impact of an attack, to identify hardest hit groups—notably immediate families and first responding rescue workers—and to develop the concentric disaster–psychopathology relationship. These World Trade Center findings, as well as those of Abenhaim et al. (1992), are suggestive of a strong relationship between degree of injury or exposure to the psychological trauma and degree of PTSD. This relationship has been labeled by Rubonis and Bickman (1991) as the disaster–psychopathology relationship. Relevant to this, Shalev et al. (2006) reported that 10 months of continuous hostilities in Israel led to comparable rates of PTSD and similar level of symptoms in two communities, one directly exposed to the terrorist attacks and the other indirectly affected. On the other hand, Vietnam veterans wounded in battle were at increased risk for PTSD (Schlenger et al., 1992, in Friedman, 2006). Recently, Hoge et al. (2007) reported higher prevalence of PTSD in veterans from the current Iraq war having persistent physical health problems 1 year after return from deployment than in wounded soldiers without chronic problems. One third of soldiers who met the criteria for PTSD had high rates of physical, somatic symptoms.

The intensity of the trauma may contribute to the development of PTSD (Shalev and Freedman, 2005). Posttraumatic Stress Syndrome rates after terrorist attacks in Israel were higher than rates after motor vehicle accidents. The authors suggest that the highly traumatic nature of a terrorist attack (exposure to death, body parts) may impact the higher rate of PTSD development. Additionally, the authors hypothesized that the longitudinal course of PTSD would be affected by the frequent versus sporadic terrorist attacks; however, this was not the case. The authors suggest that there may be some sort of resiliency that develops during an era of continuous terror. Others

(Pine et al., 2005) also report that duration and intensity of stress exposure increases the risk for development of psychological disorder. The type of traumatic event can also influence the development of PTSD (Kessler et al., 1995). Perry et al. (1992) reported that the severity of symptoms of PTSD was not proportional to the extent of injury (the disaster–psychopathology relationship), but rather was proportional to perception of injury.

B. Natural Disasters and Their Outcomes

In studies of natural disasters, it has been shown that both psychological and physical morbidity are greater in communities affected by the disaster than in unaffected controls. For example, Fairley et al. (1986) reported that a Fijian community devastated by a cyclone showed considerably more psychological and physical morbidity 2 months after the event than an unaffected, but similar community. Within 3 months, these differences were resolved. These authors found that brief, catastrophic stress without loss of life appeared to provoke these morbidities (viz., ASD and PTSD) for brief periods of time. Common measures of psychological morbidity include indications of anxiety, depression, or somatic symptoms, often captured using the General Health Questionnaire (GHQ) (Goldberg et al., 1997) or the Profile of Mood States (POMS). These measures have been used variously in epidemiological assessment of community reactions to climatic disasters (Fairley et al., 1986; Silove and Bryant, 2006), survivors of shipwrecks (Leopold and Dillon, 1963), motor vehicle accidents (Delahanty and Nugent, 2006), and the like. Somatic symptoms most commonly found are loss of appetite, dizziness, headache, and chest pain (see also Leopold and Dillon, 1963). All of these measured variables are indications of ASD or PTSD. In recent studies following the Thai tsunami, psychological disorders, including PTSD, depression, and anxiety were high, with loss of livelihood a critical factor in the prediction of continued mental disorders (Silove and Bryant, 2006). At 9 months, rates of psychological disorders declined in adults, but not in children. Several of these studies also evaluated the effects of an effective warning and response planning system on community psychological reactions to the catastrophic event (e.g., Liu et al., 1996).

C. Warfare and Its Outcomes

Warfare represents a special catastrophic situation for deploying soldiers and civilians caught up in combat. Rates of PTSD, depression, and other stress disorders are also high among all age groups, especially children who survived war (Wexler et al., 2006). The Vietnam conflict may well be the paradigmatic case of the 20th century. The psychiatric histories of the Vietnam conflict record the consequences of the psychological stresses of combat and deployment. They help us to comprehend the degree to which such consequences appear to be dependent upon sociocultural, contextual variables. The war in Vietnam caused the psychiatric community, if not the broader American society, to examine the power of cultural constructs and military organizational behavior. Engel (2004) discusses the impact of societal values and beliefs on the development of psychiatric casualties. For the first time, the medical community recognized that each military conflict provides unique causal factors for its various psychological, psychosocial, psychosomatic, and somatic consequences.

Marlowe (2001) cites reports documenting that the incidence of neuropsychiatric illness in U.S. Army troops in Vietnam was, in fact, lower than any recorded in previous conflicts. The rate in Vietnam in calendar years 1965 and 1966 of individuals hospitalized or excused from duty for neuropsychiatric reasons was 12 per 1000 troops per year. The rate during the Korean War was 73 per 1000 troops per year, and in WWII, in combat areas, the rate was as high as 101 per 1000 in the First U.S. Army in Europe. Marlowe suggested several reasons for these improved outcomes. In addition to the rotation policy and sporadic combat, he noted that morale and training were probably better than those in WWII or Korea, as was the functioning of psychiatric teams in close proximity to the combat forces.

Health officials in these medical teams agreed that cases of neuropsychiatric casualties were reduced, but they saw and reported a somewhat more complex scenario. They noted the presence of neuropsychiatric illness in support personnel, even those removed from combat by an appreciable distance. Combat stress casualties among forces engaged in moderate to heavy combat represented ''classical combat fatigue.'' These soldiers, in general, had healthy, premorbid personality structures, and combat fatigue was low in occurrence among those forces. A second group demonstrated a condition described as ''pseudo combat fatigue'' (Strange, 1968). These soldiers were premorbidly neurotic, more difficult to treat, demonstrated characterological or personality disorders, a history of poor adjustment, poor stress tolerance, and more negative involvement with alcohol. This was the first time such a dichotomy among combat stress disorders was reported.

D. Outcomes Summary: Acute Stress Disorder and Posttraumatic Stress Disorder

From the foregoing discussion, we conclude that first and foremost, a chemical attack is an act of terrorism, a potential catastrophe evoking psychological responses independent of the physiological/toxicological characteristics of the agent employed. The most prominent outcomes are ASD and PTSD. PTSD is characterized by exposure to a traumatic event that poses actual or threatened death or injury and elicits intense fear. Three symptom clusters include (1) re-experiencing the traumatic event (flashbacks, intrusive memories), (2) avoiding of stimuli associated with the event and ''emotional numbing'', and (3) hyperarousal (increased startle response, impaired sleep, and hypervigilance), with symptoms occurring for more than 1 month (APA, 1994; reviewed in Keane et al., 2006; Nemeroff et al., 2006). There is an 80% probability of comorbidity of other psychological disorders, in particular depression, with PTSD (Kessler et al., 1995). Additional symptoms of ASD and PTSD include nightmares, fatigue, depression, anxiety, irritability, loss of concentration, and memory impairment (reviewed in Smith et al., 2000; Moreau and Zisook, 2003). The major difference between ASD and PTSD is the duration of the symptoms and the presence of dissociation with PTSD. Acute stress disorder and peritrauma dissociation are predictors of the development of PTSD. PTSD has an estimated lifetime prevalence of 8% and is more common in women (Kessler et al., 1995). Note that lifetime prevalence in Vietnam veterans is 30% (see Kulka, 1990 in Davis et al., 2006). The intensity and nature of the traumatic event, the degree of morbidity and mortality after an attack, and imperiled survival resources in the aftermath can affect development of PTSD (Hoge et al., 2007; reviewed in Nemeroff et al., 2006).

IV. CHEMICAL ACCIDENTS AS CATASTROPHES: SPECIAL CONSIDERATIONS

Natural disasters have also included some well-characterized episodes of chemical spills secondary to the environmental disaster. Notably, Bowler et al. (1994a, 1994b, 1998) reported on cognitive and affective changes in exposed populations compared to controls following chemical spills in the United States. She and her colleagues used such tools as the Minnesota Multiphasic Personality Inventory (MMPI), Wechsler Adult Intelligence Scale-Revised (WAIS-R), Stroop Test, and the POMS. The prevalence of PTSD was about 20%–25% to a diverse array of industrial chemicals (Bowler et al., 1998). An accidental exposure to HD occurred in a vinyl chloride monomer manufacturing facility in Plaquemine, Louisiana in 1996, resulting from process contamination with sulfur and nitrogen impurities in a vinyl. Six year follow up of 247 workers revealed 25% incidence of PTSD (Iyriboz, 2004). Of potential relevance, initial reports were of exposure to ethylene dichloride, formic and hydrofluoric acids, isocynates, and copper chloride and sulfate and vinyl chloride monomer. Six weeks after exposure, it became known that the agent was sulfur mustard.

Investigators of public health outcomes of chemical spills postulate several factors relating specifically to the presence of chemicals, in particular: (1) the insidious and indiscriminate nature of chemicals, (2) the process of decontamination, (3) the wearing of Personal Protective Equipment

(PPE), and (4) the inability to control one's environment. We discuss each one of these factors immediately below.

A. Insidious Nature of Chemicals

The fear of chemicals in the environment, even in the absence of demonstrated exposures, can produce significant effects. Valuable insights into the production of these effects by ''threat'' of chemical exposures have come from studies of communities that had experienced railroad or industrial chemical disasters. In one such study, Bowler et al. (1994a, 1994b) reported greater levels of depression, anxiety, and somatic symptoms among residents nearby to the chemical spill site. These residents also reported greater environmental worry and lower perceived social support among residents of the affected area (level of exposure or degree of injury unspecified) versus controls matched for age, education, gender, race, and socioeconomic status.

An additional fear when chemicals are involved may be fear of congenital or genetic defects in subsequent offspring. This is a phenomenon known to many industrial hygienists. A related problem in emergency response literature is the notion of stigma. In the aftermath of incidents of ''invisible environmental contaminants,'' it is common for people in the affected areas to feel feared, rejected, unsupported, ostracized, or even ashamed (Becker, 2001). See also Bowler et al.'s findings above.

Finally, chemicals trigger a special fear to those potentially exposed because of their ''invisibility.'' Vyner (1988a, 1988b) proposes a model whereby invisible environmental contaminants produce traumatic effects on the individuals who may have been exposed to them. Invisible environmental contaminants include ionizing radiation, dioxin, carbon monoxide, and PBBs. Vyner's model consists of the following stages: (1) experienced uncertainty, (2) adaptational dilemmas (Should I evacuate? Should I have children?), (3) hypervigilance (viz., every cloud contains pernicious chemicals; every odor represents a chemical release), (4) nonempirical belief systems about the exposure, and (5) traumatic neuroses. Vyner points out that a delayed onset of traumatic stress disorder has been described among atomic veterans, residents of the Three Mile Island area, Hiroshima survivors, and workers occupationally exposed to toxic chemicals.

B. Process of Decontamination

Diamond et al. (2004) also discussed some of the psychological impacts of decontamination, focusing on loss of dignity and agoraphobia. The former includes the basic question of whether potentially exposed patients will take off their clothing in the absence of an obvious immediate danger. Interestingly, these authors suggested that decontamination personnel reported concerns about spread of contamination to family members.

C. Wearing of PPE

With respect to PPE, for example, there have been valuable insights gained through the study of training exercises, field studies, and simulation (King and Frelin, 1984). Many of the psychological effects of the use, or threat of use, of CWAs have been identified in studies of soldiers' wearing of the military's Mission-Oriented Protective Posture (MOPP) suit during training. A ''classic triad'' of symptoms including anxiety, panic, and claustrophobia has been reported (Singh, 1992). A well-known example of phobia and its treatment (since WWI) is ''gas mask phobia'' (Carter and Cammermeyer, 1985; Ritchie, 1992). Gas mask phobia refers to an extreme hyperventilation, tachycardia, and the appearances of instances of apnea and panic. Ritchie (1992) recommended a process of systematic desensitization, graded exposure, and participant modeling to ameliorate the simple phobia. Oordt (2001) suggested that many factors contribute to gas mask anxiety, including physical sensations, Conditioned Emotional Responses (CERs), and situational demands, and the condition can be managed by a cognitive-behavioral approach. King and Frelin (1984) reported that

simple performance decrements produced by the mask (restricted visual fields and diminished speech intelligibility) could be reduced in absolute, but not relative terms, by practice over several days. In King and Frelin's study, the tasks used as dependent variables were basic tasks for a combat medic, for example, immobilizing a fracture, administering care to a chemical agent casualty. A more comprehensive review of the military studies of the impact of wearing PPE upon military behavior and task performance can be found in Krueger and Banderet (1997), and a more complete discussion of PTSD treatment appears later in this chapter (see Sec. V.F.). Jones, in Chapter 20 of this book, provides a review of the types of PPE worn by civilian response elements, their protective capacities, and their impact on the performance of first responders.

D. Ability to Control One's Environment (viz., Locus of Control)

Diamond et al. (2004) commented on the special fear of chemicals with respect to two dimensions: (1) the invisibility of the agent led to an absence of visual cues for escape and (2) concern about access to treatment. Riddle et al. (2003), in their discussion of Gulf War Illness, suggested that the feelings of helplessness in the face of a ubiquitous and unseen ''killer'' were overwhelming. Ursano et al. (1989) also suggested that chemical and biological warfare create a special terror because they are invisible, indiscriminate, and seemingly uncontrollable. Howorth (2000) wrote that many observers as early as shortly after WWI emphasized the importance of helplessness and lack of control in contributing to psychological consequences.

When the stressful event overwhelms ego defenses, further negative outcomes may ensue. These include: (1) conversion disorders, (2) mistaking normal physiological stress symptoms for exposure to CWA (despite significant efforts to train soldiers in proper recognition of signs of poisoning), (3) mistaking or magnifying the symptoms of minor illnesses, and (4) deliberate faking or malingering. One might add the possibility of an additional type of self-inflicted wound to this list (i.e., the inadvertent or misguided use of antidotal compounds, e.g., atropine and diazepam). Self-administration of two nerve agent antidote autoinjectors can produce headache, restlessness, and fatigue, symptoms that can be aggravated in a tired, dehydrated, or stressed individual (Romano and King, 2002).

V. DIRECT CENTRAL NERVOUS SYSTEM EFFECTS OF CWAs AND STRESS

A. Acute Exposure to Nerve Agents

Nerve agents were designed to be extremely toxic to man, unlike the selective toxicity attributed to the development of organophosphorus insecticides. For a review of the clinical effects of these agents and the medical response thereto (Hurst et al., 2005). The problem of field management of CWA casualties is complicated by the known CNS symptoms of mild intoxication by nerve agents. Cholinergic signs and symptoms of nerve CWA exposure include miosis, headache, nausea, dizziness, anxiety, muscle fasciculations, tremor, incoordination, emesis, abdominal cramps, diarrhea, sweating, salivation, tearing, rhinorrhea, and phlegm (Brown and Brix, 1998). Symptoms may also include ataxia, confusion, slowing or loss of reflexes, slurred speech, coma, and paralysis, all of which have been seen in human studies (Sidell, 1997), and effects on learned behaviors, analgesia, and cardiac effects as observed in animal studies (Harris et al., 1984; Karczmar, 1984; Brezenoff et al., 1985; Geller et al., 1985; Wolthuis et al., 1986). Occasionally, such symptoms may resemble the acute stress response (Romano and King, 2001a, 2001b).

Severe intoxication by high doses of nerve agents may produce unconsciousness and coma, seizures, respiratory depression, and death from cardiorespiratory failure. The neurochemical changes associated with high-dose exposure to nerve CWA and subsequent seizures and brain pathology are reviewed in McDonough and Shih (1997). The early phase of nerve agent induced brain injury is exclusively a cholinergic one, in which nerve agent induced inhibition of acetylcholinesterase

(AChE) increases acetylcholine, activates muscarinic receptors, and elicits epileptiform activity. In the absence of adequate treatment with an anticonvulsant, the sustained, cholinergic-induced seizure activity can progress within 5–40 min after nerve agent exposure to an intermediate phase characterized by the overactivation of glutamate receptors and neurotoxic levels of intracellular calcium, which promotes continued seizure activity long after the cholinergic crisis has subsided. Sustained seizure activity ultimately results in physiological and metabolic disturbances that contribute to neuronal injury and cell death (McDonough et al., 1995). Nerve agent induces brain pathology in multiple limbic and cortical regions, including the amygdala, pyriform cortex, hippocampus, cingulate gyrus, as well as the thalamus (McDonough et al., 1998). Myhrer et al. (2005) observed cognitive deficits in a novelty task and in retention of a brightness discrimination task to be correlated with brain pathology in rats exposed to one LD_{50} sarin. Soman exposure that did not produce seizures did not affect behavior. In this section, we will describe the neurological sequelae to nerve agent exposure and describe efforts to differentiate these sequelae from the stress response.

There have been a number of investigations as to the visible long-term consequences of an acute exposure to organophosphorus (OP) nerve agents, to include several studies of the effects of sarin-exposure victims from Japan. In this short paper, we will only cite four of the most pertinent papers. Yokoyama et al. (1998) evaluated 18 victims of the Tokyo subway incident 6–8 months after exposure. All but three of the victims had plasma cholinesterase values below normal on the day of exposure. Sarin-exposed individuals scored significantly lower than controls on a Digit Symbol Substitution Test (see Figure 22.1); they scored significantly higher than controls on a GHQ (psychiatric symptoms) and a POMS (fatigue).

Additionally, they had elevated scores on a PTSD checklist; they had significantly longer P300 latencies on event-related brain-evoked potentials and longer P100 latencies on brain visual evoked potentials; and exposed females had significantly greater indexes of postural sway. The elevated scores on the GHQ and POMS were positively related to the increased PTSD scores and were considered to be due to PTSD. Yanagisawa et al. (2006) reviewed long-term effects of sarin exposure in the Tokyo attack. Findings from questionnaire surveys 10 years after the incident suggested high incidence of psychological problems, including fatigue, difficulty concentrating, forgetfulness, headaches, apathy, and depressive mood.

Nakajima et al. (1998) performed a cohort study of victims of the Matsumoto City sarin exposure 1 and 3 years following the incident. At 1 year following the exposure, they reported that 20 victims still felt some symptoms (fatigue, asthenopia, blurred vision, asthenia, shoulder stiffness, and husky

0	1	2	3	4	5	6	7	8	9
*	≠	Π	λ	÷	$	!	?	Σ	Δ

*	≠	÷	!	Σ	Δ	?	$	Π	≠	⊠	≠	÷	$	Σ
Δ	Σ	$	÷	≠	*	*	λ	Π	!	?	Δ	*	$	?

FIGURE 22.1 This figure is an example of a Digit Symbol Substitution Test (DSST). Typical tests require a subject to substitute up to 90 symbols using the key directly above the test table. The key performance measures are speed and accuracy. The DSST is a measure of psychomotor performance, attention, and ability to remember and operate on complex symbols. It is one of the first tests to show decline with the onset of dementia, and in some instances it may exhibit decline even before dementia is clinically manifest. Norms are based on sex and age.

voice). They had all lived close to the sarin release, and they had lower erythrocyte ChE activity than those who did not have symptoms. (Note: Not all the symptoms seen at 1 year have been related to nerve agent exposure historically.) A follow-up report (Yanagisawa et al., 2006) reviewed findings that psychological complaints continued in individuals who had been in proximity of that sarin attack, irrespective of acute physical effects of sarin exposure, suggesting that other factors such as the stress associated with CWA attack may have had prolonged psychological effects. At 3 years, some victims still complained of experiencing these symptoms, although with a reduced degree and frequency. Additionally, there have been two brief case reports of severely poisoned nerve agent victims (one sarin, one VX) in Japan, who experienced retrograde amnesia, possibly due to prolonged periods of seizures or hypoxia. Additionally, one of the Matsumoto victims who experienced prolonged seizure activity was followed for at least 1 year and was found to have sporadic, sharp-wave complexes in the electroencephalogram (EEG) during sleep and frequent premature ventricular contractions on Holter monitoring of the electrocardiogram (Sekijima et al., 1995).

B. Chronic or Repeated Exposure to Nerve Agents

Chronic or repeated subtoxic exposures to OP agents produce less consistent health effects than those observed following acute exposures. (Note: In this section the term ''subtoxic'' refers to doses which produce no clinical signs [in man] or toxic signs in animals; however, these may produce significant depression of AChE or ChE.) For example, Burchfiel et al. (1976) reported that repeated low doses of sarin administered to rhesus monkeys produced a long-term increase in relative power in the EEG beta frequency bands. This study is the most cited study in support of a long-term health effect. Conversely, further reports in the literature of animal studies show that nerve agents can be administered repeatedly with minimal overt neurobehavioral effects if care is taken in choosing the dose and the time between doses (Russell et al., 1986). Blood and brain AChE levels can be reduced to $<20\%$ of normal with no observable signs of toxicity with appropriate dosing schedules (Sterri et al., 1980). Behavioral tests that have been useful in evaluating toxic effects of CWA exposure in rodents include acoustic startle response, cognitive tests, and motor tests. Effects of nerve agents on performance tend to occur at doses that produced signs of toxicity in addition to inhibition of cholinesterase (Thomson et al., 2005, 2006). For the most part, effects that occur after agent exposure are short-lived. Sipos et al. (2005) observed a transient increase in acoustic startle response in guinea pigs exposed to low doses of soman, sarin, or VX (s.c.). Also, in guinea pigs, repeated exposure to low doses of sarin (s.c.) impaired performance on an operant progressive ratio task (Langston et al., 2005). Rats exposed to cyclosarin by whole body inhalation that displayed mild signs of toxicity had small performance deficits in an operant task; testing was initiated 2 days after exposure (Genovese et al., 2006). It is possible that greater deficits may have occurred immediately after exposure, but this was not tested. Kassa et al. (2001a, 2001b, 2004) observed that rats exposed repeatedly to asymptomatic doses of sarin had impaired spatial memory in a Y-maze test, gait abnormalities, and increased stereotypy. In a small study in nonhuman primates, exposure to sarin at levels that produced significant inhibition of ChE but did not produce toxic signs, did not impair cognitive performance on a serial probe recognition task (Genovese et al., 2007). Guinea pigs repeatedly exposed to subtoxic doses of GB failed to habituate to some measures in a functional observation battery (Hulet et al., 2002). Altered EEG, with reduced theta band power, and altered brain ChE activity in guinea pigs exposed to subtoxic doses of VX were reported (Jung and Shih, 2006; Roberson et al., 2006). No brain pathology was observed in guinea pigs exposed to low doses of sarin via s.c. exposures or whole body inhalation (Petras et al., 2006) or in mice exposed repeatedly to low doses of VX, even in animals that displayed mild signs of toxicity (Chang et al., 2007). In a rat model, low doses of VX reduced body temperature and impaired motor coordination on a balance beam in animals that did not display other signs of toxicity (Lumley et al., 2005). Decreased rearing and locomotor activity were also reported in rats exposed to doses of sarin that did not produce other signs of toxicity (Nieminen et al., 1990). Conn et al. (2002) did not see an

effect of repeated subtoxic exposure to sarin on activity. Differences may in part be related to the type of activity measured. This latter study used telemetry to grossly assess motor activity, whereas the Lumley et al. (2005) study used the balance beam test to assess motor coordination. Although the majority of findings reported here are on short-term behavioral and performance deficits following low doses of nerve agents in rodents, typically with mild signs of toxicity, a few studies report longer term cognitive impairment that continues even after signs of cholinergic toxicity have subsided (McDonald et al., 1988; Buccafusco et al., 1990; Bushnell et al., 1991; Prendergast et al., 1997, 1998).

Physiological measures of low-level nerve agent exposure may be more sensitive than behavioral changes. The most sensitive indicator of nerve agent exposure was miosis, which occurred at doses below those producing toxic signs in rats and guinea pigs (Hulet et al., 2006). In addition, loss of body weight or reduced body weight gain was commonly associated with exposure to low doses of nerve agent (Jung and Shih, 2006; Lumley et al., 2006; Roberson et al., 2006).

The most notable effect of chronic subtoxic dosing is the development of tolerance to the disruptive effects of each acute exposure on certain behaviors. In this respect, nerve agents act much like other OP compounds, and the possibility and mechanisms of tolerance development have been addressed in several studies (Russell et al., 1986).

As for the human experience, workers exposed to small amounts of nerve agents that produced mild, nonthreatening medical signs of exposure, reported CNS effects such as headache, insomnia, excessive dreaming, restlessness, drowsiness, and weakness. Clearly, exposure to nerve CWA may produce stress reactions, organic brain syndromes, or both. McDonough and Romano provide a deeper discussion of the effects of acute or chronic exposure to nerve agents in Chapter 4 of this book.

C. Acute and Chronic Exposure to Mustard Gas

In the major episodes of HD (mustard gas) use in the twentieth century, viz., WWI (Henley, 1956) and the Iran–Iraq war (Balali-Mood, 1996), neuropsychiatric symptoms following acute exposure to HD have been reported. Symptoms included depression and anxiety, insomnia, and neurasthenia. Iran sustained approximately 387 chemical attacks in the Iran–Iraq war. An estimated 60,000 survivors had high rate of morbidity and psychological distress. Hashemian et al. (2006) compared mental health among individuals exposed to (1) both high-intensity warfare and CWA, (2) only to high-intensity warfare, or (3) low-intensity warfare. A high level of mental health problems was observed in individuals exposed to both high-intensity warfare and CWA, with prevalence rates for lifetime PTSD, current PTSD, anxiety, and depression of 59%, 33%, 65%, and 41%, respectively. Low-intensity warfare resulted in 8%, 2%, 18%, and 6%, respectively, while high-intensity warfare without CWA resulted in 31%, 8%, 26%, and 12%, respectively. Compared to individuals exposed to high-intensity warfare, those exposed to both high-intensity warfare and CWA had a greater risk for lifetime PTSD, current PTSD, anxiety, and depression. There was high comorbidity with depression and anxiety in individuals with PTSD. It is unclear at this time why exposure to CWA increased risk of PTSD and other mental disorders above that in response to high-intensity warfare. Possible suggestions are an additive effect of the two stressors, negative impact of CWA (mustard gas) on pulmonary function serving as a reminder of the event, or fear of future exposure to CWA (Hashemian et al., 2006).

Schnur et al. (1996) provided a follow up of 14 U.S. soldiers who participated in mustard gas testing during WWII. They reported on instances of PTSD among those personnel. Their conclusions were reinforced by Balali-Mood (1996) who suggested that, in general, the neuropsychiatric symptoms among Iranian soldiers exposed to HD were attributable to PTSD.

Alternatively, Lohs (1975) reported on consequences of chronic exposure to mustard gas among workers in chemical munitions plants. He listed impaired concentration, diminished libido, and

TABLE 22.3
Sources of Information about Psychological/Behavioral Effects of CW Terrorism

Sources of Information	Component of the Catastrophic Event	Significant Psychological Outcome
Natural disaster, terrorism, etc.	Stress	PTSD[a]
Chemical spills	Fear of chemicals, decon, etc.	Anxiety, depression, PTSD
Military training	Wearing of PPE	Mask phobia[b]
History of chemical warfare	Direct and indirect effects of CWAs	PTSD, loss of libido,[c]
	Direct CNS effects	neurasthenia PTSD, insomnia, irritability, restlessness[d]

[a] Frequency of neuropsychological effects or PTSD directly related to extent of injury; see Abenhaim et al. (1992) as an example.

[b] Performance degradation mitigated by training; see King and Frelin (1984).

[c] Iranian casualties demonstrated various neuropsychological sequelae; see Balali-Mood and Navaeian (1986) and Hashemian et al. (2006).

[d] Effects of nerve agents linger, perhaps for months; see Yokoyama et al. (1998).

''sensory hypersensitivity'' as prominent among these workers. Results are complicated by potentially toxic coexposures to the workers. In the case of mustard gas, we do not have the same level analysis differentiating organic versus PTSD-related outcomes as exists for the nerve agents. For a more comprehensive review of the literature on neurological and psychological consequences of CWA exposure, the reader is directed to Romano and King (2002).

Table 22.3 summarizes the types of prominent psychological sequelae that have been observed following natural disasters, chemical spills, military training experiences, the history of land warfare since the beginning of the twentieth century, and the like.

D. Acute Stress Exposure: Biochemical and Neuroanatomical Correlates of PTSD

Exposure to and the threat of exposure to CWA can lead to a variety of responses including ASD, depression, anxiety, dissociative disorders, drug abuse, fatigue disorders, eating disorders, and PTSD. Somatic symptoms, including fatigue, sleep disturbance, joint pain, memory loss, depression, headache, and difficulty in concentrating are commonly reported in PTSD (reviewed in Moreau and Zisook, 2003). As PTSD is commonly observed in subjects exposed to trauma, including CWAs, we will review briefly some of the recent findings on predispositions that may contribute to the development of PTSD and on biochemical and neuroanatomical changes associated with PTSD. For a more thorough review on the pathophysiology of PTSD, see Yehuda et al. (2006), Keane et al. (2006), Nemeroff et al. (2006), McNally (2003), and Morgan and Southwick (2000).

An important feature of PTSD is that not all individuals exposed to the same stressor develop PTSD. Rather, predisposing factors (genetic susceptibility, in utero effects, early developmental effects or childhood trauma, history of psychiatric illness, gender, intelligence, and coping style) affect vulnerability to the development of PTSD (reviewed in Marmar et al., 2006). Although trauma severity is a good predictor of PTSD development (Carlier et al., 1997), individual differences in stress responses and coping mechanisms prevent a simple relationship between frequency and intensity of stress exposure and the development of psychopathology. Peritrauma or acute physiological response to a stressor, including activation of the sympathetic nervous system (SNS) and the hypothalamic pituitary adrenal (HPA) axis, are thought to be important determinants

of the development of PTSD and may serve as risk or resilient factors (Shalev et al., 1998). Activation of the SNS and the HPA following a stressor are considered important for adaptation and the development of coping mechanisms; however, excess activation of these systems may lead to impairment in CNS function (reviewed in Bohus et al., 1987, 1991; McEwen, 2004). Autonomic arousal would be expected to follow reports of a terrorist attack, and symptoms of muscle tension, tachycardia, hyperventiliation, sweating, and tremor could be incorrectly attributed to exposure to CWAs.

The acute phase of PTSD has much overlap with panic, whereas the chronic phase has many similar features to depression and is often comorbid with depression (Kessler et al., 1995). Panic reactions are associated with increased adrenergic activation, fear conditioning, and hyperarousal. Increased norepinephrine release following stress is thought to contribute to memory consolidation of the traumatic event or fear conditioning (Pitman, 1989). Hyperarousal in the weeks after trauma was one of the best predictors of PTSD development a year later (Carlier et al., 1997; Schell et al., 2004). There have been several reports of increased heart rate and lower cortisol in hours after traumatic event in those who may go on to develop PTSD (Shalev et al., 1998; Yehuda et al., 1998a; Bryant, 2006). Shalev and Freedman (2005) reported that increased heart rate, peritrauma dissociation, and early PTSD symptoms contributed to the development of PTSD. Although measurement of stress response of military subjects in the field has been limited, soldiers with PTSD who did not have secondary depression were observed to have increased norephinephrine (Yehuda et al., 1998b). Elevated 24 h urinary catecholamine excretion (reviewed in Delahanty and Nugent, 2006) and abnormal pharmacological responses to adrenergic agents have also been reported in PTSD (Friedman, 1998). Pharmacotherapy results suggest that excessive and uncontrolled activity of the SNS may play a role in subsequent development of PTSD. Thus, in a pilot study, Pitman et al. (2002) reported that the beta-adrenergic blocker propanolol, when administered within 3 h of a traumatic experience in Emergency Department patients, reduced fear responses 3 months later and prevented PTSD symptoms. Also, the postsynaptic alpha 1 adrenergic blocker, prazocin, reduced nightmares, PTSD, and other symptoms in combat veterans (Raskin et al., 2003).

The neuropeptide Y (NPY) and gamma-amino-butyric acid (GABA) may be associated with resilience to the development of PTSD. A depletion of plasma NPY and a blunted NPY response to yohimbine was reported in soldiers with PTSD when compared to nontraumatized control subjects (Rasmusson et al., 2000). NPY counters the anxiogenic effect of corticotropin-releasing hormone (CRH) and counters effects of norepinephrine in brain regions associated with fear and anxiety (reviewed in Yehuda, 2006). Recently, Vaiva et al. (2006) reported that road traffic accident victims with low levels of GABA were more likely to develop PTSD. Elevated levels of GABA 1 year posttrauma were associated with trauma recovery.

Knowledge and understanding of biochemical correlates of PTSD and neuronal circuitry involved in stress responses (adaptive versus maladaptive) has increased in the past few years. One of the main biochemical effects of stress that has been associated with the development of PTSD is dysregulation of the HPA axis. However, findings have been somewhat inconsistent. Early reports suggested low basal levels of cortisol, hypersensitivity to dexamethasone, and increased lymphocyte glucocorticoid receptors (Yehuda et al., 1991, 1998a, 2004; Marmar et al., 2006). Others reported higher 24 h urinary cortisol in patients with PTSD (Pitman and Orr, 1990). One possible explanation for the discrepant findings may be related to the high comorbidity of depression with PTSD, because cortisol levels of patients with major depressive disorder are typically resistant to dexamethasone suppression (reviewed in Rush et al., 1996), the opposite of what was originally reported with PTSD. Patients with PTSD that is comorbid with depression have elevated evening cortisol and elevated norepinephrine relative to those without depression (reviewed in Delahanty and Nugent, 2006).

One theory is that, in individuals who develop PTSD, low cortisol levels during the acute trauma phase fail to suppress the activation of the SNS and that elevated catecholamines increase

memory consolidation of the traumatic event or emotional memory, manifested as intrusive memories (reviewed in Delahanty and Nugent, 2006). This suggestion has been supported by animal studies in which epinephrine following a stress exposure increased fear consolidation (reviewed in McGaugh 1989; McGaugh and Izquierdo, 2000). In addition, the beta-adrenergic antagonist propanolol administered after posttraining reduced retention of fear-conditioned response (Cahill et al., 2000). In further support of this theory, Delahanty and Nugent (2006) observed low urinary cortisol levels in patients immediately following motor vehicle accidents to be correlated with PTSD symptoms. Additionally, it is suggested (Yehuda et al., 1998a) that effects of earlier trauma on HPA function may predispose to a maladaptive stress response to subsequent trauma (reviewed in Delahanty and Nugent, 2006). A question has been whether the reduced cortisol levels were a pre-existing trait or a result of the stress exposure. Patients treated in intensive care units (ICU) for prolonged periods of time have increased incidence of PTSD and also are at risk for corticosteroid insufficiency (Schelling et al., 2006). Cortisol administration to ICU patients following critical injury (septic shock) or major surgery (cardiac) reduced PTSD symptoms and improved health-related quality of life in long-term survivors. The authors suggest that hydrocortisone may inhibit adrenergic activity, terminate the stress response, and interfere with retrieval of traumatic memory. In a pilot clinical study, de Quervain (2006) observed that low dose cortisol administered for 1 month inhibited retrieval of traumatic memories in patients with PTSD.

Other neurochemical alterations associated with PTSD include altered serotonergic and dopaminergic system (reviewed in Southwick et al., 2005). In addition, Geracioti et al. (2006) recently reported elevated substance P levels in patients with PTSD. Substance P levels were further increased in response to a traumatic videotape stimulus.

Neuroanatomical changes in the limbic system are present in individuals with PTSD. For years it has been well established that PTSD is associated with reduced hippocampal volume and deficits in hippocampal based memory (reviewed in Bremner, 2006), and these effects were thought to be a result of the severe stress response. Recent data suggest that reduced hippocampal volume may predispose to the development of PTSD. Using a cohort of twins from the National Vietnam Veteran Registry, Pitman et al. (2006) reported that smaller hippocampal volume was a pre-existing condition to the development of PTSD. Prospective studies did not reveal changes in hippocampal volume when assessed 1 week and 6 months following trauma. These data suggest that reduced hippocampal volume may predispose individuals to develop PTSD. Individuals with PTSD are reported to have alterations in the amygdala, which is important for fear conditioning and consolidation of emotional memory. Pavlisa et al. (2006) reported interhemispheric differences in amygdalar volume in patients with chronic PTSD. It is thought that in PTSD, disinhibition of the amygdala by the medial prefrontal cortex (including cingulate gyrus and orbitofrontal cortex) induces hypervigilence such that innocuous stimuli are then interpreted as threatening (reviewed in Friedman, 2006).

Although brain structures other than the hippocampus have not been well investigated, decreased volume of the cingulate cortex was recently reported (Araki et al., 2005). In nine victims of the Tokyo subway sarin attack with PTSD and 16 matched victims of the same traumatic event without PTSD, voxel-based morphometry showed a significant gray-matter volume reduction in the left anterior cingulate cortex (ACC) in trauma survivors with PTSD compared to those without PTSD. The severity of the disorder was negatively correlated with the gray-matter volume of the left ACC in PTSD subjects. There were no significant differences in other gray-matter regions or any of the white-matter regions between two groups. These data constitute evidence for structural abnormalities of ACC in patients with PTSD. Together with functional neuroimaging studies showing a dysfunction of this region, they provide further support for the important role of ACC, which is pivotally involved in attention, emotional regulation, and conditioned fear, in the pathology of PTSD. See McDonough and Romano, Chapter 4 of this book, for further discussion of this ACC volume reduction.

E. Animal Models of ASD and Posttraumatic Stress Syndrome

Animal models can complement clinical studies of PTSD in that prospective studies can more readily be conducted, exposure and physiologic stress conditions can be more tightly controlled, and biochemical and neuroanatomical measures can be evaluated. A variety of animal models, including conditioned defeat, fear conditioning, and predator stress models have been developed to mimic ASD and features of PTSD. Animal measures that are considered useful and parallel symptoms of PTSD include fear, avoidant and hypervigilant responses. We will briefly discuss a few representative animal models of ASD and PTSD.

A social defeat mouse model of combat stress reaction has been used to assess fear responses to extreme stress (Hebert et al., 1998; Lumley et al., 1999, 2000). Combat stress reaction is considered one of the best predictors of PTSD. Recent findings from a longitudinal study indicated that veterans with combat stress reaction were 6.6 times more likely to develop PTSD (Solomon and Mikulincer, 2006). In addition, their PTSD was more severe and more easily reactivated. Acute social defeat leads to long-term fear of conspecifics, even nonaggressive conspecifics, indicated by avoidance and defensive postures (Siegfried et al., 1984, 1990; Lumley et al., 2000). Defeated mice also display avoidance of olfactory cues associated with the stressful event (Lumley et al., 2003). Similar to PTSD, in which neutral cues may elicit conditioned fear responses, defeated mice in this model demonstrated fear responses to neutral or harmless stimuli. Defeated mice also fail to display territorial scent marking (Lumley et al., 1999), which is highly correlated with aggressive behavior (Bronson, 1979).

Acute social defeat decreased levels of brain-derived neurotrophic factor (BDNF) mRNA in limbic regions, including the hippocampus and amygdala, 24 h after social defeat (Pizarro et al., 2005). Decreased BDNF has been implicated in depression and neurodegenerative diseases (reviewed in Russo-Neustadt, 2003). Decreased levels of BDNF following stress could lead to loss of synaptic function and increase vulnerability to insult and possibly contribute to psychopathology. In a mouse model of repeated prolonged social stress, impaired spatial memory (Lumley et al., 2001) and increased dendritic spine density in the amygdala were observed (Meyerhoff et al., 2005).

Although acute social defeat has been resistant to most pharmaceutical interventions evaluated, administration of a CRH antagonist immediately following defeat reduced defensive responses 24 h later, possibly through inhibition of memory consolidation (Robison et al., 2005). Further, CRH antagonism in the basolateral amygdala (BLA) reduced this fear consolidation (Robison et al., 2004). Administration of a low dose benzodiazepine, diazepam, exacerbated flight responses in this social defeat model (Lumley et al., 2000). Clinically, diazepam, one of the current treatments for nerve agent poisoning, has had negative effects against PTSD (reviewed in Friedman, 2006).

Another animal model that has been used to model symptoms of PTSD is a fear conditioning model in which a neutral stimulus (conditioned stimulus) is paired with a fear evoking stimulus, such as shock stress (unconditioned stimulus). This model evokes fear response, glucocorticoid release, and activation of the autonomic nervous system. Tronel and Alberini (2007) reported that injection of a glucocorticoid receptor (GR) antagonist into the basolateral amygdala disrupts conditioned fear and suggests that GR antagonists may be useful treatment of pathogenic memories.

The majority of individuals exposed to trauma are resilient and do not develop PTSD. As in humans, lower mammals also have individual differences and heterogeneity of stress responses. Recent animal models of PTSD attempt to take into account this heterogeneity. Cohen et al., 2006 review two measures—performance on the elevated plus maze and acoustic startle response, used to mimic symptoms of PTSD. In their model, they differentiate animals that display stress-induced ''extreme behavioral responses'' (EBR) on both of these tests from those that display ''minimal behavioral responses'' (MBR). Different types of stress paradigms caused different proportions of EBR and MBR, similar to the suggestion in clinical literature that more severe stress increases incidence of PTSD. In Cohen's study, although stress led to an EBR immediately after the stressor in 100% of the animals, only 25% of the animals continued to show EBR 30 days following stress.

The authors suggest this is analogous to the time course observed in ASD and the development of chronic disorders. Cohen et al. (2006) also evaluated Lewis rats, a rat strain with blunted HPA axis response and high baseline anxiety-like responses. Administration of corticosterone 1 h prior to predator stress dramatically reduced the number of rats showing EBR compared to control (50% versus 8%).

Other animal models not addressed here, but which have been used to model symptoms of PTSD, include predator stress models. In addition, models of early stress, such as effects of maternal separation on endocrine function, in particular the HPA axis (reviewed in Meaney, 2001), may have implications for the development of stress disorders. Other studies (Cohen et al., 2006) evaluated stress response in juvenile rats as a precursor to adult stress responses. These animal models may help understand the biology of stress, stress vulnerability, and stress resistance and may be useful to identify appropriate therapeutic targets to reduce stress pathology.

F. Treatment of PTSD

Psychotherapeutic and pharmacological treatments are used to treat symptoms of PTSD (reviewed in Davis et al., 2006; Friedman, 2006). Cognitive behavior therapy involving prolonged exposure to a traumatic memory is used to extinguish fear based memories and is the nonpharmacological treatment of choice (reviewed in Friedman, 2006). Cognitive therapy is used to challenge distorted belief systems and to reduce guilt and shame. Recently, virtual reality technology has been used to create scenarios of more realistic and intense exposures (reviewed in Roy et al., 2006). Using this technology, improvement was observed in Vietnam War veterans and World Trade Center survivors with PTSD.

The current pharmacological treatment of choice for PTSD is selective serotonin reuptake inhibitors (SSRIs), which are effective against all three symptom clusters of PTSD (intrusive memories, avoidance/numbing, hyperarousal; reviewed in Davis et al., 2006; Friedman, 2006). The Food and Drug Administration currently approves two SSRIs, sertraline and paroxetine, for short-term treatment of PTSD. Davis et al. (2006) reviewed studies of long-term treatment against PTSD and observed that SSRIs were successful at reducing relapse and reducing the symptoms of PTSD. Sertraline improved quality of life, fluoxetine reduced avoidance symptom cluster and combat-induced PTSD, and paroxetine improved verbal declarative memory and increased hippocampal volume (Davis et al., 2006; reviewed in Bremner, 2006). In general, long-term treatment further improved PTSD symptoms by 25%, depression by 40%, and quality of life by 30% over that provided by short-term treatment. Extended treatment also reduces rate of relapse by fourfold. Success has also been reported in clinical trials using tricyclic antidepressants, monoamine oxidase inhibitors, and other antidepressants. Antiadrenergic drugs, anticonvulsants, and atypical antipsychotics are also considered potential treatments against PTSD that do not respond to other treatments. Clinical trials with the atypical antipsychotics, risperidone, and clozapine, have met with some success. The anticonvulsant valproate evaluated in Vietnam veterans with PTSD improved PTSD symptoms, improving sleep quality, reducing avoidance, and decreasing hyperarousal. However, adverse CNS and GI complaints were reported in some subjects. A long-term clinical trial with nefazodone reduced PTSD symptoms, improved quality of sleep, and reduced hyperarousal. Benzodiazepines have had negative results against PTSD (Friedman, 2006), similar to what was observed in animal models (Lumley et al., 2000). The authors (Davis et al., 2006) point out the need for more long-term studies on pharmacotherapy of PTSD, as well as on studies that combine cognitive therapy with pharmacotherapy.

G. Potential Interaction between Stress and CWAs

An area of research that has not been well studied is the potential interaction between psychological stress and CWA exposure on the development of psychopathology. The cholinergic system, severely affected by nerve agents, is also affected by physical stress (reviewed in Somani and Husain, 2001). Grauer and Kapon (2004) reported that rats exposed to 1.0 LD_{50} sarin had

reduced fear conditioning 3 weeks later, relative to control. In addition, rats exposed to 0.9 LD_{50} sarin and then to prolonged intermittent stress (3–6 weeks) had less body weight gain when assessed 5 months later, relative to stress alone, sarin alone, or control. Henderson et al. (2002) observed that exposure to subtoxic levels of sarin in rats had greater effects on the cholinergic system (M1 and M3 receptors; AChE levels) in limbic and cortical regions when sarin was administered under heat stress relative to no heat stress. These effects were observed 30 days after sarin exposure. Kalra et al. (2002) observed that repeated, low levels of subtoxic doses of sarin administered by inhalation (1/day $\times$ 5 days), reduced plasma levels of the stress hormone corticosterone in rats 24 h after the last exposure. However, Kassa et al. (2004) observed increased corticosterone in rats 3 months after exposure. Romano and Shih (1983) demonstrated a relationship between analgesia, acetylcholine levels in several brain regions, physostigmine, and stress. More research is needed to better understand the potential interaction between stress and nerve agents, and to better identify effects that may be a result of CWA exposure relative to those that may be a result of stress exposure.

VI. SUMMARY AND CONCLUSIONS

Historical review of American forces' activity in WWI showed that initial experiences with chemical attacks produced large numbers of patients who thought they had been exposed to chemical agents, for example, the experience of the 3rd Division at Aisne-Marne. As training and wartime experiences accumulated, however, this proportion sharply decreased. A similar trend was seen in Israel during the Gulf War. Historically, experience suggests that training and education of the populace, as well as an effective warning system, should minimize the numbers of panicked ''worried well'' in a future incident. Defense against CWAs and TICs continues to be a focus of research, development, and acquisitions programs (Department of Defense, Annual Report to Congress, 2006; U.S. Army Center for Health Promotion and Preventive Medicine, 2007).

Both the threat of use and the actual use of CWAs will have important psychological and physiological consequences. The threat of use of CWAs can produce significant stress effects in affected populations. We conclude that first and foremost, chemical terrorism is an act of terrorism, a potential catastrophe evoking psychological responses independent of the physiological/toxicological characteristics of the agent employed and with ASD and PTSD as prominent outcomes. If CWAs are actually used, the psychological impacts of both CNS and peripheral nervous system effects of the CWAs and medical countermeasures must be considered. In the case of moderate-to-severe exposure to nerve agents, chronic neurological sequelae can be expected in some cases, as can long-term PTSD-like effects. A variety of toxic industrial chemicals and materials also represent a ''chemical'' threat and must be considered in planning. Recent experiences with terrorist use of CWAs in Tokyo, as well as modeling efforts (Kales and Christiani, 2004) or reviews of wartime experiences (Newmark, 2004), suggest that psychological CWA casualties will outnumber physical CWA casualties by ratios of approximately four-to-one. Threatened or actual use of CWAs will require substantial mental health support to health care personnel and to the populations they service, and will have major impacts on the health care system. Finally, to mitigate the early as well as the delayed psychological effects once an exposure has occurred, we should provide patients with therapeutic courses of action that enable them to maintain a positive sense of control over their health.

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23 Emergency Response to a Chemical Warfare Agent Incident: Domestic Preparedness, First Response, and Public Health Considerations

David H. Moore and Barbara B. Saunders-Price

CONTENTS

I. INTRODUCTION

The bombings of the World Trade Center in New York in 1993 and the Alfred P. Murrah Federal Building in Oklahoma City in 1995, and the attacks on the World Trade Center in New York and the Pentagon in 2001 have brought the reality of terrorism into Americans' lives with startling clarity. Over the past 10 years there has been a concerted effort on the part of the international community to limit the quantity and proliferation of chemical warfare (CW) agents; however, the proliferation of CW agents within unstable sectors of the world is a cause for grave concern because of the potential for use of such agents by terrorists. The vivid images of Japanese commuters poisoned by sarin gas in the Tokyo subway, the pictures of Kurdish women and children killed by poison gas employed by the Iraqi regime of Saddam Hussein, and the public reactions to missile attacks by Iraq against targets in Saudi Arabia and Israel during the first Gulf War demonstrate the terror of these weapons. Access to chemical-manufacturing facilities and suppliers around the globe provides terrorists the availability of precursors and chemical reagents, while faltering economic conditions can pave the way for theft or sale of the chemical agents themselves. A particularly troubling phenomenon is the availability of accurate information about the chemical properties, uses, and effects of CW agents on the Internet. This medium makes highly sensitive information available to virtually every person on earth.

The Aum Shinrikyo cult in Japan successfully manufactured the CW agent sarin and successfully deployed the agent in Matsumoto City and Tokyo, Japan. In the case of Aum Shinrikyo, the cult operated a multistory production facility with the capacity to produce up to 70 tons of sarin in a five step process. According to Kota Kinoshita, a reporter who covered the Aum Shinrikyo and the sarin incidents, his investigation of the cult led him to believe that the technology and the methods for production and purification of the agent were transferred to Japan from a sympathetic scientist from Russia. The cult had enormous amounts of money and had also used the nerve agent VX to assassinate a person that the cult believed was a spy for the Japanese Police (Proceedings of the 10th Anniversary Conference on the Tokyo Subway Incident, 2005).

Chemical weapons were used by Saddam Hussein's Iraqi government both on the battlefield in the Iran–Iraq war of the 1980s and by Saddam Hussein's troops as a terrorist weapon in Kurdish villages in the northern part of Iraq. The attack on and massacre in Halabja, Iraq, on March 16, 1988, did not at the time create concern in most countries or most people, but increased awareness of the potential consequences of chemical attacks has made the Halabja massacre and its photographs a symbol of chemical weapons' use against civilians around the world.

More recently, there have been reports that al-Qaeda operations included classes in CW at an al-Qaeda training camp in the Afghan city of Herat in 2000/2001. Professionals assessing the Islamic terrorists have noted training documents and uncovered caches of laboratory equipment and materials for the use of ricin, cyanide, and assorted toxic industrial chemicals (TICs) (Pita, 2005). Chemicals and equipment have been found in Afghanistan, Jordan, London, and Chechnya, and possible targets have been the New York City subway system, the London underground, banks, schools, churches in Muslim lands, synagogues, casinos, etc. (Salama, 2006). In 2002, Italian police arrested a group of Moroccans who were planning to use cyanide against the U.S. Embassy in Rome (Croddy et al., 2002).

The increased worldwide awareness of the need to control the production, storage, and use of CW agents has led to the formulation and signing of a treaty, outlined in the Chemical Weapons Convention (CWC), calling for the prohibition and elimination of CW agent production. The technical arm of the CWC is the Organization for the Prohibition of Chemical Weapons (OPCW), which provides inspections, monitoring, and assistance with disposal of chemical weapons. According to the OPCW, States Parties that have declared chemical weapons production facilities include Bosnia and Herzegovina, China, France, India, the Islamic Republic of Iran, Japan, the Libyan Arab Jamahiriya, the Russian Federation, Serbia, the United Kingdom of Great Britain and Northern Ireland, the United States of America, and another State Party. Of the 65 declared facilities, 55 have

been certified as destroyed or converted for peaceful purposes (Organisation for the Prohibition of Chemical Weapons, n.d.). Destruction of chemical weapons and CW agents has begun in several countries, including the United States, Russia, and abandoned Japanese chemical weapons in China.

The total world declared stockpile of chemical weapons was about 59,000 tons in early 2006. ''As of March 31, 2006 the U.S. has destroyed 10,103 metric tons of chemical agents since entry-into-force of the CWC, or 36.4% of its declared inventory of 27,768 metric tons, far more than all other declared CW possessors combined'' (U.S. Department of State, Fact Sheet, 2006). The U.S. chemical demilitarization program is projected to be completed in 2012, but it may take as long as 2020. Japan is obligated to destroy all abandoned CW agents in China by 2007, but has asked for an extension to 2012. (Guangdong News, 2006).

Although such prohibition is a noble endeavor, implementation of aggressive projects to destroy these enormous stockpiles of CW agents poses significant public health problems for civilian populations living near storage sites, destruction facilities, or transportation routes. Additionally, the manufacture of large quantities of TICs that is prevalent in most industrialized countries can present an accident threat to civilian communities surrounding such an area. Facilities of this nature are also not immune to sabotage or terrorist attack. The targeting of industrial complexes producing or utilizing TICs as a means of waging war was the topic of an international conference (Proceedings of the Chemical and Biological Medical Treatment Symposium, 1998).

Over the past 5 years, subsequent to the publication of the last edition of this text, enormous emphasis has been placed on domestic preparedness for possible use of weapons of mass destruction (WMD). CW agents, along with nuclear weapons and biological warfare agents, are included in this category. The reader is referred to the previous editions, where much of the information on medical and public health considerations of CW agents remains accurate (Somani, 1992). Moreover, recognition of the possible terrorist use of toxic industrial chemicals and materials (TIMs) has presented additional challenges. This chapter is designed to expand on the previous work and to put this information into a more current context.

II. CURRENT CLIMATE

A. National Response Plan

Since the last edition of this text, and in direct response to the events of September 11, 2001, agencies of the Federal Government were reorganized to better respond to the multiple threats to the homeland. The Department of Homeland Security (DHS) was established as a cabinet-level department made up of agencies from various other departments (DHS Web site: www.DHS.gov). Of particular importance to this discussion, the DHS assumed responsibility for the Federal Emergency Management Agency (FEMA), the U.S. Coast Guard, and the Emergency Medical Response System. Other Federal Government agencies not incorporated into the DHS were instructed to more closely coordinate national emergency response efforts with DHS, including the Department of Health and Human Services (DHHS) and the Department of Defense (DOD).

The National Response Plan (NRP), updated after the Federal Government's much criticized response to hurricane Katrina, establishes a comprehensive, all-hazards approach to enhance the ability of the United States to manage domestic incidents (DHS/National response plan-Web site). The NRP is designed to help prevent terrorist attacks within the United States, including terrorist use of chemical agents, and to minimize the damage and assist in the recovery from such incidents. The plan incorporates practices and procedures for security, emergency management, law enforcement, firefighting, public works, public health, responder and recovery worker health and safety, emergency medical services (EMS), and attempts to integrate them under a unified structure. The NRP describes of how the Federal Government coordinates with state and local governments and the private sector during an emergency. The plan organizes the federal response under 12 emergency

support functions (ESFs). Each ESF has a designated agency assigned primary responsibility, with other agencies given responsibility in support roles.

The DHHS directs and manages the federal response under ESF #8, Health and Medical Services. Response actions under this function are grouped into four general categories: prevention, medical services, mental health services, and environmental health. ESF #8 provides supplemental assistance to state and local governments in meeting the public health and medical needs of victims of an incident of national significance (DHS Web site). This support is categorized in the following core functional areas: assessment of public health/medical needs, public health surveillance, medical care personnel, and medical equipment and supplies. The Centers for Disease Control and Prevention serve as the lead in this mission to assess the health and medical effects of exposure, conduct field investigations, collect samples, provide advice on protection from the hazard, and lend technical assistance for treatment and decontamination of victims. Planning and coordination of DHHS emergency response capabilities are managed through the Office of Public Health Emergency Preparedness (OPHEP) (OPHEP Web site).

The National Disaster Medical System (NDMS) is a section within the United States. DHS is responsible for supporting federal agencies in the management and coordination of the federal, medical response to major emergencies, such as acts of terrorism, including the potential use of chemical agents. Special teams available under NDMS include Disaster Medical Assistance Teams, Veterinarian Medical Assistance Teams, Disaster Mortuary Operational Response Teams, National Nurse Response Teams, and National Pharmacy Response Teams (U.S. Department of Health and Human Services Web site).

The Metropolitan Medical Response System (MMRS) Program is currently managed by the DHS. The primary focus of the MMRS program is to develop or enhance existing emergency preparedness systems to effectively respond to a public health crisis, especially WMD events. Through preparation and coordination, local law enforcement, fire, Hazardous Materials (HAZMAT) teams, EMS, hospital, public health, and other first response personnel plan to more effectively respond in the first 48 h of a public health crisis (The Metropolitan Medical Response System, n.d.). The system has grown from one jurisdiction in Washington, D.C. in 1996 to more than 125 jurisdictions throughout the United States and consists of parts of existing local systems that can be called in to provide triage, treatment, and patient decontamination. This system transports patients who have been decontaminated at the scene to other facilities as appropriate for continued care. The system also assists medical facilities in developing procedures that ensure patients are decontaminated before they enter a facility. As part of this system, Metropolitan Medical Strike Teams ensure the continued viability of a jurisdiction's existing health system, given the added burden of a WMD incident (City of Seattle Fire Department, n.d.).

Other special teams from within the Federal Government can rapidly respond to assess an incident and help locate and examine an unknown WMD device. The DOD established the Chemical and Biological Incident Response Force (CBIRF) to respond rapidly in the event of a chemical or biological incident (CBIRF Web site). This dedicated force, under the control of the U.S. Marine Corps, is equipped with the latest current detection equipment and trained for mass casualty decontamination and consequence management. In 1998, it was announced that the nation would do more to protect its citizens against the growing threat of chemical and biological terrorism and that the DOD would form teams to support state and local authorities in the event of an incident involving WMD. As of 2006, over 30 WMD Civil Support Teams (WMD-CST) have been certified and another 70 have been formed throughout the United States (WMD-CST Web site). Their mission is to support local and state authorities at domestic WMD incident sites by identifying agents and substances, assessing current and projected consequences, advising on response measures, and assisting with requests for additional military support. These National Guard teams assist state governors in preparing for and responding to chemical, biological, radiological, or nuclear incidents as part of a state's emergency response structure. The WMD-CSTs are able to deploy rapidly, assist local first-responders in determining the nature of an attack, provide medical and

technical advice, and pave the way for the identification and arrival of follow-on state and federal military response assets. Each team consists of 22 skilled, full-time National Guard members. In addition to personal protective equipment and reconnaissance, detection and sampling kits, each team has computer modeling and response database systems. Of particular note is each team's two large pieces of equipment—a mobile analytical laboratory used for field analysis of chemical or biological agents and a uniform command suite that has the ability to provide interoperability of communications to the various on-scene responders. The capabilities and procedures for the WMD-CST are found in FM 3-11.22 (Official Department of the Army Publications and Forms Web site).

B. Strategic National Stockpile

The Strategic National Stockpile (SNS) was established by the Homeland Security Act of 2002 and is managed jointly by the DHS and the DHHS (Centers for Disease Control and Prevention, n.d.). The SNS Program works with governmental and nongovernmental partners to upgrade the Nation's public health capacity to respond to a national emergency and give rapid access to large quantities of pharmaceuticals and medical supplies that few state or local governments would have the resources to create and manage.

The SNS is a national repository of antibiotics, chemical antidotes, antitoxins, life-support medications, IV administration, airway maintenance supplies, and medical or surgical items. The SNS is designed to supplement and resupply state and local public health agencies in the event of a national emergency anywhere and at anytime within the United States or its territories (Centers for Disease Control and Prevention, n.d.).

The SNS is organized for flexible response. The first line of support lies within the immediate response, ''12 hour Push Packages''. These are caches of pharmaceuticals, antidotes, and medical supplies designed to provide rapid delivery of a broad spectrum of assets for an ill-defined threat in the early hours of an event. These Push Packages are positioned in strategically located, secure warehouses ready for immediate deployment to a designated site within 12 h of the federal decision to deploy SNS assets.

If the incident requires additional pharmaceuticals and medical supplies, follow-on vendor managed inventory (VMI) supplies will be shipped to arrive within 24–36 h. If the agent is well defined, VMI can be tailored to provide pharmaceuticals, supplies, and products specific to the suspected or confirmed agents. In this case, the VMI could act as the first option for immediate response from the SNS Program.

III. CHEMICAL WARFARE AGENTS

Medical professionals and emergency response personnel seldom see mass casualties that resemble CW agent casualties. With the increased threat of terrorism worldwide, a focus on the management of CW agent casualties is timely and appropriate. The goal of any WMD response plan should be to train teams of professionals who understand chemical agent threats and how to respond to them efficiently. A more complete description of the agents, their effects, and the medical management of casualties is presented in other texts and is only summarized in this section for general information.

CW agents are either lethal in their effects or incapacitating, depending on the class of agent, the concentration, and the period of exposure. The lethal agents, nerve, blood, and pulmonary or choking agents, and the incapacitating vesicant agents will be covered in the following sections. Excluded from this discussion are other incapacitants and riot-control agents.

CW agents are also classified as persistent and nonpersistent. The former includes the vesicants, such as sulfur mustard (HD) and Lewisite (L) and the nerve agent VX. Nonpersistent agents are more volatile and do not remain in an open environment for more than a few hours. Among these are phosgene, cyanide, and the nerve agents, tabun (GA), sarin (GB), soman (GD), and cyclosarin (GF).

TABLE 23.1
Comparison of Potencies of Chemical Warfare Agents

CW Agent	ECt_{50}	LCt_{50}
Nerve agents	3–5	10–200
Mustard	50–100	1500
Cyanide	>1000	2500–5000
Phosgene	>1000	3000

Note: *Ct* is concentration of vapor (mg/m^3) × time (minutes of exposure). ECt_{50} is the *Ct* producing clinical symptoms in 50% of the exposed population. LCt_{50} is the *Ct* that is lethal for 50% of the exposed population.

Toxicity follows exposure to chemical agents dispersed as solids, liquids, aerosols, or vapor (Table 23.1). CW agents have characteristics that make them uniquely suited to warfare. In addition to their extreme toxicity, their chemical structures are simple, and the manufacturing processes for most are relatively uncomplicated and inexpensive. Cyanide and phosgene are manufactured in large quantities for use in industry and are shipped in bulk by truck or train.

A. Means of Intoxication

Most CW agents were designed to be volatile and nonpersistent and are encountered as vapor or gas. These agents can also be dispersed as an aerosol following a detonation. The persistence of the agent is dependent on factors such as temperature, pressure, and wind speed. Thus, for some of the nerve agents, such as GA, GB, and GD, as well as phosgene and chlorine, the primary route of intoxication is through the respiratory tract. The nerve agents VX and thickened GD and the vesicant agent, sulfur mustard, are three of the most persistent CW agents and pose a threat from dermal absorption as liquids or droplets. These agents can pose vapor hazards as well.

Contamination of foodstuffs by chemical agents may occur from contact with vapor, aerosol, or droplets. The effects of the chemical agents on food depend on the nature of the agent, as well as the nature of the food. For example, foods having a low water content and a high fat content, such as butter, oils, fatty meats, and fish, absorb vesicant and nerve agents so readily that removal of the agents is virtually impossible. Chemical agents can cause the food to become highly toxic without changing the appearance of the food. Unprotected foodstuffs may be so contaminated that their consumption results in gastrointestinal irritation or systemic poisoning. Protected foodstuffs in cans and bottles or food wrapped in heavy plastic are not affected by agent vapor and can be salvaged (Moore, 1989).

Few environmental factors impact community and individual well-being more than the ready availability of adequate and safe, potable water. Surface water sources in the area of a chemical release could become contaminated. The contamination of water, whether intentional or inadvertent, may reach concentrations that could produce casualties. Deep groundwater reservoirs and protected water storage tanks are regarded as safe sources of drinking water following a vapor release of chemical agents. Although avoiding any possibly contaminated water source should be a goal, methods such as reverse osmosis are available to treat large volumes of potentially contaminated water for emergency drinking. However, these techniques may not eliminate low-dose exposure to the contaminating agent and are not readily available or are too expensive for most civilian populations. The fate and distribution of CW agents in the environment has recently been reviewed (Munro et al., 1999).

B. Chemical Agent Effects

1. Nerve Agents

Nerve agents exert their effects by inhibition of the enzyme acetylcholinesterase (AChE), leading to accumulation of excess levels of the neurotransmitter acetylcholine (ACh) at cholinergic synapses. Enzyme inhibition is both rapid and irreversible, thus making organophosphorus (OP) nerve agents highly toxic and extremely dangerous chemicals (Table 23.1). These agents were designed to kill or incapacitate enemy forces, disrupt military operations, and deny terrain to the adversary. However, they have also been effective weapons of terror.

Nerve agents gain entry by absorption through the lungs or skin and impair the activity of cholinergic synapses, including those of smooth and skeletal muscle, autonomic ganglia, and the central nervous system (CNS). Acute toxic effects of nerve agents can be elicited at very low concentrations, whereas lethal effects are observed at somewhat higher concentrations. Threshold symptoms for vapor exposure are commonly stated to be miosis, rhinorrhea, and airway constriction, generally appearing at a *Ct* (vapor concentration $\times$ exposure time) of 2–3 $mg/min/m^3$ (Sidell, 1992). Low to moderate exposure of skin to liquid nerve agent causes localized sweating, nausea, vomiting, and a feeling of weakness (Medical Management of Chemical Casualties Handbook, 1999). Lethal amounts of vapor or liquid cause a rapid cascade of events culminating within a minute or two in convulsion, loss of consciousness, apnea, paralysis, and death (Medical Management of Chemical Casualties Handbook, 1999). Toxicity is thus concentration dependent, requiring a defined minimal concentration of agent; recovery generally occurs by synthesis of new AChE.

Additionally, after both vapor and liquid agent exposure, there are CNS effects that vary in intensity and duration. After mild to moderate exposure to nerve agent, there may be forgetfulness, inability to concentrate, insomnia, impaired judgment, nightmares, irritability, and depression. These effects may be present for 4–6 weeks or longer. They may also occur on recovery from acute, severe effects of exposure. Long-term and low-dose effects of the nerve agents are the topic of Section VI of this chapter.

2. Blister Agents

Sulfur mustard (H, HD) has been a major military threat agent since World War I. Lewisite (L) also falls into this class but the effects of this agent are not discussed here. Sulfur mustard constitutes both a vapor and a liquid threat to all exposed skin and mucous membranes. The effects are delayed, appearing 2–24 h after exposure (Sidell and Hurst, 1992). Mustard reacts with tissues within minutes of absorption. In extracellular water, it rapidly forms a highly reactive cyclic compound that binds to enzymes, proteins, and other substances. Mustard is a strong alkylating agent that causes cross-linking in DNA strands leading to cell death (Smith and Dunn, 1991; Sidell et al., 1997). Blood, tissue, and blister fluid do not contain mustard and cannot cause further toxicity. Typical effects occur in the eye, ranging from mild to severe conjunctivitis, blepharitis, and damage to the cornea. Airways react with initial irritation, progressing to severe damage of lower airways with higher concentrations; respiratory failure and pneumonia, in addition to bone marrow suppression, may lead to death in sulfur mustard poisoning. Skin injury initially shows erythema, followed by formation of vesicles that later coalesce to form bullae (Sidell et al., 1997).

3. Blood Agents

CW blood agents are hydrogen cyanide (hydrocyanic acid, military designation AC) and cyanogen chloride (CK). Cyanide is a rapidly acting, lethal agent causing death in 6–8 min after inhalation of a high concentration. However, few toxic effects are seen below a lethal concentration. Once absorbed, the cyanide ion rapidly combines with the active site of the enzyme cytochrome oxidase interfering with aerobic metabolism, creating excess lactic acid and metabolic acidosis (Baskin and

Brewer, 1997). Cell death is the final outcome. The organs most susceptible to cyanide are the CNS and the heart. The onset and progression of signs and symptoms are slower after ingestion of cyanide or after inhalation of a low concentration of vapor. There may be an asymptomatic period of several minutes followed by the initial transient hyperpnea. This may be followed by feelings of anxiety, agitation, vertigo, weakness, nausea, vomiting, and trembling. Later, there is a loss of consciousness, decrease in respiration, convulsions, apnea, and cardiac arrest.

4. Choking Agents

Phosgene is a simple, highly volatile molecule ($COCl_2$) known as carbonyl chloride (CG). The odor of phosgene has been described as an odor of newly mown hay. On inhalation, CG chemically induces acute lung injury because of a reaction of its carbonyl group with groups affecting cell membrane stability (Urbanetti, 1997). This allows plasma to leak into the alveoli and produces pulmonary edema. There is a symptom-free period (10 min to 24 h) that varies with the amount of CG inhaled. Substantial toxicity can occur from levels of 1.5 to 2 ppm. Its aroma is detectable at 2–3 ppm, hence toxicity may occur without subject awareness (Medical Management of Chemical Casualties Handbook, 1999). In cases where individuals are exposed to high concentrations of phosgene, there may be initial symptoms of mucous membrane irritation followed by pulmonary edema, hypoxia, hypotension, bronchospasm, right heart failure, and death.

5. Toxic Industrial Chemicals

Industrial chemicals, including pesticides, pharmaceutical agents, and polymer and plastic components, can be toxic, but not necessarily as toxic as CW agents. There are thousands of TICs that are synthesized, manufactured, used, stored, and shipped routinely without incident. However, there is the potential for the use of these TICs by terrorists or criminals. The saying that ''the dose makes the poison'' means that the amount of the chemical a person is exposed to is as important as the toxicity of that chemical. So, although most TICs are less toxic that CW agents, their availability in large quantities can potentially have as dramatic an effect as CW agents. And as we noted in the introduction, there is a history of use of TICs to threaten and poison both civilian and military populations.

There are many programs that are addressing the possible use of TICs in terrorist and even military attacks. An international task force has examined a variety of high production volume (HPV) industrial chemicals and other classes of industrial chemicals for their possible use to cause intentional harm (Bennet, 2003; Evans, n.d.; Resta, n.d.). In addition to HPV, lists of TICs are reported in a variety of programs, for example, the U.S. Environmental Protection Agency's (EPA) Toxic Release Inventory, the Risk Management Program, the Stockholm list of persistent organic pollutants, the World Health Organization's International Program on Chemical Safety, and the Organization for Economic Co-operation and Development's Screening Information Data Sets (Chemical Safety Information from Intergovernmental Organizations, n.d.). TICs that are typically high on any list evaluating possible use include cyanides and pesticides (OP and carbamate).

C. Clues to the Presence of Chemical Warfare Agents

Should CW agents be employed in a civilian situation, for many of the agents, real-time detection and monitoring is not currently widely available. With the exception of a public announcement by a terrorist group of the employment of a toxic chemical agent, or a public announcement of an accidental release, signs and symptoms of persons exposed to the toxicant will most likely be the first indication of the presence of the agent. For this reason, it is essential that first-responders and medical personnel be familiar with the clinical aspects of CW agent intoxication. This is not only critical for treating the casualties, but is equally important to protect the responders themselves and to limit the spread of contamination. In a previous section, we have examined the clinical signs of

CW agent intoxication, and how CW agents could be dispersed in the environment. Due to their chemical characteristics, CW agent use in a domestic terrorist incident may not be associated with a high explosive event, and these agents are likely to be dispersed in such a manner that would involve primarily a vapor hazard. In the immediate vicinity of the incident, where there may be a continuing source of agent vapor, the probability of detecting the CW agent is greatest. However, due to the chemical properties (persistent versus nonpersistent) of many of the agents, detection may not be possible at the time emergency medical personnel arrive at the venue of an incident. Once casualties of a CW agent incident are removed from the area of the attack and become accessible to medical personnel, the signs and symptoms of the patients may be the only detection method available to guide incident commanders and law enforcement personnel.

Effective and efficient incident response depends on the rapid and accurate identification of the chemical agents involved. The protection of first-responders and emergency medical personnel at local medical facilities, as well as the effective treatment of casualties, hinges on this critical capability. Various devices capable of detecting chemical agents in the environment are available to civilian communities. Many devices were designed for military applications but have now been adapted for civilian use. Recently, local response units in the United States have improved their capability for CW agent detection. A complete discussion of chemical detection technologies and devices can be found in Chapter 19 of this book.

An emergency response to an incident that involves the accidental or intentional release of toxic chemicals or materials will typically be categorized as a HAZMAT incident. With the greater emphasis placed on this type of response, HAZMAT incident response plans have become increasingly standardized across the country. Specialized HAZMAT teams are routinely activated to respond in such situations. HAZMAT teams are typically part of the fire services and will possess chemical detection equipment. The first-responders, typically the police or fire department, must be capable of determining that a HAZMAT incident has occurred. Unfortunately, most emergency response vehicles do not have any chemical detection equipment, and the first-responder must make a quick judgment call whether or not to call in HAZMAT units.

The equipment needs of early responders to a domestic incident in which CW agents may be involved are significantly different than those for military personnel. The military has the advantage of intelligence information that enables the users of the equipment to predict a probable threat agent and the likely area of impact from the chemical agent. In the case of first-responders to a domestic terrorist incident, there are currently no such benefits of intelligence. The medical personnel on site will require equipment capable of detecting the widest range of chemical agents. HAZMAT teams are routinely equipped with chemical detection devices and detection kits, but these are usually chemical-specific tests indicating only presence or absence of a single suspected TIC or class of chemicals. Chemical detection equipment currently used by HAZMAT teams varies considerably by locality with many large metropolitan areas having significant technology available.

IV. PROGRAMS TO PROTECT CIVILIAN POPULATIONS

A. Civilian versus Military Response Considerations

Before plans for the destruction of the CW stockpiles of most countries, and preceding the sarin attacks in Matsumoto and Tokyo, the development and implementation of defensive measures against CW agents was primarily centered on the military use of these chemicals. However, military and civil defense planners face very different situations when planning for a potential chemical threat, mainly with respect to prior knowledge about the identity of the enemy and the time, place, and means of attack. The value of deployment of a chemical detection system and the use of highly specific antidotes, therapeutics, or pretreatment drugs diminishes considerably in the most probable civilian terrorism situation in which the enemy, the agent, the time, and the place of attack are unknown. For civil defense purposes, it is therefore more appropriate to emphasize prior planning,

medical treatment, and consequence management over prevention. The responsibility for prevention in this case is left to intelligence and law enforcement agencies. However, it would be advisable to include the medical community in the distribution of preincident intelligence in order to maximize the medical response in dealing with chemical incidents. One significant difference between military and civilian response planning for a CW incident is that the populations to be protected are fundamentally different. In military chemical defense planning, the population of interest is primarily healthy young males between the ages of 18 and 26 years, whereas the civilian community at risk includes this prior population, as a minority, as well as both genders, the very young, the very old, and the sick. Furthermore, acceptable levels of exposure for military personnel operating in a CW environment differ significantly from those of first-responders and medical personnel who might find themselves involved in a civilian emergency situation. In the first case, military standards prevail, whereas NIOSH exposure criteria should be considered when CW responses are planned or when appropriate protective equipment is designed and utilized for civilian emergencies.

B. Other Countries

In many countries, the military and civilian populations are closely coordinated and in times of crisis, as well as in national and local emergencies, the military assists the civilian police, firefighters, and other emergency workers. In many countries, the governments are preparing for possible chemical attacks that include civilians. As an example, in Croatia during the war in the 1990s, the fighting resulted in attacks on Croatian industries with the intent to release TICs that would cause casualties in the civilian population. Croatian military trained and assisted local police forces, firefighters, and emergency workers in setting up evacuation procedures for civilians, and the military assisted with medical treatments, using field hospitals and other military equipment.

1. Israeli Model

Although the Japanese have actually experienced the consequences of a CW agent incident, there is no population more aware of the present day threat of CW than that of the nation of Israel. Early in this country's history, and well ahead of the invasion of Kuwait by Iraq in 1990, Israel had implemented an aggressive program of domestic preparedness. Their program of ''Homeland Defense'' includes extensive preparations for the widespread use of CW agents against the population. Doctrine was established for the protection of the civilian population by the Ministry of Defense and is based on the concept of protected space. Protected space is a readily accessible space capable of providing occupants with protection against both conventional and nonconventional weapons for several hours. Since 1992, all new buildings, as well as additions to existing buildings, have been required to be equipped with a protective space that meets specific engineering specifications. Expedient measures to provide a protective space within a home or office are taught in civil defense training classes. Examples of such measures include the use of wet towels at the bottom of a doorway or the use of tape around door and window openings. Furthermore, every Israeli citizen is issued a protective mask and atropine injectors to be used in the event of a chemical attack. As mentioned earlier, a civilian population is not homogeneous, and the Israelis have adopted measures to meet the needs of most of the citizenry. The government has provided standard protective masks of various sizes, including a protective mask with a blower unit for those who cannot use the standard mask, a protective hood-kit designed for children ages 3–8 years old, an infant protective suit with a blower designed for infants 0–3 years old, and a medical hood-kit designed for people with impaired respiratory systems. Distribution of protective kits is administered by the Israeli Defense Force's (IDF) Home Front Command through a system of exchange centers located throughout the country (Israeli Homefront Command Web site). Hospitals and medical centers prepare for the use of chemical agents by stockpiling needed medications and by routinely conducting realistic mass chemical casualty exercises. Their experience during the Gulf War demonstrated the need for mass decontamination facilities and efficient triage. Following

missile attacks on Israel during the Gulf war, there were large numbers of panicked worried well. The presence of large numbers of patients who think they have been exposed to a chemical agent or who are presenting with psychosomatic symptoms could severely limit a medical facility's ability to respond rapidly and effectively to the needs of critical casualties. Since 1991, the IDF has made improvements in the quality and availability of protective masks and supplies. Distribution centers remain open throughout the year, and on the days following September 11, 2001, over 40,000 masks per day were issued to concerned citizens (Israelis in Preparedness Frenzy, 2001). Additionally, Israel has invested in significant improvements in its medical system to respond to a chemical attack to include support for training for CW and trauma, purchasing additional respirators and monitors, ensuring hospitals have adequate communication equipment, providing chemical protection for neonatal intensive care units, and protective equipment for hospital medical staff. Medical preparedness and training have also been improved recently with major Israeli medical centers developing a core curriculum in medical aspects of nuclear, biological, and chemical warfare (Rubinshtein et al., 2002).

C. Chemical Stockpile Emergency Preparedness Program

In the United States, the Chemical Stockpile Emergency Preparedness Program, or CSEPP, is a partnership among state, local, and federal governments. It was created as a result of a directive from Congress that chemical weapons stockpiled at eight Army installations in the United States be destroyed over the course of several years. A total of 39 counties in 10 states participate in CSEPP. The slight but real threat of emergency involving chemical agents at these sites necessitates that local officials and responders remain ready for such an emergency and involve the community in their efforts. The U.S. Army is custodian of the stockpiles, whereas the FEMA is the source of long-standing experience in planning for contingencies in civilian areas. Partnering with the U.S. EPA and the U.S. DHHS, the combined effort of CSEPP allows for the funding, guidance, resources, and training needed to effectively provide protection to communities surrounding stockpile sites. Protective measures are determined for each community based on its unique needs and considerations. The plans and procedures are appropriate for the specific agents stored at the nearby Army installation. The most common emergency protective measures are evacuation and shelter-in-place and are based on two planning zones, the Immediate Response Zone and the Protective Action Zone (FEMA Web site). The distance from the stockpile for each zone varies and is based on risk analyses. In the case of a stockpile accident, the community is informed and instructed through radio and TV emergency broadcast alert systems and over loudspeakers. Sirens and tone alert radios serve to alert and warn residents in Immediate Response Zones. Evacuation routes are designated and fully equipped shelters are identified. This provides the right atmosphere for people to stay calm and follow the recommendations of local officials and emergency managers. Public information facilities in neighborhoods serve to disseminate information and function as response command centers for questions from the media and concerned citizens. The chemical agents of primary concern to CSEPP are the nerve agents GB and VX and the blister agents H, HT, and HD. The chemical agents are stored in three basic configurations: (1) projectiles, cartridges, mines, and rockets containing propellant and explosive components; (2) aircraft-delivered munitions that do not contain explosive components; and (3) one ton steel containers. Most of the stockpile (61%) is in the latter form (CSEPP Web site).

V. RESOURCES FOR FIRST-RESPONDERS

In 1999, the Institute of Medicine of the National Academies of Science examined the impact of a chemical terrorist attack on the ability of first-responders and medical personnel to accomplish their jobs efficiently and safely. Three priorities were identified: timely and accurate communication between responder groups, a system to identify and distribute available resources including

pharmaceuticals, and operating procedures for all responsible agencies. Research and development needs for each of these areas were identified and short- and long-term projects were suggested (Chemical and Biological Terrorism: Research and Development to Improve Civilian Chemical Response, 1999).

A. Programs to Improve the Response

1. Training

The United States has placed strong emphasis on effective training for civilian response to a chemical incident within the city response structure. Accordingly, a federal interagency team coordinates the training and exercise programs with multiple U.S. cities. Courses are offered in six subjects: Awareness, Operations, Technician-HAZMAT, Technician-Emergency Medical Services, Hospital Provider, and Incident Command. City officials determine which training best fits their needs, and teams consisting of CB experts from DOD as well as professional, civilian first-responders present the training. Those receiving the training later promulgate it on a sustaining basis in their own communities. The effectiveness of the training is assessed in subsequent tabletop and functional exercises that provide direct feedback to the city for future activities and programs the city may pursue to enhance the response to chemical terrorism.

The U.S. Army Medical Research Institute of Chemical Defense located at Aberdeen Proving Ground, Maryland, in partnership with the U.S. Army Medical Research Institute of Infectious Diseases, conducts the Medical Management of Chemical and Biological Casualties Course. This course is designed for physicians, nurses, and other medical professionals and is accredited for Continuing Medical Education units through the American Medical Association. The U.S. Government Accountability Office (GAO) has identified this course as the military Gold Standard for the nation (U.S. GAO Web site). Since 1980, more than 20,000 students have been trained in this course both at the laboratory and at remote locations around the world. This and other trainings have been made available to civilian medical personnel and first-responders. An additional course offering, the Field Management of Chemical and Biological Casualties Course, provides the only course extant in the United States that focuses on advanced training for prehospital medical providers and emergency responders to efficiently and effectively manage incidents involving CW agents and biological threat agents. Accreditation for EMT/NREMT students is obtained through the Continuing Education Coordinating Board for Emergency Medical Services. Both of these courses are approved by the DHS as eligible for grant assistance. The most recent course added to the curriculum is the Hospital Management of Chemical, Biological, Radiological/Nuclear and Explosive Incidents Course. This course is designed to equip military and civilian, hospital-based medical and management professionals with skills, knowledge, and information resources to carry out the full spectrum of health care facility responsibilities required by a chemical, biological, radiological/nuclear, explosive, or mass casualty event. This course offers continuing education credits for physicians, nurses, and EMT/paramedics. Since December 2004, more than 400 students have obtained training in this course. The institute has also developed a Web site featuring a variety of instructional products in the medical management of chemical casualties (U.S. Army Medical Research Institute of Chemical Defense, Chemical Casualty Care Division Web site). Materials from the training courses offered are available for download, including PowerPoint slides from course lectures. Also available on the site is the complete *Textbook of Military Medicine, Part 1, Medical Aspects of Chemical and Biological Warfare*.

2. National Response Center

Congressional legislation has specifically directed the establishment of a designated telephonic link to a designated source of relevant data and expert advice for the use of state or local officials responding to emergencies involving a weapon of mass destruction. As part of the center,

the Domestic Preparedness Chemical and Biological Hotline (1-800-424-8802) is operated 7 days a week, 24 h a day (National Response Center Web site). It provides emergency technical assistance from a variety of federal agencies or, if warranted, an actual federal response to assist first-responders during incidents. The hotline is intended for use by first-responders as well as state emergency operations centers and medical facilities. Assistance is provided on a wide array of subjects that include personal protective equipment, decontamination systems and methods, toxicology information, and medical symptoms and treatment for exposure to chemical and biological agents.

3. Chemical Weapons Improved Response Program

To address the issue of identifying response capability shortfalls, the DOD, in the late 1990s, established the Chemical Weapons Improved Response Program (CWIRP) (CWIRP Web site). The program has formed an alliance between federal, state, and local government offices as well as various industry organizations. Through an iterative process of workshops, conferences, exercises, and technical studies, the program has structured its work around four functional working groups: Health and Safety, Emergency Management, Emergency Response, and Law Enforcement. The responsibility of each group is to identify, prioritize, and arrive at solutions pertinent to first-responders.

This program, in collaboration with other federal agencies, has conducted research studies that are of importance to first-responders. A major focus of this program is to address issues that impact the well-being of the community at large and the challenges associated with the delivery of services by local and regional public health and medical assets. Concepts for the triage and management of CW agent casualties including the concept for an ancillary medical facility to handle the large number of walking wounded and worried well casualties expected from a terrorist attack were developed (Domestic Preparedness 1997–1998 Summary Report, 1999). Applied research is another focus of this program. Examples include studies on the protection afforded by standard firefighting turnout gear from CW agents, methods for mass casualty decontamination, and the effect of positive- or negative-pressure ventilation on vapor concentrations inside structures.

Firefighters respond to HAZMAT incidents wearing what is referred to as turnout gear and self-contained breathing apparatus (SCBA). The protection afforded by these ensembles was assessed in test chambers using a chemical simulant. The equipment was found to provide the wearer sufficient protection against nerve and blister agents to allow for the reconnaissance and rescue of victims. Furthermore, the protective efficacy of the gear can be increased simply by using common, heavy-duty duct tape (Guidelines for Incident Commander's Use of Firefighter Protective Equipment with Self-Contained Breathing Apparatus for Rescue Operations during a Terrorist Chemical Agent Incident, 1999). Guidelines established from these studies are meant to assist incident commanders in making decisions to enter chemical agent vapor environments to perform rescue, reconnaissance, mitigation, or detection operations, and to establish minimum criteria for entry for first-responders. In a parallel study addressing law enforcement and EMS issues, six commercial, level-C chemical protective suits and the standard police duty uniform were assessed to determine what protection they provided the wearer (Interim Summary Report for Law Enforcement and Emergency Services Protective Ensemble Testing, 1999). The commercial ensembles consisting of the respirator, gloves, and overgarment were felt to provide adequate protection to responders in areas of low concentration as might be found at the perimeter of the incident, but not for activities in areas where the threat is expected to be much greater.

The Department of Justice has published Emergency First Responder Equipment Guides that provide information on personal protection equipment (PPE) for consideration by emergency first-responders. They contain data sheets for respiratory protection, for protective garments, and for other protective apparel and include information on duration of protection, dexterity, and mobility (U.S. Department of Justice, n.d.).

Another tool firefighters bring to an incident is fans used to blow smoke out of the building to facilitate evacuation and rescue. These fans were assessed for use in reducing chemical vapor hazards (Use of Positive Pressure Ventilation Fans to Reduce the Hazards of Entering Chemically Contaminated Buildings Summary Report, 1999). Using simulants, it was determined that dramatic reductions in vapor concentrations can be attained with these fans by creating positive-pressure ventilation in structures; fans can reduce the vapor concentration 50%–70% within the first 10 min of use. This reduction significantly increases the first-responders' protection above and beyond the adequate protection provided by standard turnout gear with SCBA.

Following a chemical terrorist attack, it can be assumed that many of the civilians who are present are not contaminated, but during the crucial minutes immediately following the event, there is currently no way of determining with certainty those that are contaminated. HAZMAT teams that respond to accidents or spills of industrial chemicals are well prepared to decontaminate themselves but have limited capability to care for mass casualties. However, a terrorist attack could well involve hundreds, if not thousands of victims, so the scale of the response must be expanded. An underlying assumption has been that decontamination should occur as soon as possible after exposure, so a recent study considered methods using equipment firefighters already bring to every incident. Firefighters have access to large amounts of water, so three water-based decontamination methods were assessed: water alone, soap and water, and bleach and water (Guidelines for Mass Casualty Decontamination During a Terrorist Chemical Agent Incident, 2001). Water alone was found to be a very effective method via physical removal. The sheer forces and dilution achieved by using high-volume, low-pressure (60 $lb/in.^2$) showering was found to be the most practical method of mass casualty decontamination. Various shower applications were examined. Some included the use of tarps and establishing decontamination corridors for privacy concerns. The use of soap provides a slight improvement via ionic degradation of the chemical agent; however, a supply of it has to be on hand. This requires that firefighters either bring it on their trucks, creating a logistical burden, or otherwise procure it on scene, which would take time. The use of bleach (sodium hypochlorite) and water was not recommended. Although these solutions react with most chemical agents, the preparation and application of the solution would take time, a distinct disadvantage when speed is critical.

Triaging casualties at an incident site may exceed emergency responders' capabilities. There may be too many people to rescue, decontaminate, and treat, regardless of exposure to the chemical agent. Victims must be prioritized into ambulatory and nonambulatory groups and further grouped based on agent signs and symptoms or the likelihood of exposure. Emergency care providers will have to decide when, for example, to perform only hasty decontamination, if at all, on a severely injured casualty who is not clearly a chemical casualty. Conversely, casualties displaying symptoms of severe chemical exposure may require antidotes and other aid before decontamination is possible. Toxic exposure issues emergency personnel could encounter on the perimeter of an incident have recently been addressed (Interim Summary Report for Law Enforcement and Emergency Services Protective Ensemble Testing, 1999). The threat to responders who perform activities on the perimeter, be they police or EMS, should be minimal. As a routine matter, no significant vapor, aerosol, or liquid danger is expected. The most likely threat will come as a result of a wind shift or off-gassing from people exiting the incident scene.

B. Exposure Guidelines

Although the CW agents and TICs are very toxic, various federal agencies have developed guidelines for possible exposures to determine safe concentrations and times for various operations for each chemical. These are based on work and exposures civilians and military personnel may be required to experience in a toxic environment. There are different exposure times for various activities and therefore for various agencies. For example, the Occupational Safety and Health Administration (OSHA) sets permissible exposure levels (PELs) for people who might be exposed

to chemicals while working 8 h days, for 40 h weeks, over a working lifetime of 30 years, that is, chronic exposures. The Emergency Response Planning Guidelines (ERPGs) were developed by the American Industrial Hygiene Association (AIHA) as planning guidelines for emergency response operations to acute exposures, not chronic exposures, to toxic chemicals. The ERPGs are three-tiered guidelines for 1 h exposures are defined as those that can be expected to have:

1. ERPG-3: severe adverse effects,
2. ERPG-2: reversible adverse effects that will not impair a person's ability to take protective action, or
3. ERPG-1: mild, transient adverse effects.

The ERPGs, as of 2006, can be found at the AIHA Web site, http://www.aiha.org/1documents/Committees/ERP-erpglevels.pdf.

Acute Exposure Guideline Levels (AEGLs) are developed by the National Research Council's Committee on Toxicology and, unlike the ERPGs, are meant for the general population. The definitions are as follows:

1. AEGL-3: The airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience life-threatening health effects or death.
2. AEGL-2: The airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience irreversible or other serious, long-lasting adverse health effects or an impaired ability to escape.
3. AEGL-1: The airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience notable discomfort, irritation, or certain asymptomatic nonsensory effects. However, the effects are not disabling and are transient and reversible on cessation of exposure.

Each of the three levels of AEGLs is developed for each of five exposure periods: 10, 30 min, 1, 4, and 8 h. The current AEGL values are listed at the EPA's AEGLs Web site, http://www.epa.gov/oppt/aegl/pubs/chemlist.htm and http://www.epa.gov/oppt/aegl/pubs/compiled.pdf.

VI. ISSUES RELATED TO LOW-DOSE EXPOSURE TO CHEMICAL AGENTS

The health effects of exposure to low doses of CW agents have been of considerable interest for several decades. During the period of large-scale production of CW agents in the United States, this subject was a particularly important occupational health issue for workers in production plants. New attention to this issue was raised when the results of human testing involving chemical agents, conducted by the U.S. Army, were the topic of National Research Council reports (Panel on Anticholinesterase Chemicals, 1982; Coordinating Subcommittee, n.d.). Interest in this issue peaked again when risk assessment and public health programs were initiated in response to the chemical demilitarization of the stockpiles of the same weapons. It was at this time that the U.S. DHHS published their conclusions in the Federal Register regarding the risk of adverse health effects to exposure to low doses of nerve agent.

Increased interest in the subject of low-level effects was generated as a result of Gulf War Syndrome. One of the suggested causes of this malady is possible exposures of soldiers to low levels of CW agents during their period of service in Southwest Asia during and after the first Gulf War. Several panels of experts have reviewed these suggestions extensively (Persian Gulf War Health Effects, 1994; Presidential Advisory Committee on Gulf War Veterans Illnesses, 1996). Current knowledge of the health effects of exposure to low doses of nerve agents has been reviewed and is the subject of Chapter 4 in this book (see also Sidell and Hurst, 1997; Moore, 1998a,

1998b). However, this issue remains critical and is of significant importance to civilian responders to a CW agent incident. The issues are basic. To respond rapidly and effectively to a chemical incident and to respond in such a way as to save lives, first-responders must subject themselves to concentrations of the agents that may exceed current occupational exposure limits. Currently available detection technology for use at the scene of an incident may not measure chemical agents at these occupational exposure levels. In the case of sarin for example, the maximum airborne exposure concentration for an 8-hour workday is two orders of magnitude lower than the detection threshold for a hand held chemical detector (Moore and Alexander, 2001). Additionally, many of the protective ensembles that will be used by first-responders will not protect down to these levels or have not been definitively tested for their protective efficacy. Although the data appear to point to no adverse health effects from an acute, low dose of nerve agent, studies are continuing, which may provide additional support to these conclusions or may find effects that have previously gone undetected.

The majority of CW agent toxicological research efforts during the past two decades have focused on developing new antidotal interventions, pretreatments, and preventive measures for nerve agent exposures (McDonough and Shih, 1997). Several recent comprehensive reviews describing the pharmacology of and general treatment principles for the major nerve agents have been prepared by Sidell (1997) and Spencer et al. (2000). Numerous recent comprehensive reviews of the health effects of low-level exposure to nerve agents are provided by Sidell (1997), Romano et al. (2001), Brown and Brix (1998), Ray (1998), and Moore (1998a, 1998b).

There is information on persistent effects following symptomatic exposure to sarin from studies of victims of the 1995 Tokyo subway attack. Eighteen victims were examined by computerized posturography 6–8 months after the poisoning. It was suggested that a delayed effect on the vestibulo-cerebellar system was induced by acute sarin poisoning, with females possibly more sensitive than males (Yokoyama et al., 1998). Another follow-up study found visual evoked potential latencies to be significantly prolonged in sarin cases compared with the matched controls (Murata et al., 1997). One subject developed neuropathy with pathological evidence of nerve fiber degeneration at death 15 months after sarin exposure (Himuro et al., 1998). Unfortunately, all these studies were accomplished on patients who received symptomatic exposures to the agents. There are no reliable follow-up studies on people who were exposed to the agent at levels for which they experienced no effects or only mild symptoms, such as miosis.

Although the data appear to point to no adverse health effects from an acute, low dose of nerve agent, studies are continuing that may provide additional support to these conclusions or may find effects that have previously gone undetected.

A. Future Research Needs

In 1998, the DOD research program was critically reviewed by the U.S. Government Accounting Office and found to be lacking a focused strategy for low-level CW agent research (U.S. Government Accounting Office Report to Congressional Requestors, 1998). Since that time, the DOD has developed a research plan and objectives and has begun to execute research outlined in the plan. For an exposed military population, the dose of a CW agent considered to be low-level is the lowest dose that results in either an immediate observable adverse health effect or that which causes operationally relevant performance decrements. The most sensitive marker of an observable health effect and the purported cause of early significant performance degradation is nerve agent-induced miosis. Although various exposure durations can be considered in the planning of future research, a one-time or continuous exposure lasting from minutes to several hours should be the primary target duration of exposure.

CW agent research relevant to the military must address the effects of low-level agent exposure on operational performance of military personnel at the time of the exposure as well as the potential delayed, adverse health effects caused by the exposure. Paramount in this effort will be arriving at

best estimates of the dose–response curve for humans so that the operational and health risks of CW agent exposure can be reliably compared with other deployment or battlefield threats. Another consideration for the military to pursue investigations of low-level CW agent exposures is based on the military's need to ensure that current doctrine, equipment, and training are adequate to protect forces from effects of exposure to low levels of nerve agents. The research required for this military requirement must address the development of best estimates of concentration or duration of nerve agents causing mild human incapacitation. Research needs included reliable and reproducible experimental systems to deliver and quantify very low levels of nerve agents in laboratory animals. If determining the lowest dose causing a significant performance decrement in humans is the objective, then studies conducted in nonhuman primates where accurate nerve agent inhalation dosimetry is combined with biochemical and physiological measurements, in addition to operant and behavioral testing, will be of particular benefit. Only under these conditions can dose–response relationships be assessed, which can be used to develop best estimates in humans for use in developing operational courses of action.

Little of this research can be applied to practical operational application for the military without the development of better detection technology (O'Hern et al., 1997). This is a topic covered in detail in Chapter 19 of this book. A practical consideration must be to ensure that greater sensitivity will not involve a higher incidence of false positive measurements. False alarms themselves distract soldiers from combat-related tasks and may initiate the requirement for wearing personal protective gear and result in a significant decrease of combat effectiveness. In other words, the presence of measurable CW agent is not the only significant health or military threat. Thus, emphasis on the development of highly sensitive and reliable field detection devices must go on in parallel to any toxicological studies of low-level effects. It is critical that military operational doctrine does not require implementation of maximum physical protective measures at exposure levels that are significantly below those likely to produce casualties or long-term disabilities. In order to do this, human toxicity must be estimated as accurately as possible, and appropriate toxicological data are required to minimize the uncertainty around these values.

In a civilian setting, such as the terrorist nerve agent attacks in Japan, a single exposure lasting from minutes to hours should be the most realistic scenario. In such a case, effects on human performance should not be an important factor. Rather, research on potential acute and delayed adverse health effects from low-level exposure to nerve agents should be of the highest priority. For civilian populations, the potential long-term health effects from an acute exposure to low levels of nerve agents must be studied. A source of potentially valuable perspective could be obtained from epidemiological study of pesticide-exposed workers. Likewise, follow-up assessment of patients surviving self-inflicted exposures would provide additional case-related information regarding delayed-onset and persistent, adverse clinical effects. The use of sensitive neurochemical and immunohistochemical methods in the examination of tissues from exposed animals is an important component of any animal study to be conducted.

VII. SUMMARY

The accidental or intentional release of chemical agents is similar to the HAZMAT incidents that metropolitan public safety personnel, and those with populations near facilities making, storing, or using TICs, contend with routinely. The emergency response to a release of CW agent can use the existing framework of response to toxic chemical incidents and can be modified and enhanced for maximum effectiveness. Additionally, poison control centers located throughout the country deal with chemical poisonings on a daily basis and can serve as the initial focus of efforts to improve the response of the medical community to dangerous CW agents. Information in this chapter has described the adverse health effects of CW agents and the threat they pose from the standpoint of protecting civilian communities in the event of their use. There is a requirement for accurate and reliable estimates of the effects of low-level CW agent exposures on human performance as well as a

need for more precise data for acute, long-term, and delayed health effects. Some estimates can be derived from the universe of existing data on studies involving animals and human research subjects; however, well-designed toxicological and behavioral studies conducted in nonhuman primates may be required to validate the estimates. Additionally, basic research designed to measure sensitive markers of nerve agent exposure should be expanded in an effort to assure that low-level exposures are not associated with long-term or delayed health effects.

Preparedness planning from the national to the local level for a potentially catastrophic event has been described. Of particular importance, however, is the need to ensure the safety of those individuals who must be called on to provide assistance to casualties and communities should toxic chemical agents be released. In this regard, these individuals must have accurate and rapid detection equipment, tested and effective physical protection, and adequate facilities and medical products to provide the care to victims of a mass casualty incident. Much has been done to address all of these areas and it is hoped that when such an event occurs, the time and resources applied to this problem payoff.

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24 Emergency Medical Response to a Chemical Terrorist Attack

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CONTENTS

I. INTRODUCTION

A true disaster is an event that overwhelms a community's ability to respond on its own. The term is used more liberally to signify highly unusual, large-scale events. If a chemical event were to occur today, it would be a disaster for a large majority of the United States, simply because most locales are woefully unprepared for this type of event. Although many areas may have a plan, training, and some equipment available, most would buckle under the weight of a truly major chemical event. This is true of the emergency medical response and for the majority of hospitals. Although this text is designed to discuss malicious intent and chemical warfare agents (CWAs), the reality is that there are many highly toxic products produced and transported on a daily basis in this country. It is imperative that the entire medical community be ready to respond to hazardous materials (HazMat) events, be they intentional or accidental, and whether they are chemical in nature, as discussed in this text, or other hazardous substances, namely nuclear and biological.

Many parts of this text are highly technical, discussing major chemical toxins, their physiologic and health consequences, and management of the toxins with antidotes and decontamination. The intent here is to create a framework whereby the emergency medical community can understand, and then employ, the basics of a large-scale chemical event. You can then turn to the other chapters for more detailed technical information to increase the breadth of your plan and subsequent

response. The likelihood of a major event is high; there are evil forces that originate from both abroad and here at home. They wish to disrupt our way of life and cause harm to the masses. There are also individuals who will try to produce chemical agents as a challenge, in a psychology similar to computer hackers and viruses, etc.

II. PITFALLS

It is important to understand the major pitfalls that frequently occur in any major operation. By keeping these failure areas in the constant forethought of consideration, you are more likely to avoid them. These major areas include command and control, communications, security, transportation and traffic, and preparation. In the 1970s, the Incident Command System (ICS) was started to deal with control of wildfires and then was expanded to increase preparation for earthquakes. This system has continued to grow and propagate to ever expanding groups of emergency service converts who see the benefit of this system for coordination and control. This is now promulgating to the entire country as the National Incident Management System (NIMS). When the spread and conversion is completed, all emergency responders will operate with identical command structures and roles (Federal Emergency Management Agency, 2006a, 2006b, 2006c). Although it is rapidly spreading in the Emergency Medical Services (EMS) world, hospitals have been slower to adopt this system. The Hospital Emergency Incident Command System (HEICS), now being referred to as the Hospital Incident Command System (HICS), is an important method of proper command and control for hospitals. Widespread adoption of it will improve the individual hospital's ability to effectively control an event. It will also improve the ability of many institutions to interact and coordinate response and resources should a large event of any etiology occur. One shortcoming, not of HICS itself, but rather its effectiveness, is a lack of full understanding of the system. It is very difficult for an institution to operate one way on a normal day and then, when stressed, be able to effectively operate under HICS. What makes more sense is for the institution to operate with a HICS framework as part of their regular operations so that emergency response is seamless and already part of corporate habit (San Mateo County Health Services Agency, 1998; California Emergency Medical Services Authority, 2006).

Communication failure is a frequent cause of response failures, and there are many reasons for this. Technology shortcomings can occur when networks and equipment fail or become overloaded. Incompatibility of systems and frequencies has been cited in many recent disasters. The ICS is standardizing and thus improving the terminology and protocols being used. Another area is lack of redundancy. Systems should be expected to fail when subjected to the stresses of a major event. It is important to build in backup and redundancy so that effective alternatives are immediately available. The communication needs to be on multiple levels and go in multiple directions. First, it is important for EMS to communicate with other emergency services and the hospitals. The hospitals need effective ways to communicate within their own institutions and with other area institutions. The hospitals should keep in mind portability, as many hospitals have had to abandon their brick and mortar due to damage. Additionally, many have had to expand operations outside to accommodate the demands. The local resources need to be able to communicate with the greater governing body and then further up the chain. Likewise, the emergency services need to be able to communicate with the lay population. The resulting ''system'' is therefore complex, employing mass media, National Oceanic and Atmospheric Administration (NOAA) alert system, hardwired and cellular telephony, the Internet, and layers of radio systems for intraorganization, local, and regional networking. One additional need is the ability to communicate with large crowds. Portable public address systems or powered megaphones will prove very useful in these situations.

When a chemical event occurs, security, controlled by the police departments, will be crucial to control the scene and support facilities. At the scene, a lack of security will allow individuals to cross into the hot zone and become victims. Likewise, it is important to control those affected to ensure they do not randomly leave the scene. The result can be overloading and result in

contamination of the closest medical facilities or other individuals. Security is also necessary at the scene as secondary devices may have been put in place to harm the first responders. Additionally, the forensic evidence must be preserved. Security is also paramount at the hospitals. You will have incoming victims and potential family members, regular patients and their families, and the media. Failure to seal your facility will yield an out-of-control sea of humanity and allow the potential for contamination of the facility and its inhabitants.

In addition to security, the flow of vehicles and people into and out of the scene must be controlled. An organized traffic plan needs to be established at the very beginning to prevent gridlock and confusion. The same is true at the hospitals, where incoming and exiting routes for emergency vehicles must be established. Civilian traffic must be kept away from these lanes. Once off-loaded, incoming routes for patients must be established so they do not mix with the regular patient population. Some larger institutions may have security forces that are large enough to perform these security and traffic management functions. For most, it is unlikely that there will be enough people. Enlistment of other hospital employees for this function can be sought. It is most appropriate for the institutions to open dialogues with their local law enforcement to define needs and roles and to evaluate the security plans.

The final pitfall area to be mentioned here is lack of preparation. This preparation includes proper training, equipment, and staffing. It also includes participation in the ICS, as mentioned above, and networking up, down, and laterally in your regional system. Once this preparation has been completed, it needs to be exercised and practiced, and then practiced again until the entire emergency service or institution on all shifts is comfortable (Auf der Heide, 1989, 2006; Rubin, 2004).

Important points to consider when planning response to a chemical event are:

1. Ratio of 5:1 is the approximate ratio of patients presenting for treatment who are *unaffected* by the chemical agent as compared to patients who are actually *affected* by the chemical agent (Lake, 2000; also see Chapter 22 of this book).
2. Walking well or minimally injured will be the *first* patients to arrive at your hospital. They will arrive by private transportation and will *not* be decontaminated.
3. Most important care that the chemical casualty receives is the care that is given within the first several minutes of a chemical attack. The conduct of care given immediately after toxic chemical agent exposure, including the administration of antidotes, can literally mean the difference between life and death or serious disability.
4. All toxic effects of chemical, biological, radiological, nuclear, and explosive agents (CBRNE) are *dose dependent*. The dose for respiratory exposure is the concentration of the agent multiplied by time of exposure. Physiological factors, such as rate and depth of respiration during the exposure period, will also determine the final pathophysiological outcome of the patient.
5. First obligation of the responder is *not* to become a victim himself. Personal protective equipment (PPE) is employed to accomplish this goal.
6. If the medical treatment facility (MTF) or hospital becomes contaminated with chemical agent, not only does it become useless to render care, but it also exposes the staff and all other patients to chemical contamination. All precautions must be used to avoid this issue. Only very limited access to the hospital or MTF should be permitted. At most, only one or two entrances to the hospital should be used by incoming patients and employees. At these entrances, proof of decontamination or noncontamination, in the form of checking the individual with a chemical agent monitor (CAM) for contamination, must be presented before admission. All other entrances must be ''locked down.'' Security guards posted on the inside of all entrances would prevent unauthorized entrance from outside. Remember, people exiting the hospital could hold the door open to admit contaminated individuals from the outside.

7. Arrival and exiting of ambulances/buses should be by one route only to keep possible contamination to a minimum. These routes should be clearly marked with yellow perimeter tape and enforced by guards. Landing zones for arriving helicopters should be far enough away from the hospital so that ''prop wash'' from their rotor blades do not recontaminate decontaminated individuals.
8. Triage and decontamination sites near the patient arrival point outside the MTF or hospital must be in place and appropriately staffed and stocked with necessary equipment. Careful note of the prevailing wind must be considered, as these sites should ideally be upwind and uphill (and upstream) from the area of the toxic chemical release. These sites should also be in shaded areas to protect workers in PPE from the warming effects of the sun's radiant energy (Jordan, 2003).
9. Decontamination teams, at least one from each shift, including weekends, should be trained in decontamination while in PPE. The Occupational Safety and Health Administration (OSHA) has specific guidelines/regulations concerning use of PPE, and noncompliance with them may bring stiff fines. Refer to the OSHA Web site, specifically 29 CFR 1910.120 and 29 CFR 1910.134. Additionally, information on CBRNE equipment may be found at 42 CFR 84.
10. Plan to have an alternative medical treatment facility (AMTF) for those patients who do not require life-sustaining levels of care.
11. Material contaminated with gaseous contamination only (no liquid contamination) will off-gas gaseous chemical agent for approximately 30 min after removal from a gaseous contaminated environment.
12. Design a mechanism by which radio communications can be maintained between the hospital, the incident commander, and the senior medic at the chemical incident site.
13. Plan to maintain tight security around the hospital in anticipation of a secondary release of a chemical agent by terrorists at the hospital site. These guards should be dressed in an appropriate level of PPE, usually level C.
14. Design a plan for the disposal of contaminated water created during the decontamination process. Have this plan reviewed by local, state, and Federal environmental protection officials. With some noteworthy exceptions, the Environmental Protection Agency (EPA) has addressed this issue in the ''good Samaritan'' clause of the Comprehensive Environmental Response, Compensation, and Liability Act (CERLA), Section 107(d) Rendering Care or Advice. ''No person shall be liable under this subchapter for costs or damages as a result of actions taken or omitted in the course of rendering care, assistance, or advice in accordance with the National Contingency Plan (NCP) or at the direction of an onscene coordinator appointed under such plan, with respect to an incident creating a danger to public health or welfare or the environment as a result of any releases of a hazardous substance or the threat thereof.'' This clause does not preclude liability for costs or damages as a result of negligence, nor does it address state and local liability issues. This is an extremely interesting and important article and should be read in its entirety (United States Environmental Protection Agency, 2000).

III. RESPONSE

When the major chemical event occurs, it will initially be known only to the victims. At some point, a call for help will be made and there will be a response. It is very likely that the extent of the event will initially be unknown as those present will most likely be victims who are unable to call, or the event will be out of direct line of sight from a safe distance. The effort has to be to prevent the proverbial mine-canary-sacrifice until others realize what is going on. Because an event can occur anywhere, at anytime, all first responders need to be trained and frequently reminded to be ever vigilant. They need to remember not to rush in where they cannot directly visualize from a safe

distance. The first responders to the scene need to be properly equipped to protect themselves so they can make an initial evaluation. If they are unable to make a safe determination, then the ideal situation would be for a rapid, fully encapsulated, Class A, protective response to ensure safe evaluation of the scene. Because lives are at stake, the details need to be established well in advance. At the hospitals, the Emergency Department (ED) personnel are also first responders (if operations need to be moved outside the ED, such as setting up triage or a decontamination zone) or first receivers (of patients that bypassed scene control and care). More people arrive at the ED via private auto or on foot than by ambulance. The same can be expected in a major event, including a chemical event. The ED personnel in general, from nurses and physicians to security and registration, need to be trained to recognize chemical events as any one of them could be the first to ''greet'' the incoming victims. The individual, who first realizes that a chemical event has occurred, be it a victim, bystander, dispatcher, or first responder, and relays that information, will be the hero that saves others' lives.

Once the initial response determines that a chemical event has occurred, a coordinated secondary response will take place, keeping in mind the pitfalls mentioned above. This is where ICS, training, and preparation are key to a successful operation. Appropriate HazMat, EMS, and law enforcement personnel are dispatched according to previously designed plans. The scene is controlled, and decontamination with initial medical care begins. At the hospitals, if the contaminated arrive unannounced; they are sequestered outside and the emergency plan is initiated. Appropriate medical, logistical, security, and leadership/ICS resources are mobilized. It is important to open communications between the institution's Emergency Operations Center (EOC) and the outside event EOC to determine what and where the event occurred and how many people are injured.

Once the preliminary steps have been completed, it is time to medically manage the event. In actuality, there are steps that can be taken during the ramping up of the operation that will decrease morbidity and mortality. The first step would be to either remove the contaminant from the victim or remove the victim from the contaminant. Along with this step are two key features. You need to be able to communicate with the victims without putting your own personnel at risk. Because a fully encapsulated responder cannot communicate effectively, the need here is for a portable, public address (PA) system. Depending on the circumstance, this might be a vehicle-mounted system; however, vehicles should not be too close to the contaminated environment. A megaphone type PA device may also be inappropriate because it would be exposed to the open environment. This leaves two options. One would be a remotely controlled, robotic type of PA device receiving the vocal input via radio. The other would be a hand-carried PA unit with the same radio input from either the fully encapsulated carrier or from the Incident Command Post. The other key feature is to have the area secured. With appropriate scene security in place, the victims are instructed to move to a secured, clean area and to strip off their clothing while moving. Those who are able would be decreasing their exposure and thus starting medical care on their own.

IV. MEDICAL TREATMENT FACILITY

Let us now consider some of these concepts for the MTF. A casualty decontamination site serving the MTF must be established because many patients will arrive via private transportation without prior decontamination. There should be only one or two arrival routes, and they should be clearly marked by signs, traffic cones, and yellow emergency marking tape. These devices should be used to funnel patients, whether arriving by ambulance, bus, car, or on foot. Perimeter guards should police this area to prevent additional contamination of the MTF by those arriving. Areas of the MTF casualty decontamination site include the following:

1. Reception or holding site, which is where new arrivals stay until triage, can be accomplished.
2. Triage site; this is sometimes combined with the reception site.

3. A contaminated emergency treatment site (to provide life-saving medical support that would allow sick, nonambulatory patients to survive decontamination).
4. A clothing-removal site.
5. A contaminated dump area approximately 75 m downwind of the arrival site.
6. Decontamination facilities.
7. A second triage and Emergency Medical Treatment (EMT) on the clean side of decontamination.
8. The hospital and alternate MTF.

Triage is a French word meaning "to sort." The purpose of triage is to do the most good for the greatest number of people when resources are overwhelmed. It should be remembered that triage is a dynamic, not static, process and that as resources change, so must the triaging technique. The number of patients, the medical condition of the patients, the number and type of medical responders, and the availability of equipment and resources all factor into this equation. Some patients initially triaged as expectant may be retriaged to a higher category as resources improve. Triage categories in the United States are divided into four classifications:

1. Immediate—requiring immediate medical intervention to save the victim's life.
2. Delayed—the patient has serious injury requiring medical care but can wait for treatment and waiting will not affect the long-term outcome of the patient.
3. Minimal—the patient has minimal injury and requires minor care, but there is nothing life threatening or capable of causing lasting injury (United States Army Medical Research Institute of Chemical Defense, Chemical Casualty Care Division, 2001).
4. Expectant—the patient needs medical care beyond that which the MTF can provide; this patient is therefore expected to die. As resources change, this patient's condition may be re-evaluated and his triage category changed to a higher category (Sidell et al., 1997).

A triage matrix for all four classes of chemical warfare agent as attached as shown in Table 24.1. Additionally, the Rule of Nines technique for assessing liquid coverage of the skin (as in contact with liquid vesicants) is shown in Table 24.2.

Triage may also employ the "START triage" system that assesses respiratory, circulatory, and neurological status to determine the need for immediate treatment. This technique is simple, requires a minimum of time, and yet is a highly effective and reproducible triage evaluation method (Super et al., 1994; Benson et al., 1996). Please refer to Figure 24.1.

Color and bar-coded, waterproof triage tags are commercially available, including tear-off tags for clothing and belongings. The advent of bar coding has greatly simplified the record keeping process, although a bar-code scanner painted with chemically resistant paint to allow easy decontamination later would also be necessary.

Who is most qualified to be the triage officer? The most qualified individual to perform this role is someone who has been specially trained for it and who has exercised that training. This individual could range anywhere from a first responder to a physician. The reality is that a minority of physicians have the training or experience to fulfill this role. In addition, his intellectual and technical skills could best be applied treating sick patients. Untrained physicians, by the nature of their training, would wish to stop and treat every individual patient and get bogged down in the treatment of one patient, while ignoring many others (Kennedy et al., 1996; Sztajnkrycer et al., 2006). There may be a subset of patients who are simply too ill to survive the delay of decontamination. An example would be a nerve agent casualty undergoing seizures. Although this patient is salvageable, treatment must precede or coexist with decontamination. An emergency MTF in the contamination reduction area (warm zone) with the medical personal in full PPE exists for this purpose. All standard equipment needed for basic CPR, chemical warfare antidotes, equipment to stabilize patients in hypovolemic shock (tourniquets, i.v. fluids), and splints to stabilize fractured

TABLE 24.1
Matrix of Triage for Chemical Warfare Agents

S/S = Signs or Symptoms	Immediate	Delayed	Minimal	Expectant
Lung-damaging agents	Acute airway problems, i.e., laryngospasm, stridor; resource dependent	Onset of s/s >4 h after exposure		Onset of s/s <4 h after exposure; resource dependent
Cyanides	Unconscious, apneic, but with heartbeat	Recovery from successful therapy; survival > 15 min post vapor; Exposure	Less than lethal dose; recovering without therapy	No circulation
Vesicants	Acute airway problem; S/S onset <4 h after exposure; stridor, retractions, RR > 30; Decreased oxygen saturation; laryngospasm; intubation to secure airway	Pulmonary s/s >4 h after exposure; Skin burn >1% but <50%; depending on time after exposure burn will not be seen, what will be seen is area of body covered by liquid agent; moderate—severe eye involvement	Minor eye irritation (eye fells "gritty"); skin burns <1% BSA in noncritical area N.B. Skin around nose and mouth are critical areas	Pulmonary s/s <4 h after exposure; resource dependent; Liquid burn >50% BSA
Nerve agents	Symptoms in two or more organs systems: airway, GI, muscular, etc. (*EXCLUDE* miosis, rhinorrhea); Unconscious, apneic with heartbeat	Recovering from moderate/severe exposure; for example, exposed to both liquid and vapor nerve agent and is recovering from vapor exposure (breathing difficulty improving) with no signs of liquid exposure	Walking and talking; may have miosis, rhinorrhea (runny nose) without any other signs or symptoms	No heartbeat; resource dependent

Source: Adapted from U.S. Army Medical Research Institute of Chemical Defense, Chemical Casualty Care Division Web site, 2006, https://ccc.apgea.army.mil/

TABLE 24.2
Rule of Nines Chart of Body Surface Area

Assume the body is made up of 11 units, each containing 9% of the body surface area:
Head and neck = 9% of total body surface, anterior trunk 2 × 9 = 18% of total body surface, posterior trunk 2 × 9 = 18% of total body surface, upper limbs = 9% each (total of 2) = 18% total body surface, lower limbs 2 × 9 = 18% each (total of 2) = 36% total body surface, genitalia and perineum = 1% of total body surface. Total = 100%

Source: United States Army, *FM-9 Handbook on the Medical Aspects of NBC Defensive Operations*, United States Army, Washington, DC, 1996, Table 6.1. This table is particularly helpful in assessing skin coverage by liquid CWAs, including the vesicants and thickened agents.

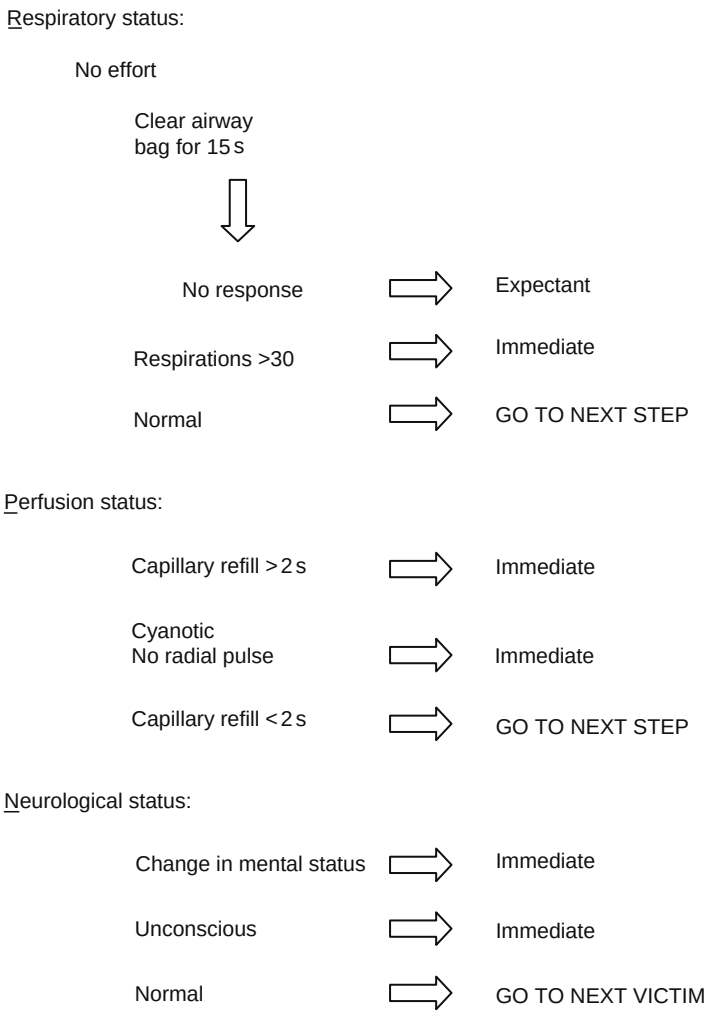

FIGURE 24.1 Simple triage and rapid treatment.

and bleeding extremities should be kept in this area. Equipment for emergency endotracheal intubation should also be kept at this location.

V. INTUBATION

Note that both liquid and vapor contamination may exist in this area. Individuals requiring a short course of artificial ventilation with an Ambu bag in this contaminated area are at risk of contaminated air entering their lungs during artificial ventilation. Manual ventilation with an Ambu bag would drive surrounding contaminated atmospheric air right into the patient's lungs. Clearly this is not a good idea. The military employs a device known as a Resuscitation Device Individual Chemical (RDIC) especially for this purpose. This device is commercially available (NSN 6665–01–338–6602) and is manufactured by the same company that manufactures Ambu bags. Indeed, it differs from an Ambu bag in only three ways. These differences include (1) a short, low-pressure hose between the outlet valve of the resuscitation bag and the mask so that it will be compatible with another military device; (2) the body of the bag is made of butyl rubber, which is highly resistant to permeation by all CWAs, including the vesicant agents (mustard, Lewisite, phosgene oxime); and

(3) the room air inlet valve is completely covered by a 40 mm gas tight, female threaded fitting. All inspired air must pass through this fitting to reach the resuscitation bag. Any standard NATO CWA filter with a 40 mm thread can be threaded into this fitting. All commercially available filters approved by the National Institute for Occupational Safety and Health (NIOSH) for CBRNE usage use the standard 40 mm fitting. Hence, contaminated atmospheric air would be filtered before entering the body of the RDIC, and therefore the patient would be resuscitated with only clean, filtered air. Every emergency treatment station in a contaminated area should be equipped with an RDIC.

Because emergency endotracheal intubation would be performed in a contaminated area, certain measures must be followed to prevent further contamination of the patient.

1. Operator should decontaminate his gloves immediately prior to performing intubation.
2. Prior to intubation, *do not* lay the endotracheal tube (ETT) on the patient's chest, on the ground, or on any other potentially contaminated area. The ETT should be kept in its wrapper, and the wrapper should ideally be kept inside a butyl rubber glove (as a protective cocoon) for as long as possible and then removed from the opened wrapper by the individual performing the intubation.
3. Do *not* touch the patient's face, lips, etc. with the ETT unless you know for a fact that this area has been decontaminated.
4. Prior to securing the tube with tape or a tube holder, be sure that this area of the skin has been decontaminated. An M291 dry decontamination kit is excellent for this purpose, as its dry, black decontamination material will dramatically show what skin area has been decontaminated.
5. *Do not* kneel on the ground while performing intubation as liquid chemical agent could be on the ground and permeate or penetrate through your PPE suit.

Ventilatory resistance postintubation will be normal for individuals intoxicated with biologicals, radiologicals, lung-damaging agents, and cyanides unless they have pre-existing airway disease. Individuals intoxicated with vesicants will probably have normal breathing, but may have increased airway resistance due to physical obstruction of the airway (denuded cells of the tracheal–bronchial tree). This may require removal by suctioning. Nerve agent exposure will tremendously increase both the airway resistance and airway secretions. A dose of atropine is frequently necessary prior to intubation to deal with this problem.

VI. DECONTAMINATION

The clothing removal station would be at the beginning of the decontamination lanes. Remember, simple removal of the patient's clothing represents perhaps 80%–85% decontamination of the individual, and clothes exposed only to vapor agent (no liquid agent) will off-gas for approximately 30 min following removal from the contaminated area. Not only does clothing removal eliminate the possible source of both liquid and gaseous contamination, it also aids in increasing evaporation of the liquid contaminant from the patient's skin should liquid contamination be present. Unlike some protocols for radiological decontamination in which a light coating of water is used to coagulate the radioactive dust to prevent spreading, *do not* wet the clothing prior to removal. Hydrated skin will absorb chemical agent much faster than dry skin. Additionally, this technique will only serve to spread liquid contamination of chemical agent to a greater degree. Approximately 75 m downwind (and ideally downhill and downstream) from this site should be a contaminated clothing and equipment dump where these items would be stored in doubled, clear plastic bags that are clearly marked with bar-coded patient identification.

At least two lanes should be used for patient decontamination—an ambulatory decontamination lane subdivided into two lanes according to gender to address privacy issues. Here, ambulatory

patients can be decontaminated with showers supplying *low pressure, high volume warm water*, and *gently* washing (avoid scrubbing, which abrades skin) with a *liquid, nonparticulate, noncaustic* soap. Soap acts as a surfactant and increases the removal of contaminant from the skin. Bleaching agents may damage the integrity of skin and are best avoided. (Although military decontamination protocols employ bleaching agents, this is only because many areas in which the military operates lack sufficient quantities of water for decontamination. In World War I, large numbers of decontamination showers were set up in field hospitals.) All of this is to prevent microscopic abrasion of the skin, which would increase the dermal (skin) absorption of chemical agents by the human body. Careful attention should be paid to the use of warm (>65°F), not cool or cold water (United States Army Soldier and Biological Chemical Command, 2002). Although conduction, convection, and radiation all contribute to heat loss or gain, evaporative cooling is by far the most efficient method of heat loss known. Evaporation of cool or cold water from the surface of the skin creates huge losses of body heat that can quickly lead to the development of hypothermia. One should also remember that in experimental animals, severe exposure to certain nerve agents resulted in lowering of the basal body temperature (resetting of the body's thermometer) in the postictal (postseizure) state.

Ambulatory decontamination is best achieved using a "buddy system"; that is, one individual will wash the other and then switch places. Ideally, one individual would stand with his or her arms extended at about a 45° angle from his or her side with legs separated like an upside down letter "V." This would allow easier access to points frequently missed during decontamination. These include the scalp and hair, behind the ears, inside the ear canal, nostrils, the axilla (armpit), the naval (belly button), groin, between buttock checks, under fingernails, back of the knee, between toes, and under toenails (New Jersey State Police, course 06013). At the end of the ambulatory decontamination process, towels must be provided for drying off, clean clothing should be provided as well. Black colored, plastic trash bags work well for this as (a) black absorbs heat best of all colors, (b) plastic has excellent heat retention properties, (c) they are readily available and inexpensive, and (d) they provide patient modesty. Furthermore, in cold weather situations, shelter from the external environment must be provided to prevent further heat loss.

In an attempt to think "outside the box," the U.S. Army Soldier and Biological Chemical Command (SBCCOM) attempted to discover possible alternative ambulatory decontamination facilities, which would be able to supplement standard shower decontamination in the event of a large scale mass casualty event. One such alternative would be to use an Olympic-sized, heated swimming pool. The SBCCOM calculated that such a swimming pool as a communal bath with soap (and the water already present) could decontaminate large numbers (well in excess of 100,000 people) of ambulatory casualties exposed to nonconvulsive doses of the nerve agent sarin (SBCCOM, January 2002). The dilution factor of the large quantity of water would keep it nontoxic for many tens, if not hundreds of thousands, of people.

A second decontamination lane should be used for nonambulatory patients. These patients constitute the bottleneck of the decontamination process. Decontamination of nonambulatory patients is best accomplished using a setup that looks and functions identically to an automatic car wash for humans. This is best done with a tent enclosure that decreases the sun's radiant energy from directly warming the responders in PPE, but provides shelter from the elements (wind, rain, etc.), aids in maintaining the patient's body heat, and provides privacy for the patient. The patient is placed on a decontaminable, monofilament, polypropylene litter. This litter possesses a honeycombed weave that creates 40% open surface, allowing liquids to pass through the litter easily. The national stock number (NSN) for such a unit is 6530-01-290-9964 (United States Army Medical Research Institute of Chemical Defense, Chemical Casualty Care Division, 2000). The litter is then rolled along a commercially available roller mechanism (like those used at super markets, etc. to unload delivery trucks), and at several locations the litter is surrounded on all four sides (above, left, right, and below) by water jets employing warm water with *high volume*, *low pressure* shower heads as previously described. At other locations, responders in PPE will be *gently* washing (carefully avoiding scrubbing) the patient with a *mild*, *noncaustic*, *nonabrasive liquid* soap. In an ideal world,

a third lane would be present for ''technical decontamination'' of responders in PPE, as well as equipment.

Remember that decontamination is a *physical* process, the purpose of which is to remove the contaminant from the patient in the shortest possible time and thus reduce the concentration of any toxic material to below toxic threshold levels. Two old time nursing principles are true of decontamination. First, ''separate the puddle from the patient.'' Second, ''the solution to pollution is dilution.'' Multiple different protocols may be devised to accomplish this goal, each having its own advantages and disadvantages. The important thing is that everyone be familiar with their particular protocol and then execute that protocol faultlessly. A particular order of decontamination is appropriate:

1. Critically ill patients (''immediate'' triage category).
2. Liquid exposed individuals.
3. Children and the elderly who would otherwise not fit into the first two categories.
4. Vapor exposure.
5. ''Walking worried.''

Special attention should be paid to hair as it will retain vapor agent and decrease the evaporation of any liquid agent (Marrs et al., 1996). This area should be gently washed with copious amounts of soap and water. All water faucets used in any decontamination facility should be of the *high volume*, *low pressure* type. The fog nozzle of a fire truck is ideal for this work. Some individuals believe shaving of hair is an appropriate decontamination method. Although this does remove the vapor and liquid-retaining properties of the hair, it also micro- or macroabrades the skin, thus increasing absorption of chemical agents.

Figure 24.2 is representative of a typical casualty decontamination facility. In the figure, the hot line laid on the ground is composed of lime or other caustic material that would decontaminate liquid chemical agent on the footwear of individuals. Downwind of the hot line, the possibility of both liquid and vapor chemical contamination exists; consequently, full PPE must be worn. On the upwind side of the hot line, only the possibility of vapor contamination exists. Hence, upwind of the hot line, only a protective mask must be worn. Of note is that in many civilian decontamination protocols, a ''hot line'' is not included. The vapor-control line (VCL) exists approximately 30 m upwind of the hot line. Downwind of the VCL, the possibility of vapor contamination exists, and a protective mask must be worn. Upwind of the VCL, no vapor (or for that matter, liquid) contamination exists, and therefore level D (street clothing) is perfectly appropriate. This area is where the hospital and alternate MTF are located. The hospital should be reserved for those patients with serious injuries, including both immediate and appropriate delayed casualties (fractures, dislocations, stable penetrating wounds, etc.). Patients belonging to the minimal and the walking worried groups should never see the inside of the hospital (MTF). They should be referred to the alternate MTF, which may be located in a large building or tent not far from the MTF.

Please note that the levels of PPE appropriate for casualty care are discussed exclusively in Chapter 20 of this book.

Before any person enters the MTF or alternate MTF, proof of decontamination must be present. Individuals may be monitored for vapor contamination by *correctly* using an electronic CAM of the point variety type (CAM, ICAM, APD 2000, and Sabre are examples). M256A1 kits, MSA, or Dragger Bubbler kits could be used, but these would consume an extraordinary amount of effort, as well as time waiting for results. Remember that the above mentioned point monitors must be held within a very few centimeters, but not touching the area to be examined, for a period of up to 30 s. Always consult the monitor's instruction manual for specifics. The presence of liquid or solid contamination can be detected with the use of M256A1 kits, M8 paper, or M9 paper, although electronic CAMs are preferred. Remember that both electronic monitors and manual methods

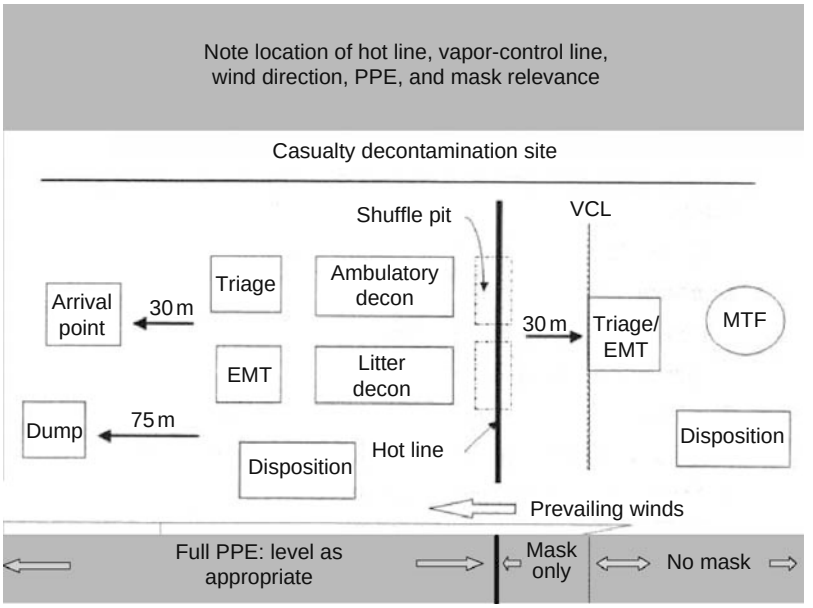

FIGURE 24.2 Representative diagram of a casualty treatment site. (From U.S. Army Medical Research Institute of Chemical Defense, Chemical Casualty Care Division, June 2001.) Hot Line = possible liquid contamination downwind (left side of line), negative liquid contamination upwind (right side of line). N.B. Possible positive vapor present on either side of hot line. VCL = vapor-control line: possible positive vapor on downwind side of line (left side of line), negative vapor on upwind side of line (right side of line). EMT, emergency medical treatment; MTF, medical treatment facility.

of chemical agent detection are subject to false positives. Read the instruction manual of your monitor to know what substances will produce false positives.

The alternate MTF should be a building or a large tent near the hospital and should be in telephone and radio contact with the hospital. Some hospitals have elected to turn the hospital cafeteria into this facility; however, this creates a problem of where to feed the hospital staff. Admission to this alternate MTF should be limited to minimal and delayed (nontraumatic injury) patients. This is the perfect place for the walking worried. Depending on the scope of the event, a second, alternative MTF may be used for expectant patients. The hospital facility can then devote its attention to the treatment of immediate and delayed (traumatic) patients.

Remember to have a media relations expert ready to deal with the media, and only that individual should release any details of the emergency, or the hospital's response to it, to the media. Guest services workers can gather lists of examined and treated individuals and telephone relatives to inform them of their loved one's status.

VII. MEDICAL APPROACH

Once the HazMat response is in place, you can move appropriately protected medical personnel in to assess the patients while decontamination commences. This is also true at the hospital. There are limited interventions that can be initiated in a contaminated environment. These revolve around Basic Cardiac Life Support, Advanced Cardiac Life Support, Advanced Trauma Life Support, and Advanced HazMat Life Support (BCLS, ACLS, ATLS, and AHLS, respectively, with trademarks) ABCDE's (Establish responsiveness, Airway/C-Spine, Breathing, Circulation/Control Hemorrhage, Drugs/Defibrillation/Definitive Care/Disability (Neurologic)/Decontamination, Exposure/Environmental/Elimination). The triage process would determine the level of responsiveness, along

with patency of airway and quality/quantity of respiratory activity. Depending on the size of event, available resources, and the agent involved, unresponsiveness with apnea may put the patient into the expectant category for no resuscitative efforts. With adequate resources and protected personnel, poor respiratory effort may be supported with bag valve mask and 100% oxygen as discussed earlier. Under circulation, a lack of pulse most likely will stop any resuscitation. However, if the event involved blast or the victim suffered blunt trauma for another reason, then the C-spine should be immobilized and external hemorrhage should be controlled. If the event is the result of a nerve agent or organophosphate, atropine and pralidoxime, and possibly valium, should be administered, preferably with Mark I autoinjector kits according to protocols discussed later in this chapter and elsewhere in this book. During the decontamination process, the environmental factors should be monitored as hypothermia can ensue quickly in the unclothed and wet victims, and hyperthermia in PPE protected rescuers. Once the victim is fully decontaminated and moved into the cold zone (sometimes referred to as the green zone), a full secondary survey should immediately be performed and the patient treated as appropriate.

VIII. TOXIDROMES

The entire HazMat and decontamination process is daunting and challenging enough by itself. Now, what do you do if the agent at work is not known? At the scene, every effort will be made to identify the chemical agent using placards, manifests, and prior knowledge (as in a known industrial plant). If this is not available, then a variety of sniffers and detectors will assist with identification (Baker, 2005). This can take a while. Meanwhile, you have severely ill patients who will do better with directed treatment. There are several toxidromes, that is, constellations of symptoms, which can help direct you to classes of agents that will allow you to start to tailor treatment. There are five toxidromes—irritant gas, asphyxiant, cholinergic, corrosive, and hydrocarbon, and each one will be discussed. Details about the chemicals involved in each of the toxidromes can be found elsewhere in this book.

A. Irritant Gases/Lung-Damaging Agents Toxidrome

Inhalation is a major route of entry into the body. Thus, it is frequently involved in toxic exposures. The irritant gas toxidrome, also known as lung-damaging agents, has two major types and an in-between, blended variety. The distinction has to do with the degree of water solubility of the involved gases. Gases that are strongly water soluble react immediately with the moist mucous membranes of the upper airway, above the vocal cords, and the eyes. Common industrial gases in this group are sulfur dioxide and ammonia. Also included are formaldehyde and hydrogen chloride. When these gases interact with the water in the mucous membranes, they cause a corrosive effect, which results in irritation and inflammation. The resulting symptoms are *almost immediate* and predictable. Starting from the exterior to the deeper, you get inflammatory changes, such as conjunctivitis, rhinitis, and pharyngitis. As this inflammation increases, there can be resulting edema that can get quite severe. In the area of the larynx, not only do you get the inflammation and edema, but you may also get laryngospasm. The end result of all this is severe irritation, such as lacrimation and blurring, rhinorrhea, sneezing, and drooling, if unable to swallow. With involvement of the vocal cords, there can be changes in phonation that range from hoarseness to loss of voice. Laryngospasm and severe edema can interfere with airflow through the upper airway, producing severe dyspnea. The exposed are very uncomfortable, and the victims will try to remove themselves rapidly from the environment.

At the other end of the spectrum of irritant gases are those that are weakly water soluble. With these, they bypass the upper airway and the immediate effects discussed above. Instead, their action is in the lower airway, and their pathophysiological action is usually manifested only *after a latency period*. Two major gases here are nitrogen dioxide and phosgene. These react with the endothelium

in the lower airways and alveolar sacs. The damage to the alveolar capillary membrane decreases surfactant. This causes the sacs to collapse and develop fluid. The resultant noncardiogenic pulmonary edema usually starts a few hours after exposure and begins to interfere with the ability to extract oxygen and dispose of carbon dioxide. The final type of irritant gas is one that is intermediately water soluble. These gases can give a mixed picture of initial upper airway irritation and inflammation, and then later, delayed, noncardiogenic pulmonary edema. A common gas in this last category is chlorine, which is used commonly in water treatment and is also a byproduct of improper mixing of commercial cleaning products.

With lung-damaging CWAs, such as phosgene, chlorine, perfluoroisobutylene (PFIB) produced from the combustion of Teflon, which possesses toxic effects 10 times more potent than phosgene, methyl isocyanate, cyanogen chloride, etc., the patient will feel much better and dramatically improve shortly after removal from the offending agent. *Do not be fooled*. This is a latency period, and symptoms and signs may reappear. The longer the latency period exists before recurrence of symptoms, the milder the ensuring signs and symptoms will be. Latency periods of <4 h are frequently associated with a grave prognosis. Once a patient is exposed to a lung-damaging agent, observe them *at bed rest* for a period of many hours afterward; the more the better. This patient must remain totally asymptomatic and have a normal physical exam (including blood gases, chest auscultation, and chest x-ray) after at least 12 h of bed rest before consideration of patient release can be made. Any signs or symptoms of respiratory insufficiency require at least 48 h of close observation (United States Army Medical Research Institute of Chemical Defense, Chemical Casualty Care Division, 2000). Any form of physical exertion MUST be avoided. Statistically, bed rest immediately after exposure to lung-damaging agents dramatically improves the outcome. The worst-case scenario, short of death, would be the development of pulmonary edema and adult respiratory distress syndrome (ARDS). This would require an ensuing need for ventilatory support; consequently, treatment should be aimed at preventing the necessity for artificial ventilatory support. In a mass casualty situation, not enough mechanical ventilators would be available. Exercise increases both the heart rate and blood pressure of the patient; this in turn increases both the pressure and the length of duration of increased pressure on blood vessel and alveolar walls at precisely the time when chemical reactions are simultaneously weakening their cellular structure and decreasing surfactant production. This is the mechanism that leads to the development of pulmonary edema when exercise or physical exertion occurs *after* exposure to lung-damaging agents. Supportive care only, as is appropriate for the pathology of the ARDS process, is the order of the day.

Lung-damaging agents, like all CWAs and all toxic industrial chemicals (TIC), produce *dose-dependent* injury. With lung-damaging CWAs (low water solubility), this damage *begins in the lower airway* (alveolus and alveolar sacs) and *progresses into the upper airway* (pharynx, larynx, trachea, bronchial tree) only as the dose increases to large quantities of agent. Should the patient present a different clinical presentation (i.e., upper airway damage first), rethink your initial diagnosis. Note the distinct contrast to the vesicant agents.

B. Asphyxiant Toxidrome

The next syndrome is the asphyxiant toxidrome. Although the irritant gas toxidrome interferes with oxygen use by affecting the flow of air in the airways, the asphyxiant toxidrome works by displacing oxygen, or at the cellular level by interfering with the transportation or use of oxygen. *Simple asphyxiants displace oxygen in a closed environment*. Examples here include high levels of methane, as in a septic system, carbon dioxide, as would occur when trapped in a small closed environment, or home fuel gases, such as natural or propane gases. Two substances interfere with the ability of hemoglobin in red blood cells to transport the oxygen to the cells. The first is carbon monoxide, which is a tasteless, odorless gas. All emergency practitioners should be well versed in this gas and its health effects as it is, unfortunately, all too common an exposure. It binds directly to

hemoglobin in place of oxygen and also makes it more difficult for the hemoglobin to release the oxygen molecules at the other hemoglobin binding sites. The other substance is methemoglobin (MetHb). You are not exposed to this substance. Rather, it is produced in the bloodstream by the chemical effects of several other substances. These include amines, nitros, nitrites, and nitrates. They cause an oxidation of the ferrous (Fe^{2+}) to ferric iron (Fe^{3+}) in the hemoglobin. Only iron in the ferrous form (Fe^{2+}) is capable of transporting oxygen. Therefore, newly produced *MetHb is not able to transport oxygen*. A major distinction between these two is coloration. The carboxyhemoglobin from carbon monoxide is bright red. Although turning cherry red is a late, premorbid finding, what will occur is a lack of blue from cyanosis. On the other hand, MetHb has a lack of oxygen and thus cyanosis is a key feature. When directly visualized, as in phlebotomy blood vials, the carboxyhemoglobin blood will appear redder, and the MetHb will appear chocolate brown.

The second type of asphyxiants is that which works at the cellular level. Here, they interfere with the mitochondrial cytochrome oxidase's function in the electron transport chain. Because this is the ''fuel cell'' for the body, energy production ceases within the cell, with cell death following close behind. The key substance implicated here is cyanide, which is usually found only in a chemical laboratory setting, but can also be a side effect of smoke inhalation. As previously mentioned, hydrogen sulfide and carbon monoxide also have some effect at this site. In addition, azides are cellular asphyxiants. The azides, along with the nitro-ate-ites, are also vasodilators and can cause headaches and hypotension.

Hydrogen cyanide and cyanogen chloride can be used as CWAs. The treatment of cyanide intoxication is usually a two-part approach. The *first part* is the pharmacologically-induced development of MetHb by sodium nitrite. This is to lure the cyanide molecule away from the P-450 cytochrome of the mitochondria and to reversibly bind it to MetHb. The *second part* of the reaction then irreversibly binds and eliminates the cyanide ion ($-CN$) by converting it to a thiocyanate ($-SCN$) through combination with sodium thiosulfate (the sulfur donor). This conversion of reaction is catalyzed by the enzyme rhodanese. The difficulty with this treatment is that for every bit of MetHb produced, that much less hemoglobin A1 is available to supply oxygen transport to cells. MetHb (which does *not* transport oxygen) levels should always be kept below 40%, and depending on the situation, levels of far less than 30% may be far more appropriate. Patients with ischemic cardiomyopathies, low cardiac ejection fractions, arrhythmias that decrease cardiac output (atrial fibrillation, etc. which abolish the ''atrial kick'' component of cardiac output), frequent ventricular arrhythmias, those with fixed lesions across any heart valve (which would severely limit the ability to increase cardiac output), those with severe acute anemia, or anemia of chronic disease, deformed hemoglobin production (such as sickle cell anemia), are already at risk for tissue hypoxia because of either low cardiac output or deficiencies in sufficient oxygen carrying capacity of the hemoglobin.

Patients who have also been exposed to carbon monoxide (as in a fire) have already increased the percentage of carboxyhemoglobin (which carries carbon monoxide tightly bound to hemoglobin) in their total hemoglobin content of whole blood, with a subsequent decrease in the percentage of oxyhemoglobin (which carries oxygen) in their total hemoglobin. Additionally, if nitrites are administered during the cyanide treatment protocol, MetHb is produced (again at the expense of decreasing the percentage of oxyhemoglobin in whole blood). Remember that cigarette smoking and exposure to automobile exhaust also raise the level of carboxyhemoglobin in the blood. Therefore, simultaneous exposure of a patient to cyanide nitrite therapy and carbon monoxide poisoning (an event not uncommon in the burning of plastics) may very seriously lower the levels of oxyhemoglobin in the blood, with resulting tissue ischemia. Furthermore, many laboratory methods of measuring oxygen saturation are inaccurate if the patient has been exposed to carbon monoxide. Because oxyhemoglobin is the only hemoglobin species (both A1 and A2) capable of transporting oxygen in adult human blood, a lowering of it significantly lowers the ability of hemoglobin to transport oxygen to the cells of the body. Additionally, the *rapid* shift from aerobic to anaerobic metabolism with the subsequent rapid production of *metabolic acidosis* produced with

cyanide intoxication depresses the heart's muscular strength and its ability to pump blood. This, in turn, lowers both the heart's ejection fraction and cardiac output, thus further decreasing oxygen delivery to the tissues.

Data in children clearly show that supportive care only, without the administration of any antidotes, increases survival after cyanide poisoning. It is suggested in children that, in the presence of high levels of carboxyhemoglobin (secondary to carbon monoxide exposure) and cyanide exposure, only sodium thiosulfate, along with aggressive supportive care, needs to be administered. Despite the inability of the mitochondria to use oxygen after cyanide exposure, treating the patient with 100% oxygen statically improves survival for unknown reasons.

Because severe exposure to both cyanide and nerve agent produces seizures in patients, a table to help clinically differentiate the two etiologies is provided (Table 24.3). Additionally, children's doses of antidotes are shown in Table 24.4.

Standard pulse oximeters (routinely found in a variety of medical settings) and standard blood gas measuring equipment will give inaccurate measurements if carboxyhemoglobin is present. This is because the extinction coefficients of light for carboxyhemoglobin and oxyhemoglobin at the 660 nm wavelength, the wavelength at which these devices measure oxyhemoglobin saturation are almost identical. Consequently, the oximeter will read the presence of carboxyhemoglobin as oxyhemoglobin and report an *inaccurately high reading* of the latter. Standard blood gas analysis

TABLE 24.3
Comparison of Clinical Features of Poisoning with Cyanide, Nerve Agent, and Botulinum Toxin

Cyanide	Nerve Agent	Botulinum Toxin
Rapid loss of consciousness	Rapid loss of consciousness	Delayed mental status change (from hypoxia)
Normal or dilated pupils	Miosis (until shortly before death)	Mydriasis with ptosis/weakness
Few secretions	Copious secretions	Normal to dry with Dysphagia
Muscle twitching present	Muscle twitching present	Progressive weakness
Fasciculations absent	Fasciculations present	Fasciculations absent
No or late cyanosis	Early cyanosis	Late cyanosis
Easy to ventilate after termination of seizure	Difficult to ventilate due to increased airway resistance	Easy to ventilate
Little or no increased sweating	Greatly increased sweating	
Lack of increased bowel sounds on auscultation of abdomen	Increased bowel sounds on auscultation of abdomen	Decreased motility
Possible cherry red color of skin and retina	—	
Initial sudden, rapid increase in depth and rate of respiration above baseline (carotid body stimulation)	Depth and rate of respirations usually not *initially* significantly increased	Normal then decreased
No response to, or initial improvement with, nerve agent therapies followed by rapid deterioration	Response to nerve agent therapies	No response to nerve agent therapies
Rapid development of metabolic acidosis	Slower development of metabolic acidosis	
May improve with just supportive therapy	Will not improve with just supportive therapy	Requires antitoxin

The above table was compiled by Michael R. Jones, M.D. and Stephen A. Pulley, D.O. from various sources.

TABLE 24.4
Nerve Agents in Children: Guidelines

Symptoms	Triage Level: Disposition	Atropine Correct Hypoxia before IV Use (Risk of Forsades, Vfib)	Pralidoxime	Diazepam May Use Other Benzodiazepines (e.g. Midazolam)
Asymptomatic	Delayed: Observe	None	None	None
Miosis, mild rhinnorhea	Delayed: Admit or Observe prn	None	None	None
Miosis and any other symptom	Immediate - Moderate: Admit	0.05 mg/kg IV or IM • repeat as needed q5-10 minutes until respiratory status improves	25-50 mg/kg IV or IM, may repeat q 1 hour. • Watch for: ⇒ muscle rigidity ⇒ laryngospasm, ⇒ tachycardia	For any neurologic effect: • 30 days to 5 years – 0.05 to 0.3 mg/kg IV to a max of 5mg/dose. • 5 years and older–0.05 to 0.3 mg/kg IV to a max of 10 mg/dose. May repeat q15-30 minutes
Apnea, Convulsions, Cardiopulmonary Arrest	Immediate - Severe: Admit intensive care status	0.05–0.1 mg/kg IV, IM, per ETT • no maximum • repeat q5-10 minutes as above	25-50 mg/kg IV or IM as above	See above

Consider other supportive agents as indicated: Oxygen, Bronchodilalors, Analgesics, Mydriatics, Environmental protection

From the Department of Pediatrics of the Walter Reed Army Medical Center. The views expressed in this chart are those of the author and do not reflect the official policy of the Department of the Army, Department of Defense, or the United States Government.

does no better because it only measures the *partial pressure* of oxygen, and then from internally stored normograms (which are based on normal oxygen hemoglobin dissociation curves for hemoglobin A1), calculates oxygen saturation. It does not *directly* measure the percentage of saturation of oxyhemoglobin, nor does it *directly* measure the percentage of oxyhemoglobin in the total hemoglobin content of whole blood. If exposure to carbon monoxide is suspected, the laboratory should be notified to run the blood gas on a CO-oximeter. This is a device that *does directly* measure the *percent concentration* (content) of each of the following hemoglobin species in whole blood: oxyhemoglobin (the only species capable of carrying oxygen), carboxyhemoglobin, MetHb, and reduced hemoglobin (Martin, 1999, 2006). Higher end models of CO-oximeters can also directly measure the percent concentration of fetal hemoglobin and sulfhemoglobin in the blood. It also *directly* measures total hemoglobin content in g/dL, as well as the percentage saturation of oxyhemoglobin. Accurate calculations of the oxygen content in the blood can be made from this data. One merely has to multiply the blood's oxygen content by the patient's cardiac output to obtain the total oxygen available to the tissues at any point of time. Keeping in mind that normal hemoglobin has 200–250 times greater affinity for carbon monoxide than oxygen, coupled with the rapid development of metabolic acidosis, which decreases the heart's muscular ability to pump blood, one can see how the body can rapidly develop a very serious deficit in oxygen-carrying capacity in the presence of carbon monoxide. Additionally, this deficit in oxygen-carrying capacity will only be further increased by increased MetHb production arising from the nitrite drugs used for treatment of cyanide toxicity.

C. Cholinergic/Nerve Agent Toxidrome

Another toxidrome is the cholinergic toxidrome, also known as nerve agent. This toxidrome is caused by organophosphates and carbamates, which are commonly used in pesticides and thus another common substance in our society. Related organophosphates are found in nerve agents. These substances bind to and thus disable functioning of acetylcholinesterase (AChE). This is needed to turn off nerve impulses at the synaptic junction once acetylcholine neurotransmitters have delivered their messages, leaving these nerve circuits fully switched on. The symptoms derive from the progressive turned on state of these nerves. The major difference between the two is that organophosphates bind irreversibly, whereas the carbamates bind reversibly. Both muscarinic and nictotinic receptors are affected. However, you will find both features present in the cholinergic toxidrome. During the middle period, there can be alteration as sympathethic and parasympathetic try to predominate.

There are common acronyms that are used in discussion of this syndrome. Muscarinic receptors cause leaking symptoms of ''SLUDGE'': salivation, sweating, airway secretions; lacrimation; urination; defecation; gastrointestinal (upset); and emesis. Nicotinic symptoms are the weekdays ''MTWHF'': miosis; tachycardia; weakness; hypertension, hyperglycemia; and fasciculations. In addition, there is a progressive spectrum of central nervous system ''C's'': confusion to convulsions to coma. As the syndrome progresses, there can at first be excitatory symptoms of tachypnea and tachycardia that later progress to bradypnea and bradycardia as weakness, then flaccid paralysis and resulting hypoxemia develop. Bronchospasm can also be a feature.

Chemical warfare nerve agents are inhibitors of cholinesterases. They prevent cholinesterases from performing their normal function of hydrolyzing (metabolizing and thereby eliminating) acetylcholine and perhaps other substances. Therefore, excess acetylcholine remains in circulation, which hyperstimulates the active site of glands, muscles, and nerves, resulting in a cholinergic crisis.

Three cholinesterases exist within the human body (1) AChE, aka true cholinesterase, red cell cholinesterase, and whole blood cholinesterase. AChE normally hydrolyzes acetylcholine to acetic acid and choline. Although AChE is found in the cells of all body organs, the amount of AChE in the cells of each organ varies in concentration. One type of cell particularly rich in AChE is the red blood cell. The AChE found at nerve endings is anchored to the plasma membrane through a

glycolipid (King, 2006), and this cholinesterase is often referred to as ''tissue cholinesterase.'' Other human cholinesterases include (2) butyrylcholinesterase (BuChE), aka plasma cholinesterase, pseudocholinesterase, and (3) carboxylesterase (CaE). The concentrations of each of these vary from organ to organ. In humans, CaE is only contained *within* cells, not within plasma, as is the case in other animal species. All three of these cholinesterases combine with nerve agents, and all are inhibited by them. Pharmacologists hope to be able to employ both BuChE and CaE as bioscavengers of NA in the future, thus decreasing the amount of nerve agent reaching the active sites of AChE.

Although the normal function of BuChE and CaE in the human body is unknown, it is known that BuChE is responsible for hydrolyzing drugs that contain ester linkages. These include ester local anesthetics, such as cocaine, procaine, chloroprocaine, and tetracaine; the depolarizing muscle relaxant succinylcholine; and the nondepolarizing, neuromuscular blocking drug mivacurium (Jensen et al., 1995). Other drugs hydrolyzed (broken down by combining with water) by plasma cholinesterase include the short-acting beta-blocker esmolol, remifentanil (an opiod), the intravenous anesthetic induction drug etomidate (often used in situations of cardiovascular instability), propanidid (not available within the United States), monoamine oxidase inhibitors (antidepression medications), and the anticancer medication methotrexate (Iohom et al., 2004). Moreover, human carboxylesterase 1 hydrolyzes heroin and cocaine (Redinbo et al., 2003).

If BuChE is inhibited (prevented from performing its usual metabolic activity) by nerve agent (NA) intoxication, the hydrolysis (breakdown by combining with water) of drugs containing ester linkages would also be impaired. Consequently, toxic levels of these drugs would be more easily achieved, and the normal metabolic (physiological) actions of these drugs would continue for a much longer period of time than usual. As an example, the duration of the neuromuscular blocking drug, succinylcholine, which produces total paralysis of all skeletal muscles, including those of respiration, is usually but a few minutes. During these few minutes, artificial ventilation is provided for the patient. With inhibition of BuChE produced by NA, this drug's effect could persist for *many hours*, greatly extending the period of time that the patient would require artificial ventilation. Unconsciousness produced by etomidate could be extended from a few minutes to hours.

One extremely well-known action of cocaine is that it prevents the presynaptic reuptake of norepinephrine and dopamine (The Answer Page, May 3, 2006). In addition to raising a patient's blood pressure, heart rate, and the strength of myocardial (heart) contraction, cocaine also causes vasoconstriction (blood-vessel narrowing), particularly of coronary arteries, thus causing increasing artery resistance and decreased blood flow to the muscles of the heart. This creates an increased need for oxygen by the heart, while simultaneously decreasing its oxygen supply. This situation is perfect for creating myocardial ischemia (lack of oxygen to the heart muscles) and a subsequent myocardial infarction (heart attack) (The Answer Page, May 4, 2006). Cocaine is normally hydrolyzed by BuChE, and the above mentioned action quickly decreases and then stops. However, if cocaine were not hydrolyzed (secondary to inhibition of BuChE by nerve agent) this action could go on for hours, and the chance of having permanent heart damage (i.e., heart attack) would grow geometrically.

In addition to the organophosphate nerve agents and insecticides (organophosphates and carbamates), certain drugs employed in clinical medicine also inhibit cholinesterases. These include edrophonium and the carbamates pyridostigmine (Mestinon), neostigmine (Prostigmin), and physostigmine, all of which are used to treat myasthenia gravis and reverse competitive neuromuscular blocking drugs employed in the operating room. The first generation of anti-Alzheimer's drugs are also cholinesterase inhibitors, including donepezil (Aricept), rivastigmine (Exelon), and galantamine (Reminyl). Memantine (Namenda), a second generation of anti-Alzheimer's drug, is not a cholinesterase inhibitor, but rather a moderate affinity NMDA receptor antagonist. The drug echothiophate (Phospholine Iodine), used in the treatment of chronic open angle glaucoma, is also an organophosphate and a cholinesterase inhibitor (Medscape, 2006). There is some evidence that phenothiazines may also inhibit cholinesterase (Keeler, 1990).

Field treatment of nerve agent exposure by U.S. Army medics employs a diagnosis and treatment matrix. The intoxication and treatment categories are vapor exposure—mild, moderate,

TABLE 24.5
Nerve Agent Diagnosis and Treatment 2 × 3 Matrix

Nerve Agent Treatment Goals Summary

Vapor		Liquid	
Symptoms	**Treatment**	**Symptoms**	**Treatment**
Mild vapor: Miosis and rhinorrhea only	Mild: None unless severe rhinorrhea then one Mark I kit	Mild: Localized twitching and sweating	Mild: One Mark I kit
Moderate: Mild symptoms plus moderate, or severe, dyspnea	Moderate: Depending upon severity of dyspnea 1–2 Mark I kits	Moderate: GI effects of vomiting, diarrhea, and abdominal cramps	Moderate: Depending upon degree of GI symptoms 1–2 Mark I kits, and observe for 18 h

Severe	
Symptoms	**Treatment**
Severe vapor or liquid: Unconsciousness or seizing or postictal or apneic, or showing effects in two or more systems (i.e., airway, GI, muscular, GU, CNS) thus showing systemic distribution	Three Mark I kits and CANA (10 mg diazepam autoinjector) and ventilation as appropriate

Source: Jones, EMS response, pre-hospital care and patient management, slide 355, 2003a.

and severe—and liquid exposure—mild, moderate, and severe. A slightly modified version of this 2 × 3 matrix is shown in Table 24.5. Employment of such a matrix is becoming more common in the civilian EMS community (Society of Michigan EMS Instructor Coordinators). The treatment of mild and moderate nerve agent intoxication will not be dealt with in this discussion. The reader is referred to Table 24.5 for treatment details.

Severe nerve agent intoxication, characterized by loss of consciousness, respiratory distress, possible development of flaccid paralysis, and the development of status epilepticus, must be treated promptly and aggressively. Again, the *A*irway, *B*reathing, *C*irculation, *D*econtamination, *D*rugs (ABCDD) approach is totally appropriate. Drugs used in the treatment of nerve agent intoxication are divided into three classes—anticholinergics represented in the United States by atropine, the cholinesterase reactivators represented in the United States by 2-PAM, and antiseizure drugs represented in most countries by the benzodiazepines (diazepam, midazolam, etc.).

Status epilepticus, more appropriately defined as excessive electrical hyperactivity within multiple and increasing numbers of nerve cells (neurons), essentially ''fries'' and destroys the cellular integrity of the neuron. ''Fried'' (dead) neurons do not function; hence, the longer status epilepticus continues, the more neurons will die and therefore lose functional capacity. This situation increases the likelihood of permanent brain damage. The take-home message is that *the longer status epilepticus continues, greater will be the degree of permanent brain damage*.

Classically, the benzodiazepine diazepam (Valium) has been employed for seizure control. However, new evidence clearly shows that midazolam (Versed) is a far superior drug for this purpose. Animal data generated in the laboratory of McDonough and Shih (1997) shows that midazolam requires a smaller effective dose on a mg/kg basis than diazepam, and the time from administration to effect is generally shorter than with diazepam. Currently, the Department of Defense is evaluating midazolam as a potential improvement over diazepam for nerve agent poisoning. Studies are currently underway in Dr. McDonough's laboratory to have midazolam approved by the Food and Drug Administration for treatment of nerve agent seizures. Once that

approval has been obtained, the military's Convulsant Antidote Nerve Agent (CANA) autoinjectors will be changed from diazepam to midazolam. One should remember that the military CANA autoinjector is *currently* diazepam, 10 mg. In a mass casualty scenario, autoinjectors of CANA might accidentally be delivered when autoinjectors of 10 mg diazepam are requested, and one should remember that they are currently one and the same. Although medics in the field are bound by treatment protocols, physicians may use drugs off-label, and consideration by physicians, based on valid animal research, in using midazolam for nerve agent seizure control would seem appropriate (Newmark, 2005). See Chapter 4 for discussion of midazolam as a replacement for diazepam.

A nerve agent seizure should be likened to a snowball at the top of a hill. Near the top of the hill, the snowball is small, and a small barrier will stop its progression down the hill (a small dose of antiseizure drug will terminate the seizure), but as the snowball proceeds down the hill, it picks up size and speed (involves more neurons in the seizure process), and at the bottom of the hill, it has grown into a large ball, which requires a large barrier to stop it (a large dose of antiseizure medication to terminate the seizures). The dose of antiseizure medication required to terminate the seizure increases as the duration of seizure increases. This increased dose follows a geometric, not arithmetic, model. The likelihood that one 10 mg dose of diazepam i.m. or i.v. (or the equivalent dose of midazolam) would be sufficient to terminate nerve agent seizures would be highly unlikely. Benzodiazepines used for this purpose must be *titrated to effect*.

Another extremely rare, but troubling clinical situation, would be the patient who presents with a nonconvulsive seizure. This patient would be unconscious, yet flaccid, and not show outward signs of convulsion. To all clinical appearances, this patient would be postictal, but would not later regain consciousness. In fact, electrical seizure activity in the brain would still be continuing, and consequently, the patient would continue to deteriorate, but for debatable reasons (depletion of ATP vs. development of a phase II block), the peripheral muscle activity known as convulsions would have terminated. Some investigators feel that examination of the vital signs (increase in blood pressure and heart rate) and eye movements might give a clue that this situation is occurring. Unfortunately, in the majority of cases, this diagnosis can only be made with the use of an EEG or other brain wave monitoring device, and in a mass casualty situation, the proper diagnosis would most likely be missed. The subsequent permanent neurological brain damage would be massive and irreversible. Perhaps modification to the computer-stored database of small, lightweight, portable brain wave monitors (to include the addition of an external coat of chemical resistant paint) used in the operating room to assess awareness during anesthesia could be used to diagnose seizure activity in the absence of convulsions. Such monitors are already being evaluated to detect seizure activity in the brains of patients who have been placed into a drug-induced coma (artificially ventilated and paralyzed) in the intensive care unit. A BIS monitor (employing a modified database) would be an example of such a monitor.

McDonough and Shih (1997) have taught us a second, extremely important lesson about control of seizures induced by nerve agents. New evidence has shown that the driving force behind the seizure quickly changes once the seizure has begun; hence, appropriate seizure control medication must also change. McDonough and Shih have presented a three-stage model. In the first stage, represented by the first few minutes of seizure activity, inhibition of brain cholinesterase by the nerve agent causes excess production of acetylcholine, which in turn overstimulates brain cholinergic receptors (principally muscarinic) and initiates the seizure. During this period of time, the anticholinergic medication, atropine, in appropriately large doses, is single-handedly capable of terminating the seizure. However, this period is very short-lived and begins to decline only a few minutes after initiation of the seizure. The second phase begins after a few minutes of seizure activity with a rapid and steady increase in the concentration of circulating excitatory amino acids (EAA). Although the production of excessive amounts of acetylcholine continues uninterrupted during this second phase, there is a concurrent decline in cholinergic stimulation as the etiology of seizure production. Moreover, there is a corresponding, steadily increasing influence of circulating EAA as the driving etiology of seizure production. During this mixed period, anticholinergic

medications progressively become less effective for seizure control; however, $GABA_A$ stimulating drugs, characterized by the benzodiazepines (diazepam, midazolam, etc.) remain effective, although requiring geometrically increasing doses for seizure control as the duration of seizure activity continues. The third phase of this model of seizure production is characterized by the complete lack of cholinergic mediated seizure production, with EAA production now accounting for 100% of the etiology of the continuing state of status epilepticus. These EAAs are principally represented by glutamate and asparate which combine with NMDA receptors. During this phase, drugs that block the receptor sites of EAA, that is, NMDA receptors, must be employed. At the present time, we lack sufficiently effective drugs in this category. Research continues in an effort to provide us with suitable NMDA receptor blocking drugs. The antiParkinsonian and antispasmodic anticholinergic drugs, aprophen, azaprophen, benactyzine, biperiden, procyclidine, and trihexylphenidyl, continue to stop seizures for a much longer period of time than atropine and scopolamine and therefore probably contain some low-level NMDA receptor antagonistic effect (McDonough and Shih, 1997).

The classical endpoints of atropine administration have been the drying of secretions and the easing of ventilation. So what is the upper dose limit of atropine? Although older literature would give some upper cumulative dose limit in mg, newer thinking has produced the answer that there is no upper limit to the dose of atropine employed (Urbanetti, *Toxic Chemical Training Course for Medical Support Personnel, September 30–October 4, 2002*, Personnel communication) and that this drug should be *titrated to effect* but avoiding toxicity. This is especially true in light of atropine's ability to stop nerve agent seizures in laboratory animals if given early enough and in a sufficiently large dose (McDonough and Shih, 1997). However, because of species specificity, what is true in laboratory animals is not necessarily true in human beings. This is where the experience of Dr. Syed Abbas Foroutan enters (Foroutan, 1996). Dr. Foroutan is an Iranian physician who had recently graduated from medical school at the outset of the Iran–Iraq War (in the l980s). He was placed in charge of the Iranian equivalent of our chemical casualty care service. The Iranians are the only people who have ever encountered *massive numbers of wartime nerve agent casualties* (approximately 40,000) in an environment where people were actually trying to kill them. Everyone else's experience is limited to accidental laboratory or rarely, field exposure, or volunteer low-dose exposure in a controlled environment. By definition Dr. Foroutan became an expert in the field of treatment of chemical casualties, and his experiences are therefore invaluable. After the war, he published a series of articles in a Farsi language medical journal in which he describes his treatment of mustard and nerve agent casualties. Although some scientists have criticized his accounts as being anecdotal and uncontrolled (it is hard to run controlled experiments when someone is attempting to kill you), his encounter with these agents was similar to what EMS personnel would encounter in the field, except that hopefully EMS personnel would encounter smaller quantities and lower concentrations of agents. At Bioscience Review 2002, Dr. Jonathan Newmark (a neurologist) presented a summary of Dr. Foroutan's writing that he later published (Newmark, 2004). Dr. Newmark compared and contrasted U.S./NATO doctrine of nerve agent treatment to the Iranian experience during the Iran–Iraq War. Dr. Foroutan employed the ABCDD principle of treatment for severely injured patients. Oximes (obidoxime) were employed only in severely intoxicated casualties and then in limited amounts (small doses), probably due to short supply. Consequently, Dr. Foroutan's drug treatment of severely injured nerve agent casualties relied principally on atropine, supplemented by a one time dose of the oxime obidoxime, and a small dose (10 mg) of diazepam. Dr. Foroutan states: ''Rapid (early) application of (high doses of) atropine has special effects on the course of the disease, e.g., prevention of delayed shock; when applied later and more slowly, atropine does not have this effect.'' He goes on to state: ''The administration of high doses of atropine must therefore be commenced as early as possible and the required dosage administered as rapidly as possible. A further advantage of this treatment lies in the fact that patient (sic) whose evacuation behind the front line lasts several hours do not require any more atropine'' (Helm, 1999). He gave a 4 mg i.v. test dose of atropine, followed by rapid administration of 5 mg i.v. over 5 min. Administration of atropine was titrated against pulse

rate using an increase in pulse of 20–30 beats/min as indicating initial atropinization. He would decrease the rate of atropine administration if the pulse rate was >110 and increase the rate of administration for pulse rates of <60–70 beats/min. In addition to the classical endpoints for atropine administration of drying of secretions and easing of ventilation, he added that the disappearance of miosis, or even the appearance of mydriasis, as an indication to decrease or stop atropine administration ''even if the patient's mouth has not completely dried.'' For severely intoxicated patients, he used between 20 and 200 mg of atropine i.v., and related giving up to 50 mg i.v. atropine every 5 min, with a total dose of up to 200 mg in 10–15 min (Newmark, 2004). These doses seem heroic by Western standards. His above quoted statements concerning the effectiveness of atropine used *early and in sufficient doses,* with a corresponding lack of effect if applied later in the course of treatment in humans, would appear to support McDonough and Shih's work. Although we are in no way advocating the use of Dr. Foroutan's protocol in the United States at the present time, his experiences in treating humans in a wartime environment with rapidly applied (i.e., given *shortly* after intoxication) high-dose atropine, low-dose oxime, and low-dose benzodiazepine therapy, coupled with McDonough's and Shih's laboratory data in animals, certainly shed light on other possible treatment modalities and warrant further clinical investigation.

Atropine has another positive effect in the cardiovascular management of nerve agent casualties. Animal studies have shown that nerve agent intoxication can cause a decrease in peripheral vascular resistance resulting in hypotension and tachycardia. Acetylcholine produced during the cholinergic crisis of nerve agent intoxication stimulates muscarinic nitric oxide synthase receptors in the endothelium of blood vessels, and this precipitates the production of nitric oxide in these same endothelial cells. Nitric oxide is well known as a vasodilator of the smooth muscle of blood vessels, as well as causing platelet aggregation (Sigma-Aldrich, 2006). Because atropine competitively blocks these muscarinic sites, its use will prevent both the smooth muscle vasodilatory and platelet aggregating effects of nitric oxide at the endothelial level.

One should note that in hypoxic animals, i.v. administered atropine has given rise to ventricular tachycardia. Although this has never been documented in humans, it has been suggested that perhaps the first dose of atropine in the hypoxic individual should be given i.m. to allow promotion of ventilation and consequently, a partial correction of hypoxia before employing i.v. atropine to decrease the remote possibility of the above phenomena occurring. This point is debatable.

It is important to realize that miosis is caused by contraction of the papillary muscles and that these muscles lack traditional capillaries. In reality, these eye muscles are isolated from the rest of the bloodstream. The nutritional needs of these muscles are supplied by the humors of the eye, not blood vessels, and the effect on them by *liquid* nerve agent not directly applied to the eyes would be dose and time dependent. Small doses of *systemically absorbed liquid* nerve agent would probably not cause the pupils to constrict; medium doses might, and large doses probably would, cause miosis. In any case, the effect would take a period of time to develop and therefore, with nonocular *systemically absorbed liquid* nerve agent exposure, absence of miosis should *not* rule out nerve agent exposure.

With liquid nerve agent exposure, moderate intoxication will yield GI symptoms of nausea, vomiting, abdominal pain and cramps from increased peristalsis, increased GI secretions and involuntary defecation, etc. If the *only* sign of GI symptoms is nausea, one should consider the possibility that this effect could also be due to vapor nerve agent exposure precipitating miosis in the eyes. It is well documented that the development of miosis can cause nausea.

A patient exposed to liquid nerve agent and not showing any signs or symptoms of intoxication should be watched for a period of at least 18 h. This is the longest reported time frame for signs and symptoms of liquid nerve agent exposure to develop. Generally, the longer it takes for signs and symptoms to develop, the milder the physiological changes will be.

Three things can occur when liquid nerve agent (or any liquid chemical warfare agent) comes in contact with the skin. It will evaporate into the atmosphere, be removed by the decontamination process, or be absorbed into the skin and then into the systemic circulation. Evaporation and

decontamination will *not* remove any liquid agent that has already been absorbed into the skin, hence the need for early decontamination. The *exact same dose* of liquid chemical agent can be applied to the skin in one of two ways, as a single larger drop, or as multiple, smaller drops. Because multiple smaller drops present more surface area than the larger single drop, evaporation of the CWA will be greater and the absorbed dose will be less with multiple small drops than the single larger drop of the same total dosage. The presence of body surface hair (Marrs et al., 1996) and covering of the skin by clothing decrease evaporation of liquid CWAs and thus increase systemic dermal uptake of these agents.

A significant difference between organophosphate insecticides and organophosphate nerve agents is that the former generally have higher fat solubility, whereas the latter generally have lower fat solubility. This means that with insecticide intoxication fat stores may give up the agent over a long period of time, and blood levels may repeatedly reach concentrations sufficient to cause bouts of acute toxicity including seizures. Severe intoxication may last for days or longer. This is usually not the case with nerve agents as they generally possess lower lipid solubility. Consequently, lesser amounts of nerve agent are stored in the fatty tissues. As a result, there is less nerve agent to be released from fat stores, and blood concentrations sufficiently high to cause development of status epilepticus usually occur only once with initial intoxication.

Exposure to low doses of nerve agent insufficient to cause physiological changes may result in psychological and mental status changes. Such patients may encounter forgetfulness, irritability, impaired judgment, agitation, anxiety, depression, decreased comprehension, nightmares, sleep disturbances, slurred speech, insomnia, expression becoming difficult, and poor concentration.

In a methodology designed to produce lower mortality and morbidity for soldiers who might become exposed to the particularly difficult to treat nerve agents, such as soman (GD), tabun (GA), and cyclohexylsarin (GF), soldiers are given oral pyridostigmine *prior* to nerve agent exposure in an attempt to hide a percentage of their cholinesterases from inhibition by these nerve agents should exposure occur. Because the binding site of the affected cholinesterases would already be bound to pyridiostigmine (a carbamate) these cholinesterases could not become combined with, and thereby inhibited by, nerve agent on exposure to it. In essence, such cholinesterases would be hidden from the nerve agent. However, unlike nerve agents, carbamates only *temporarily* combine with cholinesterases, and later the carbamate will spontaneously dissociate from these cholinesterases. This dissociation process will be complete within only a few hours. The uninhibited (protected) cholinesterases liberated from carbamates can then resume their normal physiological functions. In the case of AChE, this would include the hydrolysis of acetylcholine, thus terminating the cholinergic crisis. On the other hand, once a patient has *already been exposed to nerve agent*, giving the patient pyridostigmine (or any other carbamate) *after* exposure will only *increase* the cholinergic crisis. This occurs because *none* of the cholinesterase would be protected from inhibition by the previously administered nerve agent. The administered carbamate would be read by cholinergic receptors as additional receptor site stimulating material, thereby further hyper-stimulating these receptors. Consequently, giving carbamate *after* nerve agent exposure should be avoided at all costs.

Oxime therapy is aimed at reactivation of cholinesterases. Atropine will only work against muscarinic sites and not against nicotinic sites. The neuromuscular junctions in skeletal muscle and the sympathetic chain (preganglionic) ganglions exemplify human nicotinic sites. Therefore, oxime therapy is all that is available to combat the signs of nicotinic intoxication including fasciculations (irregular movements of small groups of muscle tissue, which appear as worm-like, irregular muscle movements just below and easily visible through the skin), skeletal muscle weakness, and skeletal muscle flaccidity. Antinicotinic treatment of severe intoxication would include the immediate injection of three autoinjectors of 2-PAM Cl (600 mg $\times$ 3 = 1800 mg), or 1–2 g of 2-PAM Cl (aka pralidoxime or 2 pyridine aldoxime methyl chloride) diluted in 100 cc of 0.9% NaCl and infused intravenously over 20–30 min. More rapid intravenous infusion of 2-PAM Cl may result in hypertension that would then be treated with intravenous injection of 5 mg of phentolamine. Signs

of 2-PAM Cl toxicity (overdose) include dizziness, blurred vision, diplopia, nausea and vomiting, circumoral paresthesias, and muscle weakness. More serious complications include unconsciousness, seizures, apnea, and flaccid paralysis (State of New York, unknown year). 2-PAM Cl is rapidly excreted in the urine, probably by tubular secretion. Heat, exercise, and thiamine, and decreased renal function all decrease renal excretion of 2-PAM Cl.

Experimental pharmacological models show that with the intermittent i.v. dosing protocol as noted above, blood levels of 2-PAM Cl will fall below therapeutic levels in 90–120 min after administration of the drug. Researchers suggest a new i.v. dosing schedule, confirmed in human volunteers, which significantly increases and maintains (by approximately a factor of 3) the blood level of 2-PAM Cl. This new dosing regimen suggests 1–2 g administered i.v. as described above, followed by 500 mg i.v. every hour, though some patients may require higher doses. Although the duration of oxime therapy is controversial, these authors (toxicologists) suggest continuing oxime therapy for at least 24 h after the symptoms resolve (Wiener and Hoffman, 2004).

Based on multiple factors (total dose of atropine required; aging half-times of the nerve agent; 2 vs. 4 position of the oxime group ($-C=N-0H$) on the pyridine ring; the number of oximes, both experimentally and commercially available, capable of reactivating cholinesterase; and the percent of total cholinesterases that the oxime will reactivate) sarin (GB) and VX usually respond best to treatment. Tabun (GA) and cyclohexyl sarin (GF) are less responsive to treatment, and soman (GD) is the most difficult (least responsive) to treat.

D. Corrosive Agent/Vesicants Toxidrome

Corrosive agents yield the corrosive toxidrome, also known as vesicants. These agents are the acids and bases. Two additional substances are white phosphorous and oxidizers. Any agent that reacts with the skin is corrosive. A major difference between how acids and bases affect the skin is in the type of reaction. When an acid reacts with the skin, it creates a hard coagulum, or scab, that limits deeper penetration of the acid and thus the degree of injury. On the other hand, bases do not create this scab, so the damage can continue to the deeper tissues. Instead of the hard coagulum, the base injury is slippery, actually soapy. Oxidizers react with the skin to create a rapid exothermic reaction and tissue destruction. White phosphorous reacts spontaneously with the air to create a fire burn injury. Generally, the injuries will be cutaneous. However, should the chemicals enter the body through respiration or ingestion, they will cause the same corrosive effects with the tissues they come into contact with.

Two corrosive agents require special mention. The first is sulfur mustard, which has been used extensively in chemical warfare. This agent exerts its effects in a delayed manner; therefore, symptom onset may occur many hours later. As with any chemical agent, higher the environmental temperature, more the agent will vaporize, mustard is no exception. This allows for greater involvement of the mucous membranes and lungs through ingestion and inhalation. It is believed that the effect is at the DNA level, culminating in cell death and inflammation. One initially sees skin erythema that progresses to blistering, followed by necrosis. The same can occur in the pulmonary tree. The other agent is hydrofluoric acid. It is commonly used in the electronics industry. Although as a weak acid it can cause chemical burns, its more important property is that it binds up intracellular calcium and magnesium. It causes severe, neuropathic type burning pain and interferes with muscle function, to include the cardiac myocardium. Though an acid, it can burn deeper because it is weak and does not cause the dense scabbing.

Unfortunately, vesicant exposure creates a huge problem because microscopic cellular damage occurs within very few minutes of exposure, but at a minimum, outward physical signs of that damage does not normally occur for at least 2 or 3 h after exposure, though usually longer. While phosgene oxime produces *immediate* pain on skin contact and Lewisite produces pain usually within *1 min* after skin contact, sulfur mustard produces absolutely *no* pain on contact with the skin. As much as people do not like pain, pain is an extremely important protective mechanism for

the body. If one places one's hand on a hot stove, the pain generated causes the individual to withdraw his hand before serious cellular injury can occur. However, without pain, there is no impetus to withdraw the hand from danger, or to even know that such danger exists. Such is the case of sulfur mustard; if contact with it exists for more than 2 min, irreversible structural damage within the body's cells will take place. Indeed, an old U.S. Army Chemical School rite of passage was to place dilute sulfur mustard on both forearms near the hand. This diluted drop was placed on the skin over either the ulnar or radial artery. One forearm was decontaminated within 2 min, and no residual cellular damage became evident, either immediately or at a later time. On the opposite forearm, decontamination was delayed for 15 min, and although there was no initial hint of cellular damage present, within a day the cellular damage became evident in the form of vesicles and bullae. Indeed, within only 15 min of exposure to the significantly diluted agent, tissue damage was 90% as great as if no decontamination of the forearm had been accomplished (Hurst, *Vesicant Lecture, Field Management of Chemical and Biological Casualties Course*, June 2001, Personnel communication). After a latency period of a few hours in which no change in the skin occurred, erythema (redness) of the skin became evident. This was followed by the appearance of multiple small vesicles (tiny fluid filled elevations of the skin) that later joined to form bullae (large fluid filled elevations of the skin). Frequently, these bullae would rupture, and if improperly treated, become infected.

Vesicles and bullae created by Lewisite *do* contain small amounts of arsenic, but according to classic teaching, vesicles and bullae from sulfur mustard *do not* contain any mustard agent. Intercellular chemical reactions, which damage the anatomy and physiology of cells, also rapidly degrade the mustard into thioglycol, a component of ballpoint pen ink. Hence, according to this classic teaching, all mustard bullae and vesicles contain only sterile ''tissue fluid,'' which is in no way harmful to patients or responders. However, new research with in vitro human breast skin demonstrates that exposure to *liquid* mustard leaves a constant reservoir of sulfur mustard representing a substantial proportion (14%–36%) of the applied dose for up to 24 h (Chilcott et al., 2000). Therapeutic implications of this finding would stress the need to *not occlude* any skin exposed to liquid sulfur mustard during this period, as up to 80% of the applied dose may vaporize before penetrating the skin (Chilcott et al., 2000). Clearly, only IMMEDIATE decontamination (within a very few minutes) in the field will prevent cellular damage caused by vesicant exposure from occurring.

The only patient exposed to vesicant that may be classified as needing immediate treatment is that individual whose airway is compromised. A hoarse, high-pitched, crowing sound (croup-like) on inspiration, accompanied by retraction of the neck and accessory muscles of respiration, coupled with unusual inspiratory airway sounds, and possibly cyanosis (blue color) of tissues, indicates airway compromise at the level of the glottic opening. This can only be remedied with immediate securing of the airway with endotracheal intubation.

A pre-existing game plan should exist for field EMS/EM to aid them in caring for patients exposed to vesicants. This should include the following:

1. Assurance that no airway obstruction is present.
2. Prompt decontamination.
3. Prompt flushing of the eyes with copious amounts of saline.
    a. Consideration may be given for a new experimental therapy, which has shown great promise in experimental animals in reducing eye pathology. This experimental therapy is accomplished by placing two drops of prednisolone acetate ophthalmic suspension in the eye every 10 min for 2 h. Within the 2 h period, the patient should be seen by an ophthalmologist who can inject a single, 1 ml subtenons injection containing 20 mg of triamcinolone and 50 mg of cefazolin. Experimental rabbits followed for 16 weeks after mustard exposure experienced a significant decrease in the early stages of corneal pathology, as evidenced by decreased corneal thickness, a decreased modified ocular

severity score (MOSS), and only two of seven experimental animals developed neovascularization, as compared to seven of seven in the controls, when this regiment was followed (Babin et al., 2002). The development of neovascularization of the cornea resulted in significantly decreased vision or blindness.

b. Spasms of eyelid muscles are common, but care should be taken not to allow the eyelids to close and stick together, as pus can accumulate under the closed eyelid and place pressure on the eyeball, that over time will decrease blood flow to the eye and may result in blindness. This can be accomplished by applying a light coating of petroleum jelly or Vaseline along the lid edges (Jones, 2003b).

4. Sunglasses should be provided for photophobia, which may develop.
5. If exposure to liquid mustard has taken place, an attempt to estimate the percentage of skin coverage by the agent using the Rule of Nines should be made (see Table 24.2).
6. Provision of general supportive care.

For unknown reasons, mustard exposure may give rise to a cholinergic effect *occurring shortly after exposure*. This cholinergic effect may cause miosis (narrowing of the pupil) resulting in eye pain and dimming of vision, exactly like nerve agent exposure. Gastrointestinal symptoms of nausea, vomiting, and diarrhea occurring within minutes to a few hours (early symptoms) of exposure to mustard may also have been produced from this cholinergic effect. Gastrointestinal symptoms occurring a day or more after exposure (late symptoms) are attributable to destruction of the cellular lining of the GI tract, as well as other etiologies. Painful miosis is treated with topical homatropine eye drops, and the *early* GI symptoms are relieved with a small dose of atropine, that is, 0.4–0.6 mg, although occasionally, greater doses are necessary.

The initial skin presentation following exposure to vesicant agents looks identical to a severe case of skin contact with poison ivy or poison oak. Hence, a key teaching point of vesicant exposure is that erythema of the skin *always precedes* the production of skin vesicles and bullae. If the patient presents with vesicles or bullae *without* preceding erythema of the skin, another diagnosis should be considered (McIntosh, *Toxic Chemical Training Course for Medical Support Personnel, September 30–October 4, 2002*, Personnel communication). As with all mustard damage, damage to the airway is dose dependent, and damage *begins* in the upper airway and *progresses* into the lower airway *as dosage increases* (as contrasted to lung-damaging CWAs). If the patient has only lower airway damage (without upper airway damage), consider a diagnosis other than mustard. For unknown reasons, mustard-burned victims require less fluid replacement than thermally burned victims with the same percentage of skin burns. Laboratory evaluation of a potential sulfur mustard victim should include serial complete blood counts (CBCs), as a shift to the left would imply bacterial infection. It is extremely important to follow white blood counts, as traditionally, white blood cells are the first type of blood cells to decrease due to the bone marrow suppression initiated by mustard exposure. Moreover, lymphocytes are the first of the white blood cells to decrease secondary to bone marrow suppression by the radiomimectic effect of mustard agent; that is, its cellular actions mimic those produced by ionizing radiation. A series of urinary assays for thiodiglycol should be obtained, as this metabolite can be seen in the urine for approximately 7–8 days postexposure. Finally, a punch biopsy of the effected skin should be obtained, looking particularly for histopathological changes indicating separation of the epidermis from the dermis (lack of intracellular bridges) and pyknotic nuclei of the cells.

There is one other key point concerning sulfur mustard. Pure sulfur mustard (HD) freezes at about 58°F. Once frozen, essentially no mustard vapor is released. Consequently, electronic monitoring relying on detection of released mustard vapor would be useless, and contamination of persons exposed to frozen sulfur mustard would not be detected by this type of monitoring. Upon rewarming, both liquid and vapor sulfur mustard would be present on the contaminated clothing.

The Iran–Iraq War (1983–1988) provides us with data on long-term complications of sulfur mustard exposure in the modern era (availability of ICU and antibiotics). In an extremely well

designed and controlled study, patients with severe complications following mustard were eligible to be included. Long-term complications occurred principally in four organ systems: lungs 95%, peripheral nerve 77.5%, skin 75%, and eyes 65% (Balali-Mood et al., 2005). Lung complications included COPD (45%), bronchiectasis (27.5%), asthma (25%), and large airway narrowing (17.5%). Nervous system complications included nerve conduction velocity (NCV) disturbances that were more common in sensory than motor nerves. The two nerves most commonly involved were the sensory tibial (70%–75%) and the sural (65%–72.5%). Electromyography was abnormal in 40% of patients studied. Long-term skin abnormalities included hyperpigmentation (55%), erythematous popular rashes (42.5%), dry skin (39%), multiple cherry angiomas (37.5%), atrophic scar (27.5%), hypopigmentation (25%), hair loss (10%), and hypertrophy (2.5%). Light microscopic examination of the skin revealed epidermal atrophy, hyperkeratosis, basal membrane hyperpigmentation, and nonspecific dermal fibrosis. Electron microscopic examination of skin tissue revealed increased melanocytes and melanosomes within the epidermis and increased collagen fibers and mononuclear inflammatory cells within the dermis. Long-term complications in the eye included prelimbic hyperpigmentation (17.5%), vascular tortuosity (15%), corneal thinning (15%), corneal opacity (10%), corneal vascularization (7.5%), and corneal epithelial defect (5%). In the hematological/immunological system patients having undergone severe mustard exposure showed significantly higher WBC, RBC, HCT, IgM, C3, and CD3+ lymphocytes counts, whereas CD16 + 56 positive (NK) cell count was significantly decreased. A total of 40 severely exposed patients were extensively studied (Balali-Mood et al., 2005).

Key teaching points of Lewisite exposure, in contrast to mustard exposure, include immediate pain on contact (within 1 min), the agent smells like germaniums, vesicles and bullae contain arsenic, and there is *no* radiomimectic effect of bone marrow suppression. However, it should be noted that Lewisite, unlike sulfur mustard, directly damages capillary permeability, giving rise to pulmonary edema, hypovolemia, and circulatory failure. Instability of the circulatory system due to capillary leak syndrome and ensuing hypovolemia are major problems with Lewisite (McIntosh, *Toxic Chemical Training Course for Medical Support Personnel, September 30–October 4, 2002*, Personnel communication). If *liquid* Lewisite contacts the eye, severe eye damage will occur. This eye damage is more severe than that caused by mustard, and a real possibility of blindness exists. Lewisite produces an inflammatory reaction much faster than mustard, but Lewisite lesions heal much faster than mustard lesions. There is less increase in skin pigmentation upon healing of skin burns produced by Lewisite than by mustard. Arsenic contained in Lewisite can give rise to arsenic poisoning, including peripheral neuropathy, nephritis with proteinuria, and encephalopathy (heavy metal intoxication). Unlike mustard, a specific therapy aimed at removing the arsenic is available. A heavy metal chelating agent capable of removing arsenic is used for treatment. Initially, British Anti-Lewisite Agent (BAL) was developed, but it has not been manufactured in years. Other heavy metal chelating agents, however, could be used to reduce the degree of arsenic intoxication. Lewisite, like phosgene and methyl isocyanate, is easily hydrolyzed and consequently, destroyed by water. The ability to produce mass casualties decreases during a rainy day.

E. Hydrocarbon Toxidrome

The last of the toxidromes relates to hydrocarbons. The hydrocarbon toxidrome is primarily from inhalation of the hydrocarbons. During attempted ingestion, those that are more volatile can find their way into the respiratory system through aspiration. There are three principal effects of exposure to hydrocarbons in gaseous, or aspiration of liquid, states. First, as mentioned in the asphyxiant toxidrome earlier, hydrocarbon gases can displace oxygen and are therefore simple asphyxiants. Once in the bloodstream, they can act as anesthetics, thereby depressing central nervous system function, including breathing. The last principal effect is on the heart. Hydrocarbons can sensitize the myocardium to circulating catecholamines. The result can be a worsening of the fibrillation threshold and resulting premature contractions, tachydysrhythmias, and finally,

ventricular fibrillation. One final property of the hydrocarbons is related to the fact that many are organic solvents. They will defat the epidermis. This will decrease its pliability and cause cracks, which can allow the chemical and other agents or bacteria to enter the skin. If contact is long enough, a chemical burn can also result.

IX. BIOLOGICAL TOXINS

Even though this book is about CWAs, we would remiss if we did not mention three technically biologic agents that produce toxins with significant effects. The first of these is the botulinum toxin, which is produced from the anaerobe *Clostridium botulinum*. It is one of the most toxic substances, over 5000 times more toxic than nerve agents. Though you can have a primary infection causing symptoms, such as that which occurs when infants ingest honey, toxicity is usually from ingestion of the toxin from spoiled food products. It permanently blocks the release of AChE at the nerve endings. Symptoms start with drying of oral secretions, followed by a bilateral progressive bulbar palsy and descending muscular weakness. The corresponding symptoms include oropharyngeal signs of dysarthria and dysphagia, ocular weakness with diplopia and ptosis, and progressive muscle weakness that involves respiratory effort. There is an antitoxin that is available by contacting your local Department of Health, who then contacts the CDC. This is a toxin that should also be considered when entertaining the thought that a nerve agent may be involved (CDC, 2006).

Next is ricin, which is a byproduct of castor oil (from Castor Beans, *Ricinis communis*) production. The directions for preparing ricin are readily available from a number of sources. Only minute amounts are required if ingested. However, inhalation of a large amount can also cause toxicity. It inhibits protein synthesis with a constellation of symptoms that culminates in multiorgan failure and death. There is an onset of symptoms including fever, pulmonary, and GI, followed by arthralgias within 4–8 h. This progresses to mental status changes from drowsiness to confusion, coma, or convulsions. There is then progressive diffuse weakness to cardio–pulmonary–vascular collapse. Treatment is supportive, though mortality is high.

The final biological toxin is Staphylococcal Enterotoxin B, or SEB, from *Staphylococcus aureus*. Aerosolized SEB could render over 80% of those exposed clinically ill. It is a potent immune stimulant that binds to monocytes and provides direct stimulation of T-helper cells and cascade of proinflammatory agents. There is a latent period of 3–12 h after inhalation or 4–10 h after ingestion. Symptoms include nonspecific fever, chills, headache, and myalgias. If ingested, GI symptoms predominate with nausea, vomiting, and diarrhea. If inhaled, you will see respiratory symptoms of nonproductive cough, retrosternal chest pain, and dyspnea. Lung exam and x-ray are typically normal unless there is development of noncardiogenic pulmonary edema and ARDS. Treatment is supportive with a symptomatic phase of 2–4 weeks (U.S. Army Medical Research Institute of Infectious Diseases, 2001; Fry, 2006).

One additional point with ricin and SEB is that the clinical syndrome will vary depending upon the route of entry into the body. If primarily inhaled, the presenting clinical picture will be one of pneumonia and ARDS. If ingested, the initial presentation will be gastrointestinal. If injected, then you will see principally muscular necrosis.

X. SYSTEMATIC APPROACH

The above mentioned symptoms are all the early, classic findings for these toxic syndromes. Later symptoms occur with the onset of hypoxia or inability to use oxygen. At first, the body will sense that there is something wrong in the form of lower oxygen. Stress and intrinsic need for more oxygen will cause an acceleration of respiratory and heart rates and increased anxiety in the victim. If not corrected, the hypoxia will worsen and the patient will flip to slower respirations and heart rate, along with slowing of mentation. Again, if not corrected, death will soon follow.

A rapid, head-to-toe evaluation can lead you to rapidly narrow the differential list of chemical causes. You would evaluate for the ABCDEs:

TABLE 24.6
Head-to-Toe Evaluation to Narrow Differential List of Chemical Causes Areas to Focus On

Head-to-Toe Survey	LDA	Hypoxia	Asphyxiant	HC	Corrosive	NA	BOT
Changed mental status		Late	CN	X		X	Late
Secretions from head	High					X	
Drying/Dysphagia							X
Eyes: red/irritated	High						
Eyes: miosis					SM early	X	
Eyes: mydriasis (dilated)			CN				X
Secretions from torso			CN			X	
Weakness			X			X	
Fasciculations						X	
Seizing			CN			X	
Skin: erythema/no pain					SM early		
Skin: blistered/scabbed					Acid/HM		
Skin: slimy/soapy					Base		
Skin: hot/smoking					Oxidizer/WP		
Skin: dry/cracked/burned				X			
Skin: severe neuropathic pain (skin-minimal)					HF		
Shortness of breath	X					X	X
Rales	Weak						
Irregular heart		Late	X	X		X	
Increased bowel sounds						X	
Accelerated vitals		Early	CN			X	
Cyanosis, pulse ox upper 80s			MetHg				
Cyanosis, changed MS	Late	Late				X	
Pink/red, depressed MS, weak, normal pulse ox			CN/CO				
HA/hypotension			Azides/nitrates				

LDA, Lung-damaging agents/irritant gas; High, Highly water soluble hits upper airways/mucous membranes first; Weak, Weakly water soluble hits lower airways first; HC, Hydrocarbons; NA, Nerve agents/cholinergics; BOT, Botulinum toxin (biologic); Corrosives/vesicants: HF, Hydroflouric acid; SN, Sulfur mustard; WP, White phosphorous; Asphyxiants: MetHg, Methemoglobinemia; CN, Cyanide; CO, Carbon monoxide.

Information for above compiled from: Baker, 2005; Mokhlesi and Corbridge, 2003; Sather and Tantawy, 2006.

1. Establish responsiveness
2. Airway/C-Spine
3. Breathing
4. Circulation/Control Hemorrhage
5. Drugs/Defibrillation/Definitive care/Decontamination/Disability (Neurologic)
6. Exposure/Environmental/Elimination.

While doing this, you would look specifically for features that can clue you into a chemical events presence. The first clue would be having a group of simultaneous victims, especially if they have the same symptoms complexes. The areas to focus on are provided in Table 24.6.

There are few available antidotes that are used in HazMat/chemical weapons medical care. When you have identified a toxidrome with an antidote, it is important to start that antidote

as quickly as possible to prevent worsening of symptoms and to reverse the clinical syndrome (Table 24.7).

This is especially true of organophosphate/nerve agent and cyanide poisoning, as these toxic chemicals can have irreversible effects on the human organism. Oxygen is used liberally to reverse any hypoxic state. It will also reverse carbon monoxide poisoning, but much more slowly than the preferred hyperbaric oxygen therapy. Another hemoglobin asphyxiant, methemoglobinemia, is corrected using methylene blue. Cyanide, a cellular asphyxiant, requires three substances to reverse its toxicity. These are amyl nitrite (not used by the military due to difficulty with controlling the dose), sodium nitrite, and sodium thiosulfate. For organophosphate/carbamate and nerve agent poisonings, immediate agents to use are atropine, pralidoxime (2-PAM Cl), and a benzodiazepine, such as midazolam. There are a couple of other antidotes for syndromes not mentioned above. Hydrofluoric acid exposure requires calcium gel and i.v. calcium chloride to stop the pain and damage. The anticholinergic syndrome caused by hydrazine requires pyridoxine for treatment. Details on some of these antidotes can be found earlier in the chapter. Refer also to Table 24.7.

Methods of drug (antidote) administration would include i.v. or i.m. with a standard syringe, i.m. with an autoinjector, and interosseous in children. Interosseous injection of antidotes has never been studied in children (Rotenberg, 2003). After spot decontamination of the i.v. site has been accomplished (M291 dry decontamination kit is ideal for this purpose since the black color of the dry material will clearly delineate the decontaminated area), an i.v. may be started. Intravenous administration will increase blood levels of drug (antidote) the quickest; however, these blood levels begin to decrease almost immediately as redistribution to tissues begins to take place, and thus, a continuous infusion will be needed to maintain blood levels of antidote. Autoinjectors will more efficiently administer any medication i.m. than conventional i.m. injection. This is because the autoinjector injects under pressure as it advances, separating the individual tissue layers and depositing drug over a much wider area. The medication is evenly distributed throughout multiple layers of muscle tissue. Conventional i.m. relies on aspiration before injection at only one level, and the injection then results in pooling of the drug in one spot. Studies have repeatedly shown that autoinjectors produce higher blood levels of the same dose of drug when compared to conventional i.m. injection; these blood levels are achieved faster; and these blood levels will last longer than conventional i.m. injection. Pediatric autoinjectors of 0.5 and 1 mg of atropine have been introduced in Israel and are currently being introduced into the United States.

XII. PENETRATING/EMBEDDED AGENTS

Patients with open penetrating wounds with embedded material will eventually be treated in either the ER or the OR. Patient clothing contaminated only with gaseous (not liquid) CWA will ''off-gas'' chemical vapor for approximately 30 min after removal from the contaminated area; thereafter it will no longer pose a significant vapor contamination hazard.

If liquid nerve agent is present within the wound embedded material, symptoms may at first respond to medical treatment, then reoccur as additional liquid agent is absorbed into the bloodstream. A search for retained, wound embedded, contaminated material should be started if the clinical course of a patient follows this presentation. This may be either as a single piece, easily seen, or as multiple, small, ''shredded'' pieces along the trajectory of the wound. Removal of this contaminated clothing material should always be accomplished with the use of forceps, never with your hands, as surgical gloves may be easily torn and easily permeated and penetrated by CWAs. Irrigation of the wound should be with sufficient saline (keeping in mind that this irrigation could potentially spread CWAs to chemically uncontaminated areas within the wound). This saline is then removed by a large bore sucker, not with surgical sponges, to minimize the possibility of the surgeon having direct contact with CWAs. Vapor hazard from contaminated pieces of clothing in the wound would only be possibly significant if a large wound was severely contaminated with ''thickened'' (micropulverized acrylate which has absorbed liquid chemical agent) mustard. Wounds

TABLE 24.7
Antidotes Used in HazMat/Chemical Weapons Medical Care

Treatment	Supportive (Fluids/O_2)	Observation	Absolute Rest	Antidote	Irrigation/ Removal[a]
LDA	X	12–48 h	X		
Hypoxia	X				
Asphyxiant:				X	
MetHg	X			Methylene blue	
CO	X			HBO	
CN	X			Amyl nitrite[b]	
				Na nitrite	
				$NaThioSO_4$	
				(Methylene blue)	
Corrosive:					
General/SM	X				X
Lewisite				Chelators	
HF				Ca gluconate	
HC	X				X
NA				Atropine	
				2-PAM Cl	
				Benzodiazepine	
BOT	X			Antitoxin	

Information for above compiled from: Lee, 2006; Shochat, 2006; Leybell, 2006; Fitzgerald, 2006; Wilkes, 2006; Huebner and Arnold, 2006; CDC, 2006.

[a] All get standard decontamination.
[b] Amyl nitrite not used by the military due to concerns about dose control.

LDA, Lung-damaging agents/irritant Gas; HC, Hydrocarbons; NA, Nerve agents/cholinergics; BOT, Botulinum toxin (biologic); Corrosives/vesicants: HF, Hydroflouric Acid; SM, Sulfur mustard; WP, White phosphorous; Asphyxiants: MetHg, Methemoglobinemia; CN, Cyanide; CO, Carbon monoxide; Chelators, Heavy metal chelators.
Doses of the above-mentioned antidotes are as follows:

1. Methylene blue: 1% solution (10 mg/mL). Adult dose 1–2 mg/kg (0.1–0.2 mL/kg) i.v. over 3–5 min; repeat dose in 1 h if continued symptomatology or significant methemoglobinemia. Maximum dose 7 mg/kg.
2. HBO—Hyperbaric oxygen: At sea level (1 ATM) the elimination half-life of carbon monoxide is 320 min, on 100% O_2 at 1 ATM, it is 30–90 min, and at 3 ATM it is 15–23 min.
3. Amyl nitrite: Inhale 1 ampule every 30 min until i.v. established (not used by military).
4. Na nitrite—Sodium nitrite: Adult dose: 10 mL of 3% solution (300 mg) slow i.v. push over 2–5 min. Pediatric: Initial dose: 0.33 mL/kg (10 mg/kg) slowly over 5–10 min, and repeat (1/2 of initial dose) 0.165 mL/kg (5 mg/kg) in 30 min, to a maximum of 10 mL (300 mg) total. (Lower doses if Hgb <12 g/100 mL.) (Note: Hydroxocobalamin is used in other countries: 70 mg/kg [not to exceed 5–10 g] i.v. over 30 min; may be administered more rapidly in cardiac arrest. Maximum dose 15 g.)
5. NaThioSO4—Sodium thiosulfate: Coadminister with or after sodium nitrite or hydroxocobalamin. Available as a 25% solution. Adult dose 12.5 g (50 mL) i.v. at 3–5 mL/min, repeat at 1/2 initial after 1 h if symptoms persist. Pediatric dose 412.5 mg/kg i.v. (1.65 mL/kg) at 3–5 mL/min. Also repeat at 1/2 initial after 1 h if symptoms persist.
6. Dimercaprol (BAL in oil): 300 mg/3 mL. Dose 4–5 mg/kg up to 400 mg. Repeat every 4 h $\times$ 4 doses. (Not manufactured any more in the United States. Use other heavy metal chelators.)
7. Ca gluconate—Calcium gluconate: 10 ml of 10% solution. Gel: 2.5%–5% beyond affected area (2.5% = 3:1 surgilube to 10% solution). SQ: 0.5 ml of 10%/cm^2 affected. i.v.: Signs/measured hypocalcemia—10 ml of 10% i.v.
8. Atropine: Adult dose: 2 mg i.v./i.m./ETT every 2–5 min. Pediatric dose: 0.02 mg/kg i.v./i.m./ETT every 2–5 min. Endpoints: drying of secretions, reversal of bradycardia, and easing of breathing.
9. 2-PAM CL (Pralidoxime chloride): Adult dose: 1–2 g i.v./i.m. Pediatric dose: 15–25 mg/kg i.v./i.m.
10. Benzodiazepines: Diazepam: Adult dose: 5–10 mg i.v. every 10–20 min, repeated as needed. Pediatric dose: 0.05–0.3 mg i.v. over 2–3 min every 15–30 min, repeated as needed.
11. Botulinum antitoxin: Contact local or state Department of Health to pass request to CDC. Skin test first. If no anaphylaxis, one vial i.v. of the antitoxin (one time as there is a long half-life).

contaminated with clothing containing thickened nerve agent (Cooper et al., 1994), as well as ''unthickened'' mustard and nerve agent, are unlikely to produce any significant vapor hazard in the surgical wound. The vapor hazard from a wound may be evaluated using a CAM held close to the material in the wound for a sufficient period of time (up to 30 s lag time) (Cooper et al., 1994).

A single, large piece of clothing material is more likely to retain liquid contamination with chemical agent than multiple smaller shredded pieces of clothing within the wound. ''En block'' removal of chemically contaminated clothing should be the order of the day. No attempt should be made to remove clothing in multiple small pieces, as this would spread contamination. Because the vapor hazard is very low, a respirator (gas mask) need not be worn, with the possible exception of thickened mustard. However, liquid splash from chemical agent contaminated irrigation fluid is possible, and for this reason, goggles or glasses should be worn (Cooper et al., 1994). The contaminated clothing material, the forceps, and all suctioned irrigation solution should immediately be immersed in a pail of 10% hypochlorite solution (Cooper et al., 1994). The ensuing detoxification (chemical neutralization) of CWAs by chlorine denaturing of the CWA usually takes a few minutes, depending on the strength and pH of the hypochlorite solution. Freshly prepared hypochlorite solutions tend to be more alkaline, and this higher alkalinity increases their effectiveness. The degree of vapor ''off-gassing'' from a small piece of wound imbedded clothing material contaminated with liquid CWA will be extremely slight, and vapor hazard should not present a significant contamination hazard to personnel in the ER or OR not wearing chemical PPE.

Remember that latex, nitrile, and vinyl gloves frequently used in emergency and operating rooms are rapidly permeated or penetrated by CWAs (Cooper et al., 1994). Permeation times of GREATLY DILUTED (<1% CWAs mixed with >99% solvent) CWAs sarin (GB), sulfur mustard (HD), soman (GD), and VX through gloves made of latex, nitrile, and butyl rubber, in thicknesses worn in the field and in emergency and operating rooms, have been measured in a report issued by the Battelle Medical Research and Evaluation Facility (Miller et al., 1999). Observations made include that the solvent in which the CWA is carried *greatly* altered the permeation time of each CWA through each glove material. Test solvents included saline, hexane, and chloroform. Glove configurations employed in these tests included (1) a double layer of latex glove material; (2) a double layer of nitrile glove material; (3) a single layer of butyl glove material; and (4) a multilayer glove configuration prepared by sandwiching a single layer of butyl glove material between two single layers of nitrile glove material. Three observations are patently obvious from this report. First, the type of solvent material *SIGNIFICANTLY* alters CWAs permeation times; saline had the longest permeation time, whereas those of hexane and chloroform were *very much shorter*. Second, the multilayer glove configuration offered *significantly* more time to agent permeation than other glove configurations. Finally, permeation times of agents and solvents through various materials must be measured by experimentation, they cannot be extrapolated. Moreover, great variability exists in the permeability of gloves to nonCWA chemicals within the same lot. Permeability of these gloves to chemicals increases over time. Gloves exposed to petroleum products will increase their permeability to CWAs and nonCWA chemicals. It is suggested that under conditions described above, DOUBLE gloving is always appropriate. Alternatively, sterile butyl rubber gloves may be employed, although this may cause degradation in surgical technique (Cooper et al., 1994).

XIII. SUMMARY

In summary, it is important to approach any major event with the potential pitfalls foremost in your mind. By proper command and control through ICS/NIMS and effective, redundant, lines of communication, you will have gone a long way toward management and coordination of the emergency. Controlling traffic and security ensures that the control is maintained and that the resources can interface efficiently without worsening the event. During a chemical event, the security is even more important to first, contain the contamination, then prevent secondary exposures, and finally to provide the most effective medical response to save the most people.

When dealing with large populations of injured, you need to have an effective public address system so that your instructions can be heard by all. In a chemical event, clear lanes that separate hot, warm, and cold decontamination zones are imperative. This needs to be appropriately staffed and equipped. The faster the victims can be separated from the contaminant, the better off they will be. Each victim needs to first be approached with rescuer safety and victim decontamination in mind, along with the Advanced Life Support ABCDEs for cardiac, trauma, and hazardous materials. If a toxidrome is found where an antidote exists, this should be started immediately when possible. After the victim is clean, then a secondary survey exam needs to be performed to look for the severity and distribution of injury with continuing care as appropriate.

An absolutely superb field and hospital reference for medical management of chemical casualties is the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD), *Medical Management of Chemical Casualties Handbook*, Chemical Casualty Care Division, third edition, July 2000. A fourth edition of this book is soon to be released. This 290 plus page, pocket-sized book contains a gold mine of clinically relevant information and should be in every ambulance and hospital emergency room. The pocket-sized format makes it easily transportable to the scene. It is also downloadable from the Internet at http://ccc.apgea.army.mil. Multiple computer programs contain much information on all aspects of CWAs. The principal problem with such programs is that they require a computer at the scene. This computer would later have to be decontaminated, an extremely expensive and time-consuming proposition. Handbooks are relatively cheap and readily available, do not rely on power supplies that could fail, do not undergo electronic malfunctions, and can be burned when contaminated.

Although most emergency workers are used to crisis situations, a smaller number has the experience to function within a very large-scale operation. Even fewer have had HazMat training or experience. For these reasons, practice and exercise are paramount until all staff, in all organizations, on all shifts, acquire enough comfort that the functioning becomes second nature. Like general fitness, this can only be accomplished through regular exercise in a manner that is as close to real as possible. In our society, the likelihood of a malicious chemical event is a real threat. The chance of a general HazMat toxic event is almost guaranteed for the nation as a whole, and high for any individual community, even in seemingly remote areas. It is imperative that all medical personnel develop a working knowledge of toxic exposures and, in particular, for the emergency medical community to be fully versed in working in and through toxic events. The best offense is a strong national defense. This is equally true in the emergency medical community when it comes to major chemical events.

Finally, the purpose of this chapter was not to just regurgitate standard treatment protocols that are readily available from other sources, but rather to introduce important concepts and clinical perils and pitfalls of treatment not generally discussed in these protocols. Hopefully this has been accomplished.

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Index

A

B

C

D

E

F

G

H

I

K

L

M

N

O

P

Q

R

S

T

U

V

W

Y

Z

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