Edited by **Shayne C. Gad**

Animal Models in Toxicology

Second Edition

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Preface

Biomedical sciences' use of animals as models to help understand and predict responses in humans, in toxicology and pharmacology in particular, remains both the major tool for biomedical advances and a source of significant controversy. On one hand, animal models have provided the essential components for research and serve as the source that has permitted the explosive growth of understanding in these fields, with a multitude of benefits to both humans and other animal species. At the same time, the benefits of such use, balanced against costs in terms of animal lives, potential suffering, and discomfort, have been subject to continuous criticism and questioning.

The questioning has stimulated significant and continuous advances in the humane use of animals and understanding of the relevance of findings to what might happen in similarly exposed people. These advances are reflected in this new edition of *Animal Models in Toxicology.* Every section has been updated, and more guidance has been directed to specific uses of animals in toxicology units.

Scientists have used animal models for so long that there is truth in the belief that many researchers employ animals primarily out of habit, with little or no thought as to what the best tools and the optimum ways of using them are. At the same time, although there are elements of poor practice that are real, by and large animals have worked exceptionally well as predictive models for humans—when properly used (Gad 1990; see also chapters 13 and 14 in this volume).

Regulations governing the purchase, husbandry, and use of animals in research have continued to change over the course of the 21st century. Indeed, in some countries (and even cities in the United States), such use has been banned and some sources made unavailable.

The real and most apparent problems underlying the failure of animal models arise primarily from selecting the wrong model, in not using an animal model correctly, or in extrapolating results to humans poorly. In addition, most graduate degree programs do not currently address these issues well (if at all) in their curricula. Indeed, broad training in animal model selection and use, and the techniques involved in such research, is currently available but not well utilized. This text originally was developed to address these needs. Indeed, it is essential to the performance of good science that the correct species be used as a model, and that data be analyzed appropriately.

Chapter 1 presents a historical review of the use of animal models and an overview of broad considerations of metabolism and relevance to use in toxicology. The core of the book, however, is in chapters 2 through 10. Each of these chapters represents the joint efforts of experts in toxicology (addressing techniques for animal use and husbandry and peculiarities of the species as a toxicological model), toxicological pathology, and species-specific metabolism. For an investigator who is not well versed in the use of a particular species, each of these chapters provides an excellent introductory "course," along with guidance to the literature for more detailed understanding. All the major species used (and strains or breeds within these species) are addressed in these core chapters.

Chapter 11 presents the case for a range of species (fish, pigs, earthworms, etc.) that are not commonly used for safety assessment studies but that might provide useful alternative models for some specific endpoints.

In chapter 12, Robert L. Hall presents and discusses the special considerations regarding the evaluation and interpretation of clinical pathology of the eight major model species.

Chapter 13 addresses in detail the general case of how to select a model species and how to extrapolate the results to humans. Chapter 14 details the pitfalls in the process—the situations that cause either human or model to be significantly more sensitive than the other or totally irrelevant to each other in specific cases.

Chapter 15 presents an overview of the regulations that govern how laboratory animals are obtained, maintained, and utilized. Such laws have become increasingly complex, and an understanding of what can (and cannot) be done is essential for the modern researcher.

The Appendix provides a quick guide to the major commercial sources of laboratory animals, whether common (rats and mice) or harder to come by (Chinese hamsters and primates). Information on the selection and use of common anesthetics, drugs, and pharmacological agents for use in laboratory animals is available in Borchards' *Drug Dosage in Laboratory Animals: A Handbook* (Telford Press).

The aim of this volume is to provide a single source reference for the use of animal models in toxicology.

Shayne C. Gad

REFERENCE

Gad, S. C. (1990). Model selection in toxicology: Principles and practice. *J. Am. Coll. Toxicol*. 9, 291–302.

The Editor

Shayne C. Gad, Ph.D., DABT, has been the principal of Gad Consulting Services since 1994. He has more than 30 years of broad-based experience in toxicology, drug, and device development; document preparation; statistics; and risk assessment, having previously been director of toxicology and pharmacology for Synergen (Boulder, Colorado), director of medical affairs technical support system services for Becton Dickinson (Research Triangle Park, North Carolina), and senior director of product safety and pharmacokinetics for G.D. Searle (Skokie, Illinois). Dr. Gad is past president of the American College of Toxicology, a board-certified toxicologist (DABT), and fellow of the Academy of Toxicological Sciences. He is also a member of the Society of Toxicology, Teratology Society, Society of Toxicological Pathologies, Biometrics Society, and the American Statistical Association. Dr. Gad has previously published 29 books and more than 300 chapters, papers, and abstracts. He has contributed to and has personal experience with IND (he has successfully filed more than 75 of these), NDA, PLA, ANDA, 510(k), IDE, and PMS preparation, and has broad experience with the design, conduct, and analysis of preclinical and clinical safety and pharmacokinetic studies for drugs, devices, and combination products. He is also a retired Navy captain with extensive operational experience overseas.

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Introduction

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CONTENTS

The use of animals in experimental medicine, pharmacology, pharmaceutical development, safety assessment, and toxicological evaluation has become a well-established and essential practice. Whether serving as a source of isolated organelles, cells or tissues, a disease model, or as a prediction for drug or other xenobiotic action or transformation in man, experiments in animals have provided the necessary building blocks that have permitted the explosive growth of medical and biological knowledge in the later half of the 20th century and into the 21st century (Meier and Stocker 1989;

Nevalainen et al. 1996). Animal experiments also have served rather successfully as identifiers of potential hazards to and toxicity in humans for synthetic chemicals with many intended uses.

Animals have been used as models for centuries to predict what chemicals and environmental factors would do to humans. The earliest uses of experimental animals are lost in prehistory, and much of what is recorded in early history about toxicology testing indicates that humans were the models of choice. The earliest clear description of the use of animals in the scientific study of the effects of environmental agents appears to be by Priestley (1792) in his study of gases. The first systematic use of animals for the screening of a wide variety of agents was published by Orfila (1814), and was described by Dubois and Geiling (1959) in their historical review. This work consisted of dosing test animals with known quantities of agents (poisons or drugs), and included the careful recording of the resulting clinical signs and gross necropsy observations. The use of animals as predictors of potential ill effects has grown since that time.

CURRENT ANIMAL STUDIES

The current regulatory required use of animal models in acute testing began by using them as a form of instrument to detect undesired contaminants. For example, miners used canaries to detect the presence of carbon monoxide, a case in which an animal model is more sensitive than humans (Burrell 1912). In 1907, the U.S. Food and Drug Administration (FDA) started to protect the public by the use of a voluntary testing program for new coal tar colors in foods. This was replaced by a mandatory program of testing in 1938, and such regulatory required animal testing programs have continued to expand until recently.

The knowledge gained by experimentation on animals has undoubtedly increased both the length and quality of our lives, an observation that most reasonable people would find difficult to dispute, but it (as reviewed by Ewald and Gregg 1983) has also benefited animals. As is the case with many tools, animals have sometimes been used inappropriately. These unfortunate instances have helped fuel the actions of a vituperative animal "rights" movement. This movement has encouraged a measure of critical self-appraisal on the part of scientists concerning the issues of the care and usage of animals. The Society of Toxicology (SOT) and the American College of Toxicology (ACT) have both established Animals in Research Committees, and these have published guidelines for the use of animals in research and testing. In general, the purpose of these committees is to foster thinking on the four Rs of animal-based research: reduction, refinement, (research into) replacements, and responsible use. This new edition is, in part, a response to these continued concerns.

The media frequently carry reports that state that most (if not at all) animal testing and research is not predictive of what will happen in people, and therefore such testing is unwarranted. Many animal rights groups also present this argument at every opportunity, and reinforce it with examples that entail seemingly great suffering in animals but add nothing to the health, safety, and welfare of society (e.g., Fano 1998). This is held to be especially the case for safety testing and research in toxicology. Animal rights activists try to "prove" this point by presenting examples of failure (e.g., thalidomide*). In light of the essential nature of animal research and testing in toxicology, this is equivalent to seeking to functionally disarm us as scientists. Our primary responsibility (the fourth R) is to provide the information to protect people and the environment, and without animal models we cannot discharge this responsibility.

Confronted with this argument, all too many toxicologists cannot respond with examples to the contrary. Indeed, many might not even fully understand the argument at all. Very few are familiar enough with some of the history of toxicity testing to be able to counter with examples where it has not only accurately predicted a potential hazard to humans, but where research has directly benefited both people and animals. There are, however, many such examples. Demonstrating the

* Where the lack of adequate testing (or interpretation of existing test results) prior to marketing is not pointed out.

actual benefit of toxicology testing and research with examples that directly relate to the everyday lives of most people and not esoteric, basic research findings (which are the most exciting and interesting products to most scientists) is not an easy task. Examples that can be seen to affect neighbors, relatives, and selves on a daily basis would be the most effective. The problem is that toxicology is, in a sense, a negative science. The things we find and discover are usually adverse. If the applied end of our science works correctly, the results are things that do not happen, and therefore are not seen.

If we correctly identify toxic agents (using animals and other predictive model systems) in advance of a product or agent being introduced into the marketplace or environment, generally it will not be introduced (or it will be removed) and society will not see death, rashes, renal and hepatic diseases, cancer, or birth defects, for example. As these things already occur at some level in the population, it would seem that seeing less of them would be hard to firmly tie to the results of toxicity testing that relies on animals. In addition, the fact that animals are predictive models for man is controversial.

ORIGINS OF PREDICTIVE ANIMAL TESTING

The actual record of evidence for the predictive value of animal studies and how they have benefited man and domestic animals is reviewed in the following two sections. However, the negative image needs to be rebutted. First, it must be remembered that predictive animal testing in toxicology, as we now know it, arose largely out of three historical events.

The "Lash Lure" Case

Early in the 1930s, an untested eyelash dye containing p-phenylenediamine (Lash Lure) was brought onto the market in the United States. This product (as well as a number of similar products) rapidly demonstrated that it could sensitize the external ocular structures, leading to corneal ulceration with loss of vision and at least one fatality (McCally et al. 1933).

The Elixir of Sulfanilamide Case

In 1937, an elixir of sulfanilamide dissolved in ethylene glycol was introduced into the marketplace. One hundred and seven people died as a result of ethylene glycol toxicity. The public response to these two tragedies helped prompt Congress to pass the Federal Food, Drug, and Cosmetic Act of 1938 (Pendergrast 1984). It was this law that mandated the premarket testing of drugs for safety in experimental animals. The most compelling evidence that should be considered is "negative": Since the imposition of animal testing as a result of these two cases, no similar occurrence has happened, even though society uses many more consumer products and pharmaceuticals today than during the 1930s.

Thalidomide

The use of thalidomide, a sedative-hypnotic agent, led to some 10,000 deformed children being born in Europe. This in turn led directly to the 1962 revision of the Food, Drug and Cosmetic Act, requiring more stringent testing. Current testing procedures (or even those at the time in the United States, where the drug was never approved for human use) would have identified the hazard and prevented this tragedy. In fact, it has not occurred in Europe or the United States except when the results of animal tests have been ignored. Table 1.1 presents an overview of cases in which animal data predicted adverse effects in humans, and table 1.2 provides some examples of known toxic reactions to substances in animals and humans.

Table 1.1 Animal Models That Predicted Adverse Effects of Xenobiotics on Humans

Agent	Effect	Animal Species	In Man
Phenacetin	Neurotoxicity, carcinogenicity	Rat	Y
Thalidomide	Phocomelia	Rat	N/Y
Accutane	Developmental toxicity of central nervous system (neural tube defects)	Rat, rabbit, dog	Y
AZT	Bone marrow depression	Dog, rat, monkey	Y
Valproic acid	Cleft palate	Rat, mouse, rabbit	Y
Cyclosporine	Nephropathy Reversible immune response suppression (essential aid to organ transplantation)	Rat, dog Rat, monkey	
Benoxaprofen (Oraflex)	Hepatotoxicity Photosensitivity	No Guinea pig	Y
Zomepirac	Anaphylactic shock/allergy	No	Y
MPTP	Parkinsonism	Monkey	Y
Cyclophosphamide	Hemorrhagic cystitis	Rat, dog	Y
Mercury*	Encephalopathy	Rat, monkey	Y
Diethylene glycol*	Nephropathy	Rat, dog	Y
Razoxin	Myelomonocytic leukemia	Mouse	Y
Benedictin	Birth defects (?)/Litogen	No	Y
Triazolam (Halcion)	Behavioral disturbances and amnesia	No	Y
Quinalones	Phototoxicity	Guinea pig, *in vitro*	?/Y
Temafloxcine	Hemolytic anemia	No	Y
Diazepam (Valium)	Development abnormalities	Rat	Y
Fialuridine (FIAU)	Nephrotoxitcity, hepatotoxicity		Y

* Not drugs.

Table 1.2 Selection of Toxic Reactions Occurring in Animals and Man

Substance	Reaction	Substance	Reaction
Acetaminophen	Hepatic necrosis	Isotretinoin, prenatal	Multiple malformations
Acrylamide	Peripheral neuropathy	Kanamycin	Cochlear toxicity
Aniline	Methemoglobinemia	Methanol	Blindness (monkey)
Asbestos	Mesothelioma	Methoxyflurane	Nephropathy (Fischer rat)
Atropine	Constipation	8-Methoxypsoralen	Phototoxicity
Benzene	Leukemia	Methyl mercury	Encephalopathy
Bleomycin	Pulmonary fibrosis	Morphine	Physical and psychological appearance
Carbon disulphide	Nervous system toxicity	MPTP	Parkinsonism
Carbon tetrachloride	Hepatic necrosis	Musk ambrette	Photosensitivity
Cis-platinum	Nephropathy	2-Naphthylamine	Bladder cancer
Cobalt sulphate	Cardiomyopathy	Neuroleptic drugs	Galactorrhoea
Cyclophophamide	Hemorrhagic cystitis	Nitrofurantoin	Testicular damage
Cyclosporin A	Nephropathy	Paraquat	Lung damage and fibrosis
D & C Yellow	Eczema	Phenformin	Lactic acidosis
Diethylene glycol	Nephropathy	Phenothiazine NP 207	Retinopathy (pigmented animals)
Diethylaminoethoxy-hexoestrol	Phospholipidosis of liver	Penicillamine	Loss of taste
Doxorubicin	Cardiomyopathy	Pyridoxin	Sensory neuropathy
Emetine	ECG abnormalities	Scopolamine	Behavioral disturbances
Ethylene glycol	Obstructive nephropathy	Slow release potassium	Intestinal ulceration
Furosemide	Hypokalemia	Thalidomide, prenatal	Phocomelia (monkey, rabbit)
Gentamycin	Nephropathy	Triothocresylphosphate	Delayed neuropathy
Hexacarbons	Peripheral neuropathy	Triparanol	Cataract
Hexachlorophene	Spongiform encephalopathy	Vinyl/chloride	Angiosarcoma of the liver
Isoniazid	Peripheral neuropathy	Vitamin A	Osteopathy
Isoproterenol	Stenocardia	Vitamin D	Nephrocalcinosis
Isothiocyanates	Goiter		

For example, birth defects have occurred with isotretinoin (Accutane) where developmental toxicity had been clearly established in animals and presented on labeling, but the drug has continued to be used by potentially pregnant women.

Research into replacements such as cellular cultures, organs harvested from slaughterhouses, computer modeling, and physical and chemical systems has been extensive (Frazier 1990; Gad 2000). Although each of these have its own utility (Gad 1989, 2000), they will not replace animals for the foreseeable future. Some degree of animal use will continue. We hope that this book will assist the responsible investigator in designing and interpreting appropriate experiments (refinement) that will require fewer animals (reduction) in which the animals are appropriately husbanded and utilized (responsibility).

SELECTING AN ANIMAL MODEL

Choosing the appropriate animal model for a given problem is sometimes guesswork and too often a matter of convenience. One often uses a species with which one is most familiar, with little consideration as to whether the chosen species is actually the most appropriate for the problem at hand. For example, the rat is probably a poor model for studying the chronic toxicity of any new nonsteroidal anti-inflammatory drug (NSAID) because the acute gastrointestinal (GI) toxicity will probably mask any other toxic effects. The guinea pig is less sensitive to most NSAIDs than the rat, and would therefore be a more appropriate species for investigating the chronic (nongastrointestinal) toxicity of an NSAID. This practice of not rationally choosing an appropriate species for an experiment undoubtedly results in imprecise or questionable science. This alone should be considered a waste of animals and resources. It also results in additional, and sometimes duplicative, experiments. We hope that this book will contribute to the reduction and refinement of the use of animals by helping to alleviate this practice. The core chapters (2–11) include discussions of the strengths and weaknesses of each of the common laboratory species, and recommendations for potential appropriate uses. Chapter 12 directly addresses the issue of how to select the best practical model.

HUSBANDRY AND CARE

The quality of an experiment often hinges on the details of animal husbandry and care. At one extreme, inappropriate handling could result in unhealthy animals and an experiment yielding variable and irreproducible results. All animals have optimal temperature, humidity, light cycle, light intensity, cage size and bedding, and dietary requirements. Rabbits, for example, have a different optimal temperature range than rats. Rats and ferrets have completely different dietary requirements. Albino rodents have very sensitive eyes, and lights of too high a candle power can cause incidental ocular damage, especially in those animals on the top row of a cage rack. Infrequent changing of indirect bedding materials can result in exposure of rodents to a high airborne concentration of ammonia, which can cause ocular damage. Recently, it has become clear that ad lib feeding of rodents in chronic or carcinogenicity studies both shortens their lives and alters the patterns of spontaneous tumors that occur. These are all examples of how inattention to the details of animal care can compromise an experiment, particularly a long-term one. A refined and responsibly designed experiment accommodates these details. It is hoped that this book will provide a convenient source of husbandry procedures for the more common animal species used in toxicological and pharmacological research.

Caging

Caging deserves special mention for two reasons. First, not all animals can be group housed. Hamsters, for example, are notoriously antisocial. Even breeding pairs cannot be left in the same

small cage together for protracted periods. Guinea pigs, on the other hand, thrive when group housed. Obviously these factors need to be considered when designing an experiment. In modern toxicology practice, animals are seldom group housed during chronic studies to maintain identification, facilitate clinical observations, and ensure necropsy of moribund or dead animals (mice, in particular, are very cannibalistic). With short-term experiments, however, group housing of certain animals might not compromise a study and might decrease the amount of housing space needed. This book discusses appropriate housing, including instances when animals should or should not be group housed.

Second, cage size is important because the animal rights movement has made it important. Although most investigators (and cage manufacturers) have long recognized that cages have optimal sizes, the 1989 proposed Animal Welfare Codes (which became law in 1991, and have been revised since) attempted to specify somewhat larger cages with several size cutoffs mediating cage changes. For example, there are three to four different cage specifications for guinea pigs depending on their age and/or weight. Many caging systems currently in use would no longer be permitted and their replacement would be very expensive. There is no scientific basis for believing that these changes will improve animal husbandry or quality of life. This is just an example of how the animal rights movement, and the resultant animal care laws, could affect the conduct of pharmacologists and toxicologists. This book contains in-depth discussion on current animal welfare laws (chapter 15). The investigator needs to be aware of not only the four Rs, but also the relevant laws and regulations governing animal experimentation.

CHOOSING SPECIES AND STRAINS

Not only is it important to pick the correct species for an experiment, but sometimes the correct strain as well. For most of the species discussed in this book there are a handful of commonly used strains. In some cases, an inbred strain might provide qualitative and specific characteristics that make it a good disease model, such as the spontaneously hypertensive rat. There are other more quantitative strain-related differences such as size, color, temperament, and background disease. For example, the Fischer 344 rat is smaller than the Sprague-Dawley rat. The CD-1 mouse is shorter lived than the C57B6/F_1 hybrid. These differences might make a particular strain more appropriate for one experiment than others. For example, the Fischer 344 rat has a high rate of spontaneous Leydig cell tumors as compared to the Sprague-Dawley rat, which would make the latter less appropriate for determining if a chemical is a testicular carcinogen. For these reasons, this book includes discussions of strain-related differences. Rats and mice provide the greatest array of strains from which to choose, including outbred and some inbred. There are literally hundreds to choose from, but the majority are specialized-use animals, such as the athymic nude mouse. For the majority of generalized pharmacology and toxicology testing, a relatively small handful of rat and mouse strains are used and the emphasis in the relevant chapters is on those more commonly used strains. Many chapters include some mention of strain; however, the situation with dogs is somewhat different.

All domestic dogs belong to the same family, which is subdivided by breed. Only the beagle breed is purposely raised for biomedical research; otherwise one uses mongrel or random-source dogs (obtained from pounds and used without regard to breed). Hence, the chapter on the dog focuses on the beagle. There might be supplier-related differences in beagles, but these have not been systemically studied.

DOSING

To study the effects of a drug or other chemical in an animal, the two have to be brought together; that is, the animal has to be dosed. Dosing is the act of introducing a drug or chemical

into a living organism. It requires active interaction between man and animal. There are, however, passive dosing techniques that are also used frequently in which the chemical is placed in the animal's air, water, or feed, and the animal doses itself by breathing, drinking, or eating. Hence, administering an antibiotic intravenously is active dosing; giving it in the feed is passive dosing. In the former case, dosimetry (i.e., calculating milligrams per kilograms of exposure) is generally intuitively simple (an exception being for the dermal route). In the latter case, other measurements must be taken (e.g., feed consumption) and a variety of formulas are used in dosimetry. The main routes used for active dosing are oral, intravenous, intraperitoneal, dermal, and subcutaneous. Other routes are sometimes used, and these are mentioned where appropriate (for a complete discussion of different routes, see Gad and Chengelis 1998, Chap. 10; Gad 2000, 2002). For oral dosing, for example, one might have a choice of using capsules or gavaging. However, capsules are rarely used with rats and gavage is seldom used with dogs. When necessary, a dog can be gavaged, but the technique is different from that used with rats. Intravenous dosing of ferrets is especially difficult, but can be done. This book presents the appropriate techniques, "tricks of the trade," so that animals can be appropriately and humanely dosed.

Second, some of the information (e.g., average feed consumption) and formulas needed to calculate or estimate dosimetry in passive dosing procedures are presented and discussed. With regard to dosing and dosimetry, it should be kept in mind that the terms *dose* and *dosage* are not synonymous. The dose is the total amount of test article given, such as 1,000 mg. The dosage is a rate term and is the dose divided by the weight of the test animal; for example, 1,000 mg/10 kg (for a dog) = 100 mg/kg. For some agents (particularly oncology drugs), this is presented in terms of quantity per meter squared (m^2) of body surface area. In most instances, when one speaks of a dose–response curve, a dosage–response curve is being described.

ANIMAL PHYSIOLOGY

All animal species and strains have their own distinctive physiology. As a result, values pertaining to blood pressure, breathing rates, ECGs, rectal temperatures, and normal clinical laboratory parameters often vary between species. Clearly, appropriate interpretation of an *in vivo* experiment requires a firm understanding of these baseline data. For example, there are well-established differences between species with regard to red blood cell size: What is normal for a dog would be high for a rat. The converse is true for breathing rates. This book provides a convenient source for these important background data.

Background Incidence of Disease and Neoplasia

All animals also have their own baseline, or natural incidence, or diseases that complicate the conduct and interpretation of chronic toxicity experiments. The background incidence of liver tumors in C57B6/F_1 mice is quite high. It would, perhaps, be prudent to investigate a suspect hepatocarcinogen in a species with a lower spontaneous incidence than these. Ferrets in the United States are currently contaminated by the Aleutian mink virus, which could make this species inappropriate for chronic experiments. The background incidence of these diseases and pathological lesions are discussed to aid the investigator in choosing the most appropriate species for an experiment and in the interpretation of the results.

Responses to Biologically Active Agents

An animal's responses to drugs or other biologically active agents might be just as important as the background incidence of disease, and species-related differences in sensitivity are important for two reasons. First, animals will often have to be anesthetized or receive other treatment such

as antibiotics during an experiment. Appropriate dosages vary among species. Thus, this book presents the appropriate dosages of common anesthetics for the model species discussed here.

Second, the other reason species-related differences are important is that in toxicity testing, these differences are the major hurdle in applying toxicity data to human hazard assessment. This is perhaps too broad a topic for a single book, but mention is made so that an investigator is aware of such differences. Cats, for example, are far more sensitive to digitoxin ($LD_{50} \cong 180$ µg/kg po), than other species, such as the rat ($LD_{50} \cong 56$ mg/kg po, as reported in National Institute of Occupational Safety and Health 1980).

There can also be qualitative differences among species. Morphine, for example, is infamous for causing different clinical signs in different species: straub tail in mice, catatonia in rats, and extreme reactivity in cats. Some of the more frequent examples of these distinctions are mentioned in the core chapters. The salient message is that species often differ both quantitatively and qualitatively in their responses to drugs or chemicals. These differences must be investigated and considered in choosing a species for an experiment and in interpreting the results. Incidentally, cats (with the exception of veterinary products intended for use in cats) are seldom used in toxicity testing and are used in pharmacology mainly for acute, terminal neurophysiological experiments. For these reasons, an in-depth discussion of cats is not included in this book.

Absorption, Distribution, Metabolism, and Excretion of Chemicals

When studying the effects of drugs and other chemicals on intact animals, it is also vitally important to investigate the processes of absorption, distribution, metabolism, and excretion (ADME). These have been intensely studied widely, and space does not permit a review of this large body of work. Some basic degree of knowledge must be presumed. We have compiled a list of references to which the reader can refer if additional information is needed (table 1.3). For the remainder of this chapter, we touch on some basic principles that apply across all species. In each individual core chapter (2–10) of this book, some basic information on ADME is presented on a species-specific basis. The emphasis is on providing the information necessary to assist one in (a) the appropriate selection of an animal model, (b) the design of the experiment, (c) the interpretation of resultant data, and (d) the applicability of the results to humans.

The principles that govern absorption and distribution apply fairly equally across all species (Pratt and Taylor 1990; Washington et al. 2001), and therefore are not discussed to any great extent on an animal-by-animal basis. It is most difficult to predict species differences in bioavailability (absorption across GI tract into the blood) or systemic bioavailability (bioavailability + first-pass metabolism) of a specific chemical. Species differences in gastric or intestinal pH, for example, may dictate species differences in GI permeability to specific chemicals, but will not account for differences in GI transit time or hepatic metabolism. Assumptions based solely on phylogenetic grounds can be quite misleading. We had recent experience with a drug found to be bioavailable in the rat and dog, but not at all absorbed in the monkey. In fact, the dog was the species most similar to the human. One needs to strive to ascertain test article bioavailability experimentally for any specific chemical, as general principles always come encumbered with exceptions.

Absorption

After dosing, a chemical must be absorbed to cause an effect. Absorption is the process of the chemical passing through a barrier to gain access to the general systemic circulation. The most common dosage routes are oral, inhalation, topical, intraperitoneal, intravenous (IV), subcutaneous (SC), and intramuscular (IM). Absorption is not generally a problem by the latter three routes as the test substance is introduced directly to the body. It is normally a foregone conclusion that drugs

Table 1.3 Summary of General Reviews of Xenobiotic Metabolism

Topic	Source
General reviews on process of drug metabolism and disposition	LaDu et al. (1972) Goldstein et al. (1974) Klaassen (1986) Sipes and Gandolfi (1986) deBethizy and Hayes (1989) Rozman (1988) Levy et al. (2000)
Cytochrome P-450	Gonzales (1988) Black and Coon (1986) Kadlubar and Hammons (1987) Lewis (2001)
Flavin-dependent microsomal mixed function oxidase	Ziegler (1988) Tynes and Hodgson (1985)
Epoxide hydrolase	Seidegard and DePierre (1983) Oesch (1972)
Glutathione S-transferase	Jarina and Bend (1977) Pickett and Lu (1989)
UDP-glucuronosyl transferase/glucronidation	Boutin (1987) Mulder (1986) Siest et al. (1989)
PAPS-Sulfotransferase/sulfate formation	Singer (1985) Jacoby et al. (1984)
Amino acid conjugations	Hirom et al. (1977)
Acetylations	Lower and Bryan (1973)
Esterases	Leinweber (1987)
Alcohol metabolism	Hawkins and Kalant (1972) Crabb et al. (1987)
Billiary excretion	Klaassen and Watkins (1984) Levin (1978)

so administered will reach the systemic circulation. Plasma concentrations will depend on rates of delivery (IV), or rates of diffusion (IM, SC). Although there are some technical concerns, the principles are either independent of species, or the species differences are obvious. For example, because of relative small muscle mass and rapid circulation time, drugs given intramuscularly will more rapidly equilibrate in rats than in monkeys. Via the intraperitoneal route, systemic availability will depend not only on the rates of diffusion, but also on the first-pass metabolism effect. There are no known species differences with regard to intraperitoneal absorption, but there are species differences with regard to rates of hepatic metabolism, which may dictate the degree of first-pass metabolism. Interestingly, first-pass effects are generally of greater concern in smaller species, such as rats and mice, where the intraperitoneal route is more commonly used.

With regard to the oral, dermal, and inhalation routes, there are very real species differences. For example, thickness and length of the small intestine, size of cecum (if indeed there is one), and gut transit time all play a role in gastrointestinal absorption.

Species differences in facilitated or active transport might also play a role in absorption. Whether an animal is an obligate nose breather or not, the structure of the nasal turbinates, respiration rate, and minute volume will all influence the size and number of particles reaching the alveoli by the inhalation route. The rat is a poor model for inhalation pharmacokinetic studies in extrapolating the results to humans for these reasons. There are well-described differences in skin structure that control dermal absorption and result in species differences. Such species differences vary with chemical class.

This book might help one sort through this maze, but there are few scientifically sound generalizations. Our best recommendation is that investigators substantiate their assumptions on dermal

or inhalation absorption before rendering any conclusion on studies conducted using these routes of administration.

Distribution

After gaining access to the systemic circulation, the toxin or drug is distributed among the organs. Distribution depends on these factors:

- Blood flow to the organ
- Extent and avidity of binding to plasma proteins*
- The "natural" affinity a particular organ might have
- The degree and extent to which the chemical crosses barriers such as the blood–brain barrier or the placenta
- The extent to which clearance (metabolism and/or excretion) competes with these processes

There are probably species differences with regard to all these processes, but not all have been vigorously explored. For example, there are few comparative studies on the blood–testes barrier, or comparisons on plasma protein binding of different chemicals in the monkey, so the database in this area is surprisingly small. A few transspecies comparisons of plasma protein binding have been done. As a broad generalization, binding is most extensive in humans and least extensive in mice. Such information is presented and discussed in the core chapters, but the reader should be aware of the gaps in the available knowledge.

Metabolism

In the area of ADME, the processes of metabolism or bioconversion are of greatest concern with regard to species-specific differences. Indeed, species differences in metabolism are a leading cause for species differences in toxicity. First, very few administered xenobiotics are excreted unchanged. Therefore, rates of metabolism often dictate the time length of a pharmacodynamic response. Second, metabolism of a xenobiotic might result in metabolites of similar potency or produce metabolites that are responsible for toxicity. For example, most genotoxic carcinogens require metabolic activation. Finally, because the metabolism of xenobiotics is an enzyme-based phenomenon, it shows a great deal of species differences. For example, Williams (1972) examined the metabolism of phenol, a relatively simple chemical, in 13 different species and found that no two species provided the same spectrum of metabolites. Species differences can be either quantitative (differing amounts of the same metabolites) or qualitative (different metabolites). Because of the importance of metabolism in toxicity testing, each individual animal chapter contains in-depth discussion of xenobiotic metabolism.

Xenobiotic Metabolism

The area of species differences in xenobiotic metabolism is not new. Some of the efforts in this area are summarized in table 1.3. It is not the objective of this book to provide yet another interspecies comparison, but rather to present information on a species-specific basis. For example, what type of regimen is required to induce increases in microsomal multifunction oxidase (MMFO) activity in the dog? The metabolism of xenobiotics by mammals is a phenomenon that has been recognized since 1842 when Keller (Mandel 1972) identified that benzoic acid was excreted in the urine as the 1 cine conjugate (hi uric acid). As a modern science, drug (or xenobiotic) metabolism was formalized in the late 1940s when Williams (1947) published the first text on the

* Interestingly, it has recently become clear that even proteins (such as some of the biotechnologically generated pharmaceutical agents) can be bound to plasma proteins.

subject. Williams (see Williams 1972, 1974, for reviews) has been particularly instrumental in the area of species differences in metabolism. Early works in this area tended to concentrate on isolating and identifying various conjugations of simple chemicals given to intact animals. Mueller and Miller published on the importance of liver microsomes in xenobiotic metabolism in their studies on the oxidative metabolism of aminazodyes (Mannering 1972; Mueller and Miller 1949). The field has grown explosively since the mid-1950s, catalyzed by the studies of Brodie and colleagues in the United States (Brodie et al. 1955; Quinn et al. 1958) and Remmer in Germany (Mannering 1972; Remmer and Merker 1963). Their works confirmed the quantitative importance of the liver in xenobiotic metabolism, and that the major underlying enzymes were located in the microsomal fraction. It was during this period that the practice of naming an enzyme by its activity, such as aminopyrine demethylase or aniline hydroxylase, was adopted. It was only later that it was recognized that all these activities are catalyzed by the same enzymes (or family thereof); that is, the cytochrome P-450-dependent microsomal mixed function oxidase system (Gonzales 1988; Guengerich 1988). Cytochrome P-450 was discovered almost a decade after Mueller and Miller described microsomal metabolism requiring NADPH (Coon 1978; Klingenberg 1958; Mannering 1972). The importance of the identification and characterization of the cytochrome P-450-dependent MMFO system to the fields of biochemistry, pharmacology, and toxicology cannot be understated. The reader is referred to any one of several reviews of the system (see table 1.1 and table 1.4).

The process of xenobiotic metabolism has traditionally been divided into Phase I (oxidative) and Phase II steps. In general (as reviewed more extensively by deBethizy and Hayes [1989]), all mammalian processes are designed to convert lipophilic chemicals to more polar and more easily excreted metabolites. In reality, the process can be more complicated than two steps because the products of Phase I oxidation can be (a) further hydroxylated at different sites, (b) further oxidized at the same site (by a different enzyme such as alcohol dehydrogenase), or (c) conjugated with glutathione or glucuronic acid, sulfate, or one of several amino acids. This process is discussed in greater detail elsewhere (see table 1.1 and table 1.2). The result is that any one xenobiotic can have an astonishing spectrum of metabolites. For example, benzene is a relatively simple chemical, yet over 15 different metabolites have been described.

Enzymes Involved in Xenobiotic Metabolism

The main enzymes involved in xenobiotic metabolism are fairly uniform across species. In all mammalian species, the liver is quantitatively the most important site of xenobiotic metabolism, and the MMFO system is the most important enzyme. Although this system is ubiquitous, there are species differences in isozymic characteristics, substrate specificity, activity, and inducibility. More recently, Davin-dependent microsomal multifunction oxidase, which is distinct from the MMFO, has been identified (Ziegler 1988), and has been shown to play a role in the metabolism of many chemicals. There are also differences in Phase II enzyme activities and cosubstrate availability (Gregus et al. 1983). Seldom do two species dispose of the same chemical that way. Each species produces a spectrum of metabolite or chromatographic “fingerprints” that are often distinct. The characteristics of the MMFO for each of the most highly used species are discussed in this book in some detail. Other enzyme systems such as the flavin-dependent (noncytochrome P-450) monoxygenase can also be involved in xenobiotic oxidative metabolism, and are discussed where available information permits. The species characteristics of other important enzymes such as epoxide hydrolase and UDP-glucuronosyl transferase are also discussed. Some enzymes are ubiquitous, such as the alcohol dehydrogenase and carboxylesterase. All species metabolize primary alcohols to aldehyde, and subsequently to carboxylic acids. This is only discussed, therefore, when there is some species-specific characteristic. This is also true for esterases, as all species rapidly hydrolyze esters.

Table 1.4 Compilation of Selected Papers That Compare Xenobiotic Metabolism in Different Species

Species Compared	Parameters Examined	Comments	References
Dog, guinea pig, rat, rabbit, monkey, human, mouse	Gastrointestinal differences that affect absorption; plasma and tissue binding; drug metabolism in liver and intestine	Rhesus monkey best predictor for ADME in man Excellent bibliography	Rozman (1988)
Rat, mouse, guinea pig	GSH-T	With CDNB total activity mouse > guinea pig > rat. Parallel to AFT sensitivity. Quantitative differences in isoenzymes.	Neal et al. (1987)
Rat, hamster, mouse, guinea pig	Induction of P-450 and MMFO activities (AP demethyl, BP-OH, EC-deethyl) by 2-AAF and 3-MC	In general, MMFO activity, rat had lowest, but BP activity most inducible. In some species, induction had no effect or decreased some activities.	Astrom et al. (1986)
Mouse, guinea pig, rabbit, hamster	GSH-T (1-chloro-2,4-dinetrobenzene)	Hamster > rabbit = gp > mouse > rat. S-Sepharose elution patterns different.	Igarashi et al. (1986)
Rat, rabbit, dog, mouse	Chlorfenvinphos deethylation (*in vitro*)	Dog > rabbit = mouse > rat with same order to LD_{50}.	Hutson and Logan (1986)
Rat, dog, monkey	Metabolism and kinetics of Tolrestat	Highest bioavailability in rats. More unchanged drug in dogs and monkeys.	Cayen et al. (1985)
Rat, mouse, guinea pig	Induction of MMFO, GSH-T, EH	EH-M gp > rat > mouse, but gp is less inducible. For GSH-T: mouse > rat > gp and gp not induced. For MMFO: gp > mouse > rat.	Thabrew and Emerole (1983)
Rat, dog, monkey	Inducing effect of hexahydroindazole (P-450, AP-demeth, AN-OH)	Increases in relative liver weights in all species. For P-450, monkey > rat > dog; for AP, monkey > rat = dog; for AN, rat = monkey > dog (gram basis, different if on protein). Best induction in dog.	Lan et al. (1983)
Rat, mouse, guinea pig, dog, monkey	Conjugation reactions	A review.	Caldwell (1982)
Rats, rabbits, hamster, guinea pig, ferret	Metabolism of glyceryl trinitrite	Species differences in plasma half-life a function of body weight.	Ioannides et al. (1982)
Various: emphasis on dog, mouse, rat, rabbit, monkey	Various aspects of metabolism covered: spectrum of metabolites, plasma half-lifes, developmental differences, inhibitors, inducers	Excellent comprehensive review with emphasis on mixed function oxidase activity.	Kato (1979)
Rat, mouse, rabbit, hamster, guinea pig	Changes in iron "spin-state" as determined by EPR induced by different binding spectra	Proportion of high spin P-450 *in vivo*: rabbit > gp = hamster = mouse > rat	Kumaki et al. (1978)
Rat, rabbit, guinea pig	Different inducing agents	Glucuronide formed only by liver, other organ involved in sulfation. Both produced in liver of rat, rabbit and gp, only sulfate formed by mouse.	Wong (1976)
Rat, mouse, rabbit, hamster, guinea pig	Protein, P-450 content, reductase concentrations, model substrates, GSH and UDPG transferase activities, lung, liver, kidney	For all species, liver most active with lung and kidney 15%–40% of liver. No species superior in all activities, but hamster tended to have greatest activities.	Litterst et al. (1975)

Table 1.4 Compilation of Selected Papers That Compare Xenobiotic Metabolism in Different Species

Species Compared	Parameters Examined	Comments	References
Rat, mouse, guinea pig, rabbit	Microsomal protein, BP-OH, UDPG transferase, small intestine vs. liver	No real differences in microsomal protein (30–35 mg/g). For BP, liver > gut for all species. For liver gp = mouse > rat > rabbit; for gut, gp > rabbit > rat = mouse; for UDPG liver, gp > rabbit = mouse > rat; for UDPG-gut, rabbit > rat > mouse = gp.	Haietanen and Vainio (1973)
Rat, mouse, guinea pig, hamster, rabbit, dog, pig, monkey	Metabolism of [^{3}H]styrene oxide: EH and GSH-T in liver, lung, and kidney	For EH, liver > kidney > lung. In general, mouse is lowest and primate is highest. For GSHT, liver > kidney > lung. GP is highest and primate is lowest. Includes lit comp. of EH activities.	Pacific et al. (1981)
Rat, mouse, guinea pig, rabbit, pig, monkey	P-450 b5, cytochrome c reduct, K_m and V_{max} of various substrates	Not big differences in micro: P-450 ranges 0.38 (pig) to 0.75 nmol/mg (gp). b5 ranged 0.20 (mouse) to 0.49 nmol/mg (gp). Cytochrome c red ranged 115 (rabbit) to 136 (gp) nmol/min/mg	Amri et al. (1986)
Rat, mouse, guinea pig, hamster	Induction of EH UDPG transferase, and GSH-T by 2-AAF or 3-MC	Large species variation (3- to 12-fold) in control activities. Except for EH in guinea pig, enzymes induced only in rat by 2-AAF. Except for GSH-T in hamster, enzymes induced only in rat by 3-MC. Rats not representative of activities or inducibility of other species.	Astrom et al. (1987)
Rat, mouse, guinea pig, hamster	Effects of DDT on AHH activity and cytochrome P-450	Induction in hamsters, decreases in other species. Acute toxic effects depend on route and species.	Haietanen and Vainio (1976)
Rat, mouse, guinea pig, hamster	Total GSH, γ-GTP, GSH synthetases, peroxidease, and reductase	GSH lowest in guinea pig, highest in mouse. Synthesis lowest in hamster, highest in rat. γ-GT much higher in gp. GSH-T highest in gp, lowest in rat and not affected by fasting. GR lowest in rat = mouse, highest in hamster, fasting effects species dependent. Px highest in mouse = hamster, lowest in rat and mouse, dependent on substrate and fasting state	Igarashi et al. (1983)
Rat, mouse, rabbit, guinea pig	GSH-T activity with different substrates, different age animals, and with different inducing agents	Wide range of activities, depending on species and substrate. Age-related changes evident in all species, peak (up to 120 days) varies with species. Activity inducible in all species, but extent depends on inducer and substrate. Rat, mouse most inducible.	Gregus et al. (1985)
Rat, mouse, hamster, guinea pig, monkey	Review on role of intestinal microflora in drug metabolism; excellent bibliography with references to primary articles and other reviews	Gut flora (β-glucosidase and β-glucuronidase; nitro, nitrite, and azo reductases) have large species difference, guinea pigs tend to have lowest amounts, mice the highest.	Rowland (1988)

Table 1.4 Compilation of Selected Papers That Compare Xenobiotic Metabolism in Different Species

Species Compared	Parameters Examined	Comments	References
Rat, mouse, hamster, rabbit, guinea pig, dog, primate, others	Excellent review; qualitative differences (lack of specific enzymes) and quantitative differences; emphasis on *in vivo* data; species differences in the metabolism of [1-14c-acetyl]phenacetin	Examples: Only in the rat is aromatic hydroxylation the major route of amphetamine metabolism. Dogs and guinea pigs have a defect in N-acetylation. Guinea pigs do not make mercapturic acids.	Williams (1972, 1974)
Rat, rabbit, guinea pig, ferret	Case reviews on how species differences in drug metabolism lead to differences in toxicity	Deacetylation highest in rat and ferret, aromatic hydroxylation high in ferret, low in others. Glucuronide formation dominant in rabbit, guinea pig, ferret; sulfation is dominant in rat.	Smith and Timbrell (1974)
Rat, mouse, guinea pig, hamster, rabbits, dogs, primates	Review chapter in monograph; species differences in biotransformation, plasma protein binding, biliary excretion, and pharmacokinetics	Example: Dog has increased risk of bladder cancer (in response to) aromatic amines because of ability to N-hydroxylate but limited capacity to acetylate.	Calabrese (1988)
Rat, mouse, guinea pig, hamster, rabbits, dogs, primates		Example: In general, protein binding is highest in primate and lowest in mouse.	Cayen (1987)
Rat, mouse, rabbit, guinea pig, dog	A citation classic; species and other factors, in the metabolism of four different chemicals explored	In general, most rapid half-life in mice, longest in dogs. Correlates with rates of microsomal demethylation.	Quinn et al. (1958)
Rat, mouse, guinea pig, rabbit, rat, dog, quail, trout	Cytochrome P-450, MMFO, EH, UDPG transferase, PAPS-sulfotransferase, N-acetyl transferase	A good basic comparison. Convenient source for species comparison.	Gregus et al. (1983)
Rat, mouse, guinea pig, rabbit, dog	Drug metabolism by nasal tissue *in vitro*	Highest activities in hamster, lowest in dog.	Hadley and Dahl (1983)

Abbreviations: AP = demeti-aminopyrine demethylase; BP-OH = benzo(a)pyrene hydroxylase; EC = deethyl-7-ethoxycoumain deethylase; 2-AAF = 2-acetylaminofluorene; 3-MC = 3-methylcholanthrene; EH = epoxide hydrolase; AN-OH = aniline hydroxylase; GSH = glutathione; UDPG = UDP-glucuronic acid; γ-GTP = γ-glutamyltranspeptidase; gp = guinea pig; GSH-T = glutathione s-transferase.

Excretion

The processes of elimination is not dealt with in great detail in the core chapters. This is not to say, however, that excretion is not important. Like absorption, elimination can be both active and passive, and most xenobiotics are passively excreted. In most cases, conjugated metabolites are actively excreted. The process of xenobiotic metabolism can be viewed, to a certain extent, as packaging for the excretory process. Across species, the active excretion of a metabolite by the liver into the bile is probably (quantitatively) the most important active excretory process concerning xenobiotic disposition. Glucuronide conjugates (as reviewed by Levine 1978; Klaassen and Watkins 1984; Williams 1972) are actively excreted by the liver into the bile and ultimately into the feces. Amino acid conjugates, in contrast, tend to be excreted by the kidney into the urine. These are

definite species-related differences in the molecular weight cut-off between 300 and 500 for the biliary transport of the metabolite that dictate whether a metabolite will end up in either the feces or the urine.

Species differences do not follow any particular phylogenetic lines. For example, rats and dogs effectively excrete phenolphthalein glucuronide (50% in bile), whereas guinea pigs and monkeys do not (< 10%; Williams 1972). Although there are species differences in excretions, these tend to be overshadowed by the species differences in metabolism. That is, a particular species might not need an efficient biliary excretory process because with a particular chemical, it might produce glucuronides sparingly or not at all. This information might be of interest to the pharmacokinist in determining where to look for a metabolite. Generally, however, such information is of academic interest to the pharmacologist or toxicologist in interpreting an experiment because glucuronides are generally inactive end products, and it does not really matter whether they end up in the urine or feces. (As with any rule, there are exceptions: Mulder [1986] cites several examples of glucuronides being active metabolites; i.e. causing toxicity.) There are several instances, however, in which biliary excretion actually influences the toxicity of a chemical, such as with some of the cardiac glycosides or heavy metals where biliary excretion occurs without metabolism and biliary excretion is the "detoxification" mechanism (Klaassen and Watkins 1984). Cayen (1987) pointed out that species-related differences in indomethacin-induced intestinal damage directly correlates to the degree of exposure of the mucosa owing to biliary excretion and resultant enterohepatic circulation. Such instances are discussed on a species-specific basis where such data permit.

SUMMARY

I have attempted to assemble a source of basic information on laboratory animals, and trust that this book will provide a convenient source of information for either the skilled or novice investigator to aid in the design and interpretation of *in vivo* pharmacological or toxicological studies.

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CHAPTER 2

The Mouse

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TOXICOLOGY

History

The domesticated mouse of North America and Europe (*Mus musculus*) is the most widely used animal in medical research. The mouse is a member of the order *Rodentia,* family *Muridae,* and subfamily *Murinae.*

The use of the mouse in biomedical research has been chronicled for several hundred years. William Harvey (1578–1657) published the results of his work on animal reproduction and blood circulation based in part on his work with mice (Harvey 1616, cited in Morse 1981). Joseph Priestly (1775) used mice in exploring the phlogiston theory, and Antoine Lavoisier (1777) used mice in his studies of the physiology of respiration (both cited in Morse 1981).

Mice were selectively bred for coat color for many centuries, but in the early 1900s efforts turned to breeding strains of mice that might mimic human disease states. Subsequently, inbred strains were derived that were particularly susceptible or resistant to various types of cancers and viruses. A strain is considered to be inbred when it has been derived by brother × sister matings for 20 or more consecutive generations (F20), and can be traced to a single ancestral breeding pair in the 20th or subsequent generations. Certain other breeding systems (e.g., parent × offspring) can be substituted as long as the inbreeding coefficient achieved is at least equal to that at F20 (Lyon 1981). The genetic groundwork was laid for most of the strains of inbred mice currently in use by researchers such as William E. Castle, Clarence C. Little, and Leonell C. Strong during the period of about 1900 to 1930 (Morse 1981).

Although highly inbred strains have proven invaluable in fields such as genetics and histocompatibility research, a school of thought developed that random bred or specifically outbred strains might more closely represent man in many areas of medical research. A random breeding program attempts to achieve a level of genetic variability similar to the initial (noninbred) population, and in so doing, preserve the “hybrid vigor” associated with heterozygosity.

Many of the inbred and outbred strains of mice currently in use are referred to as *Swiss strains*. All of these Swiss strains are traceable to a group of two male and seven female albino mice obtained from the noninbred stock of Dr. A. de Coulon of Lausanne, Switzerland, and imported

into the United States by Dr. Clara Lynch of the Rockefeller Institute in 1926 for use in cancer research (Lynch 1969, cited in Hill 1983).

Choice of the Mouse in Toxicological Research

As discussed in chapter 1, the choice of a species for toxicity testing is based on consideration of a range of variables. Ideally, if toxicity testing is intended to provide information on the safety of a test article in or by humans, the species chosen for testing should be most similar to the human in the way it handles the test article pharmacodynamically. Substantial differences in absorption, distribution, metabolism, or elimination (ADME) between test species and the target species (e.g., the human) will reduce the predictive value of the test results. From a practical standpoint, often the pharmacokinetics are unknown in humans or the variety of available test species at the time of species selection. For this reason, testing is usually conducted in at least two species. Generally, one of those species is usually a rodent and one a nonrodent. The two most commonly used rodent species are mice and rats, and often toxicity testing is conducted in both of those species.

Mice have many advantages as test animals for toxicity testing. They are small; relatively economical to obtain, house, and care for; and generally easy to handle. Mice are generally more economical than rats in these respects. Although mice might attempt to escape or bite handlers, with regular, gentle handling they are easily managed. Other advantages of the species include a short gestation period and a short natural life span. These characteristics allow studies that include evaluation of reproductive performance or exposure to a test article for periods approaching the expected life span (e.g., evaluation of carcinogenic potential) to be conducted in a practical time frame. High-quality, healthy mice are available from reliable commercial suppliers. Many genetically well-defined, highly inbred, specifically or randomly outbred strains are available. Mice have been used in biomedical research for hundreds of years, and because of this, many technical procedures have been developed for use with the species, and a vast body of historical data is available for most strains. This historical database includes information on optimal nutritional and housing requirements in addition to data such as the expected background incidence of various diseases and types of tumors in untreated animals, and it is continuously being added to (Blackwell et al. 1995).

There are some disadvantages to using mice, and most are related to the small size of the animal. The smaller size and higher metabolic rate compared to the rat renders the species a bit less hearty than rats. Deviations in environmental conditions such as an air conditioning failure or failure in an automatic watering system typically have more severe effects on smaller species such as mice than they do on rats. Owing to their high level of natural activity, most strains of mice will not become as docile or easy to handle as rats that have received equivalent handling. Small size often precludes or renders more difficult a number of procedures that are commonly conducted in toxicity testing, such as the collection of large samples or repeated samples of blood and urine, electrocardiographic evaluation, and some necropsy evaluations.

This section provides brief summaries of some of the normal physiological values and salient features of the species and some of the specific strains that might be useful in selecting an appropriate species and strain for toxicity testing.

Normal Physiological Values

Selected normal physiological values for mice are shown in table 2.1 and table 2.2. Median survival of a number of groups of Charles River CD-1 outbred mice is shown in table 2.3.

These normal values will vary depending on the strain of mouse, supplier, condition at arrival, type of feed, environmental and housing conditions, and in some cases, time of year. These data should be considered as a reference, but will not necessarily represent experience in any particular laboratory.

Table 2.1 Normal Physiological Values: General and Reproductive

General	
Life span	
Average	1–3 years
Maximum reported	4 years
Adult weight	
Male	20–40 g
Female	18–40 g
Surface area	0.03–0.06 cm^2
Chromosome number (diploid)	40
Food consumption	4–5 g/day
Water consumption	5–8 ml/day ad libitum
Body temperature	36.5°C
Oxygen consumption	1.69 ml/g/hr
Reproductive	
Age, sexual maturity	
Male	50 days (20–35 g)
Female	50–60 days, (20–30 g)
Breeding season	Continuous, cyclic
Estrus cycle	4–5 days
Gestation period	
Average	19 days
Range	17–21 days
Litter size	
Average	12
Range	1–23
Birth weight	1.5 g
Age begin dry food	10 days
Age at weaning	16–21 days (10–12 g)

Source: Data derived from Jacoby and Fox (1984), and from the *Animal Diet Reference Guide,* Purina Mills, Inc. (1987)

Table 2.2 Normal Physiological Values: Cardiovascular and Respiratory

Cardiovascular	
Heart rate	
Average	600/min
Range	320–800/min
Blood pressure	
Systolic	133–160 mm Hg
Diastolic	102–110 mm Hg
Blood volume	
Plasma	45 ml/kg
Whole	78 ml/kg
Hematocrit	41.5%
RBC life span	20–30 days
RBC diameter	6.6 microns
Plasma pH	7.2–7.4
Respiratory	
Rate	
Average	163/min
Range	84–230/min
Tidal volume	
Average	0.18 ml
Range	0.09–0.38 ml
Minute volume	
Average	24 ml/min
Range	11–36 ml/min

Source: Data derived from Jacoby and Fox (1984), and from the *Animal Diet Reference Guide*, Purina Mills, Inc. (1987)

Table 2.3 Median Survival of 16 Groups of Control Mice

	Period of Time on Study (Months)			
Sex	**6**	**12**	**18**	**21**
Male	98%	91%	63%	46%
Female	98%	95%	74%	68%

Data represent median survival of Charles River CD-1 outbred albino mice enrolled in 24-month chronic toxicity studies at pharmaceutical or contract toxicology laboratories. *Source:* Adapted from Lang (1989b).

Species Differences

Mice are similar to other common laboratory animal species and to humans in many ways, yet the differences should not be underestimated. Mice have a high metabolic rate compared to other species. This fact alone could result in increased or decreased toxicity of a test article, depending on the specific mechanism of intoxication. In many cases, high metabolic rate may be associated with rapid ADME of a test article. Mice are obligate nose breathers, and have more convoluted nasal passages than humans. This might result in an excess of respirable test article deposited in the nasal passages, causing either increased or decreased relative toxicity, depending on the most critical site of absorption. The small size of the mouse compared to other common laboratory species offers a significant advantage if the test article is expensive or in short supply. As an approximation, a mouse weighs about 10% as much as a rat, about 5% as much as a guinea pig, about 1% as much as a rabbit, and less than 1% as much as a dog or primate. Material requirements to administer equivalent dose levels are usually proportional to body weight, so the test article savings associated with the mouse are evident. The small size of a mouse results in high surface area to body mass ratio, which in turn causes the mouse to be relatively intolerant of thermal and water balance stresses. The kidneys of a mouse have about twice the glomerular filtering surface per gram of body weight as a rat, and owing to the specific architecture of the murine kidney, they are capable of producing urine that is about four times as concentrated as the highest attainable human concentrations (Jacoby and Fox 1984). These characteristics of renal architecture and function might be important to the toxicity of some test articles. Mice differ from most species by the formation of a persistent vaginal plug after mating. The presence of a vaginal plug is easily detected, is considered evidence of mating, and is a useful characteristic during the conduct of reproductive studies.

It is also frequently the case in pharmaceutical research and development that the nonclinical efficacy model for a new drug is in the mouse, making it the natural choice for rodent evaluation of the drug.

Strain Differences

In addition to differences between mice and other species, there are important differences among different strains of mice. The appropriate choice of a strain of mice for a particular toxicity study should consider the specific objectives of the study and the specific characteristics of candidate strains that might assist or hinder in achieving those study objectives.

One difference among strains is in the normal body weights of various strains at different ages. These differences are summarized for selected strains available from the Charles River Breeding Laboratories in table 2.4. Outbred strains tend to be larger at maturity than inbred strains, with the CD-1 strain reaching the highest mean weights at 56 days of age of those strains in table 2.4. The CF-I strain has been reported to be highly resistant to mouse typhoid and to be relatively resistant to salmonellosis (Hill 1981). Nude or athymic strains of mice are more sensitive to tumor development than heterozygous strains. These sensitive strains develop the same types of tumors as those

Table 2.4 Normal Body Weights in Grams of Selected Strains of Mice

Age (days)	Outbred Strains						Inbred Strains						Hybrid	
	CD-1		CF-I		CFW		C3H		C57BU6		BALB/c		B6C3FI	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F
21	12	11	12	11	9	9	—	—	—	—	—	—	—	—
28	20	18	18	17	16	13	17	16	14	13	16	14	16	14
35	27	22	24	21	19	17	18	17	17	14	17	16	20	17
42	30	24	27	22	24	20	20	18	19	16	18	17	22	18
49	33	26	28	24	27	22	24	23	21	17	20	18	24	19
56	35	27	30	26	28	23	27	26	22	18	21	19	26	21

Source: Data derived from Charles River Growth Charts, Charles River Laboratories, ca. 1975.

seen in more conventional strains, but the incidences are higher and the latency periods shorter. There is a wide spectrum of susceptibility to spontaneous lung tumors in various strains of mice, and evidence suggests that there is a high correlation between spontaneous incidence and chemical inducibility in those various strains (Shimkin and Stoner 1975).

The inbred strain A mouse appears to be the most susceptible to lung tumors, and forms the basis of a lung tumor bioassay, with tumors inducible within 8 weeks or less of treatment. Susceptibility of various strains to the initiation or promotion of skin tumors has also been shown to differ greatly (Chouroulinkov et al. 1988; Steinel and Baker 1988). The incidences of selected spontaneously occurring neoplastic lesions in CD-1 (outbred) and B6C3F1 (hybrid) strains are compared in table 2.5.

The number of strain-related differences in susceptibility to various test articles and environmental conditions exceeds the scope of this chapter, but additional information is available (Nebert 1981).

Husbandry

Facilities

Facilities used to conduct toxicology studies in mice must have separate and adequate areas for the required laboratory procedures and for the housing and treatment of study mice. This discussion is limited to facilities for the housing and treatment of mice. Several characteristics for an adequate facility for the conduct of toxicity studies intended to support regulatory approval of new drugs are listed in the Good Laboratory Practice regulations (CFR 1988, para. 58.43). Such a facility should include enough animal rooms or areas to properly separate different species and projects. In addition, there should be facilities for the quarantine of incoming or sick animals, and for the isolation of any studies that involve use of hazardous materials. In practice, animal rooms used for toxicity studies in mice should not contain any other species of animals. Isolation of individual projects is generally interpreted to mean that an animal room should be dedicated to a single toxicity study. One exception to the requirement to conduct only one study per animal room is the case of acute or very short-term toxicity studies, each of which is limited to a small number of animals. Another exception is dermal carcinogenesis, or "skin painting" studies, which generally involve a relatively small number of animals treated for 30 to 40 weeks. A number of acute or dermal carcinogenesis studies can be run concurrently in a single room. For practical reasons, adequate isolation for acute or dermal carcinogenesis studies is typically interpreted to mean separate cage racks for each study or isolation of multiple studies within the room by geographical location within the room. The intent of the requirements to isolate species and, in most cases, studies is to reduce the probability of cross-contamination between studies with the various test chemicals or disease entities, and to minimize the opportunity for accidental administration of the incorrect test substance to a group of animals. The concept of quarantining incoming animals can be met by having new animals delivered into a sanitized room that contains no other animals, then allowing the new mice an

Table 2.5 Incidence of Spontaneously Occurring Neoplastic Lesions Lesions Occurring at Spontaneous Incidence of ~1% in Either Sex of Charles River CD-1 or B6C3F1 Mice Strain

	CD-1		B6C3F1	
Location and lesion	**M**	**F**	**M**	**F**
Lymphoreticular tumors				
Lymphosarcoma			6.0	12.0
Lymphocytic leukemia		1.3	1.4	
Lymphoma	3.7	9.9		
Histiocytic lymphoma		0.5	1.4	
Histiocytic sarcoma		0.1	1.4	
Lymphoblastic lymphoma	1.1	1.7	0.2	0.1
Lymphocytic lymphoma	2.6	1.7	0.6	1.7
Reticulurn cell sarcoma	2.6	5.1	0.4	0.2
Skin/subcutis				
Fibrosarcoma	0.2	0.5	1.0	0.5
Mammary gland				
Adenocarcinoma		1.7		0.9
Lung				
Bronchiolar/alveolar adenoma	4.0	2.9	8.3	3.3
Bronchiolar/alveolar carcinoma	3.5	3.1	1.9	0.6
Alveolar type 11 carcinoma	11.7	13.9	0.2	0.1
Alveolar type 11 adenoma			2.5	1.2
Adenoma	1.2	0.7		
Liver				
Nodular hepatocellular prolif.	5.4	1.7	0.5	0.1
Hepatocellular adenoma	5.6	0.8	17.2	7.1
Hepatocellular carcinoma	7.3	1.0	13.2	2.4
Hemangioma	1.0	1.2	0.7	0.3
Hemangiosarcoma	1.0	0.2	0.5	0.1
Reproductive system				
Ovary				
Cystadenoma		1.1		0.3
Uterus				
Endometrial stromal polyp		3.3		2.9
Endometrial sarcoma		1.9		0.6
Leiomyorna		1.0		
Leiomyosarcoma		1.0		0.3
Hemangioma		1.0		0.9
Pituitary				
Adenoma		3.4	0.3	7.9
Thyroid gland				
Follicular cell adenoma	0.2		0.8	2.4
Adrenal				
Cortical adenoma	8.6	1.0	0.4	0.3
Harderian gland				
Cystadenoma			1.9	1.6
Adenoma			1.5	0.6

Source: Data from Charles River Breeding Laboratories, compiled from control animals on 24-month studies completed between 1978 and 1986.

adequate acclimatization period prior to initiation of the toxicity study. This procedure minimizes the risk of exposure of either new or existing animals to incoming or endemic diseases.

The physical conditions in an animal room are referred to as the macroenvironment. These conditions include such things as the temperature, relative humidity, lighting, ventilation, and concentrations of various gases (e.g., CO_2, ammonia). Many of the macroenvironmental parameters

are routinely monitored in facilities that conduct toxicology studies. Abnormal fluctuations in some of these parameters can have a deleterious effect on the validity of toxicity data generated.

Temperature and Relative Humidity

Mice are quite sensitive to variations in temperature, and respond to those variations with important physiological changes. This sensitivity is caused by the large surface area to body weight ratio in mice, which causes them to radiate heat quickly in a cold environment. Mice respond to low temperature by nonshivering thermogenesis, and resting mice can generate heat at a rate about triple their basal metabolic rate. Group-housed mice can compensate for low temperatures by huddling in a group, a practice that is more effective in a solid-bottom cage containing bedding. Mice have a limited capacity to compensate for excessive heat, and do so primarily by vasodilation of the ears to increase heat loss, and by increasing body temperature by several degrees. In the wild, mice adapt to excessive temperatures by moving to cool burrows. Mortality is often observed if the ambient temperature reaches 37°C or higher. The range of environmental temperatures where an animal's oxygen consumption is minimal and virtually independent of changes in ambient temperature is called the thermoneutral zone. The thermoneutral zone for mice is one of the narrowest of species studied, and is about 30.0°C ± 0.5°C. Mice seem to be generally healthier at a temperature of about 21°C to 25°C than they are within the thermoneutral zone (Jacoby and Fox 1984). The recommended dry-bulb temperature for a mouse housing room is 18°C to 26°C (ILAR 1985).

Variations in environmental temperature can affect the results of toxicity studies in unpredictable ways. Muller and Vernikos-Danellis (1970) found that the acute toxicity of dextroamphetamine was reduced by tenfold when the temperature was reduced to 15°C from a normal of 22°C, but was increased by two to threefold when the temperature was increased to 30°C. Conversely, the acute toxicity of caffeine was increased when the temperature was altered either up or down from 22°C. Food consumption is inversely related to ambient temperature, and the secondary effects of changes in food consumption can be complex.

The relative humidity in a mouse room is a significant factor in thermoregulation for the mice housed. At constant temperature, mice are more active at lower relative humidity than at higher humidity. This difference is believed to be a function of the mouse's ability to better dissipate heat under conditions of lower relative humidity (Stille et al. 1968). Low ambient relative humidity has been associated with increased transmission of a disease called ringtail in mice. High relative humidity leads to increased production of ammonia in the urine and feces. Increased ammonia concentrations have been associated with the development of respiratory diseases in rodents (Broderson et al. 1976). The recommended relative humidity for a room housing mice is 40% to 70% (ILAR 1985).

Lighting

Any description of lighting in an animal facility must include a description of the intensity, wavelength or spectrum, and photoperiod of the light.

Light intensity is expressed in footcandles (ftc) (lumen/ft^2) or lux (lumen/m^2). Historically, lighting intensity was selected for convenience of the researchers, based on the assumption that what was good for people was good for mice. In fact, the *Guide for the Care and Use of Laboratory Animals* as recently as 1978 recommended light intensity, of 100 to 125 ftc. Mice are nocturnal, however, so it is not surprising that their eyes are better adapted to lower light levels. Continuous exposure to light at 100 to 125 ftc for 6 days has been found to cause loss of 90% of the photoreceptors in albino-beige mice (Robison and Kuwabara 1978). Light levels of 25 ftc or lower have been recommended for mice (Robison et al. 1982). In addition, the specific design and location

of individual cages within an animal room can have a substantial effect on the light level at the animal's level. Owing to the nocturnal nature of mice and the attenuation of light by the structure and location of individual cages, there is probably little or no actual injury to an animal housed in a room lighted to 100 to 150 ftc on a 12-hr light, 12-hr dark cycle.

The wavelength or spectrum of light found in a mouse room is generally a function of the type of fluorescent lighting in common use at the animal facility. Although the lighting spectrum might not be of the highest concern in designing toxicity studies, Spalding et al. (1969) showed that mice exhibited the highest level of voluntary wheel running activity under red light or in darkness; an intermediate level of activity under yellow; and the lowest under green, blue, or daylight. Recalling the nocturnal characteristics of mice, the proximity of red light to dusk or darkness and of blue light to ultraviolet and daylight on the visible spectrum is probably not a coincidence.

The photoperiod for mouse rooms used for toxicity studies is typically diurnal, with a cycle of about 12 hr light to 12 hr darkness. The photoperiod influences circadian rhythms, and is probably most often thought of in the context of influence on the estrus cycle. Whereas the estrus cycle of the rat is synchronized by the photoperiod, the estrus cycle of the mouse is less easily influenced by photoperiod (Campbell et al. 1976).

Ventilation

Ventilation in an animal housing facility should be designed to provide sufficient fresh air to remove the thermal load generated by the animals, lights, and equipment; maintain acceptable levels of dust and odor; provide adequate oxygen; and contain any biohazards or intercurrent disease in the animal colony.

A common practice for animal facilities has been to design the heating, ventilation, and air conditioning system to provide 10 to 15 air changes per hour. In reality, this approach can be quite misleading because it depends on the total volume of the animal room rather than the biological and thermal load. An animal room with 12-ft ceilings would require 50% more cubic feet of fresh air per minute to achieve 15 air changes per hour than would a room with 8-ft ceilings. Clearly the more important factor in this example is the number of cubic feet of fresh air per mouse (or kilogram of animals) housed rather than the number of air changes per hour. As a guide, an adequate ventilation rate for a mouse room is about 0.147 ft^3/min of fresh air per mouse, with a heat removal capacity of about 0.6 BTU per hour per mouse (Runkle 1964).

If at all possible, supplied air should be 100% fresh outside air, introduced into the animal room at ceiling level, with exhaust air picked up at or near the floor to eliminate a maximum amount of heavier-than-air ammonia vapors. Supply air should minimally be drawn through a particle filter, and many facilities now include high-efficiency particle (HEPA) filters on supply air. Addition of HEPA filtration to an existing system usually requires a substantial increase in air conditioning blower power or capacity to overcome the added resistance of the filters. Supply of 100% fresh air at the ventilation rates discussed entails significant energy costs for heating and cooling. These costs can be reduced by the installation of a heat (cool) recovery system between the exhaust air and the incoming fresh air. The efficiencies of such a system are accrued during both the heating and the cooling seasons. If, for some reason, 100% fresh air cannot be supplied, any recycled air must be passed through a complex filtration system to prevent reintroduction of (and cross-contamination by) biological or chemical contaminants and odors.

Relative air pressure between animal rooms and corridors should be considered as a prime mechanism for control of cross-contamination and communication of disease between animal rooms. In theory, if all animal rooms are either at positive pressure relative to the corridor, or if all are negative relative to the corridor, there should be no communication of airborne contamination between rooms. If any hazardous substances or human pathogens will be used within the animal rooms, those rooms should be maintained negative to the corridor to prevent contamination of people.

Noise

There are no specific regulations or guidelines governing noise to which mice can or should be exposed. There are, however, numerous reports in the literature of adverse or abnormal effects of "stressful" noise on mice, including reduction in body weight (Fink and Iturrian 1970); changes in immune response and tumor resistance (Jensen and Rasmussen 1970); audiogenic seizures (Mokler 1973), which are seen in genetically susceptible mice and can also be induced in normal mice; unexpected responses to certain drugs (Iturrian and Johnson 1975); actual hearing impairment; and others. In view of the variety of effects associated with noise, control of environmental noise in a facility used to conduct toxicity studies in mice should be addressed.

Noise should be described in terms of two dimensions: intensity and frequency. Sound intensity (sound pressure level or loudness) is measured in decibels (dB) and frequency (pitch) is measured in hertz (Hz). Hertz is the standard unit for cycles per second. Although a "safe" intensity of sound has not been described, intensities of 90 to 100 dB have produced adverse effects, including inner ear damage, and it has been recommended that noise levels be maintained below 85 dB (Anthony 1962). It is especially important to consider the frequency of environmental noise because the frequency spectrum for hearing in mice differs substantially from that in humans. High-frequency noise that is inaudible to humans can be disruptive or injurious to mice. The average human ear can hear sounds in the frequency range of about 20 Hz to 20 kHz, with maximum sensitivity at about 2 kHz. In contrast, mice cannot hear sounds with frequencies as low as 1 kHz, clearly hear sounds at 50 kHz, and probably have an upper limit in the range of 60 to 70 kHz (Clough 1982). Mice emit sound in these upper frequencies, apparently as a means of communication between mothers and young, and associated with mating and aggression. Environmental noise near these frequencies might disrupt normal behavior. Devices that emit sound in this spectrum include ultrasonic motion detectors (used for door openers and intrusion alarms), ultrasonic cleaning and mixing equipment, high-speed homogenizers, dropped or banged metallic devices (e.g., cages, pans, covers), and many others. Dogs and nonhuman primates create noise of an intensity and frequency that can be disruptive to mice.

Environmental noise should be controlled at two levels: the design and selection of facilities and equipment and establishment of procedures for animal husbandry and the conduct of laboratory activities that might produce noise at a disruptive intensity or frequency. As the noise of dogs and nonhuman primates is disruptive to rodents (ILAR 1985), mice should not be housed in close proximity to these species. Loud noises (e.g., barking of dogs) can be transmitted from room to room, sometimes significant distances, through the ventilation system ductwork. This transmission can be reduced by installing labyrinthine configurations and commercially available acoustic attenuators in the ductwork that services noisy rooms. Mice should not be housed in close proximity to essentially noisy operations such as cage washing. Intense, high-frequency noise can be generated by the movement of cage racks, equipment carts, and so on, in hallways adjacent to animal rooms. A variety of design considerations can improve this situation. A cage rack moving on rubber or synthetic cushioned wheels with well-lubricated bearings over a monolithic flooring will be much quieter than a similar rack on steel wheels with squeaky bearings moving over quarry tile or even concrete flooring. Procedures should be devised and personnel should be trained with an appreciation for the deleterious effects excessive noise could have on the well-being of mice, and consequently on the results of toxicity studies conducted with those animals.

Construction Parameters

Clearly a detailed discussion of the architectural and engineering aspects of a facility intended to house laboratory mice is beyond the scope of this text. Our experience, however, has suggested two areas that should receive top priority when new construction, renovation, or even routine maintenance is required.

Cleanability. Animal rooms are exposed to a wide variety of soiling agents on a continuing basis. Sources include such things as feed dust, spilled water, animal waste, bedding, parasites (e.g., mouse pinworms), and various bacterial and viral strains that might infect the species housed. Consequently, animal rooms must be swept and mopped frequently, typically using a chemical detergent and disinfectant, and should be sanitized at least before each new study goes into a room, and on a regular basis if a long-term study is in progress. One of the most effective processes for sanitization uses a pressure sprayer to clean ceilings, walls, and floors with an effective disinfectant. This process requires that all surfaces (ceilings, walls, and floors) be coated with a durable, waterproof, chemical-resistant finish. That finish should withstand impacts, such as cage racks colliding with walls, and various objects being dropped on floors without damage to the flooring. In addition, all lighting fixtures, electrical outlets, switches, computer connections, thermostats, and so on, should be either of waterproof construction, or should be equipped with waterproof covers that can be closed during sanitization.

Vermin Resistance. Animal rooms are notoriously attractive to insects such as cockroaches, flies, and other pests. The ready access to feed and water that is essential for the mice is an ideal environment for insect infestation as well. The use of toxicants to control pests in rooms housing animals for toxicity studies is discouraged for several reasons. Many common pesticides are known to induce the synthesis of hepatic microsomal enzymes, which in turn could alter the apparent toxicity of the substance being tested. As most toxicity studies are conducted on test substances with unknown or incompletely understood pharmacology, the possible interactions with a particular pesticide are unpredictable. Pests might consume a toxicant, then enter animal cages prior to dying, and be ingested by the study mice. This could lead to indirect intoxication of the study animals.

One environmental requirement for a successful insect population explosion is the availability of a concealed harborage for breeding. We have had remarkable success in controlling insect infestation in our facility by the simple process of sealing, caulking, closing, or eliminating every crack, crevice, hole, wall penetration, electrical outlet, and floor drain in our animal rooms. For this practice to be successful, contractors and maintenance people who work in these rooms must understand the purpose and importance of their task. Cracks in a wall behind a sink are just as useful as harborages as cracks in the middle of the wall, so each contractor and maintenance person must watch for and eliminate these problems when they are found.

If elimination of harborage has not been implemented or is incomplete, it might be necessary to employ a toxicant on a carefully controlled basis. The study toxicologist should participate in the selection of a suitable toxicant, factoring in all that is known about the pharmacology of the substance being tested in the toxicity study and providing a best estimate of possible interactions with the toxicant. Accurate records should be kept of what toxicant was used, where and how much was applied, and how often it was applied. These factors should be reviewed and considered when the toxicity study is completed and the results are being interpreted.

Caging

The physical conditions in an animal cage (primary enclosure) are referred to as the microenvironment. These conditions (e.g., temperature, relative humidity, lighting, ventilation, concentrations of various gases [e.g., CO_2, ammonia]) might differ substantially from the conditions in the macroenvironment, depending on the specific design and placement of the cage within the animal room. Microenvironmental parameters should be evaluated for various cage designs and locations within animal rooms, but are not routinely monitored in facilities that conduct toxicology studies.

Any caging used for mice in toxicology studies should be designed to provide adequate space for freedom of movement and a comfortable environment in terms of temperature, humidity, and ventilation that will minimize stress on the animals. The caging should be cleaned regularly to allow the mice to remain clean and dry. Caging should be as resistant to escape as possible to

preserve the integrity of the study as well as the health and safety of the animals. Even if an escaped animal can be recovered and returned to its cage, the health of the animal and resultant impact on the integrity of the toxicity data generated remains in question, as the animal might have contacted toxic or interfering substances during its travels.

Cage Type

The two types of mouse caging most commonly used for toxicology studies are wire-bottom cages and solid-bottom cages.

Wire-Bottom Cages. Wire-bottom cages are typically suspended in rows on a movable rack with between 10 and 70 cages arranged on a side. Racks can be single sided or double sided, depending on the configuration of the animal room and the number of animals that need to be housed. Most contemporary wire-bottom cages are fabricated of stainless sheet steel backs and sides, with stainless steel wire mesh fronts and floors. Sides and backs might have holes or slots stamped into them at manufacture to allow improved ventilation and access to an automatic watering system if one is available. Mesh fronts allow observation of the animals. Wire-bottom cages are also fabricated of polycarbonate or other rigid plastic products. Transparent plastic cages provide easier observation of animals, but typically offer reduced ventilation through the cage itself. Mesh floors allow urine, feces, and spilled food to drop through, typically to a waste pan or absorbent paper, which can be cleaned or replaced easily and regularly. Waste pans should be cleaned or papers replaced at least three times a week. A wire-bottom design has the advantage of keeping animals relatively free of contamination from urine and feces, and also providing ease of cleaning. Wire-bottom cages should be rotated out of use and washed at least once every 2 weeks. This washing procedure should be monitored regularly, and should be adequate to produce negative results on microbiological swab testing. A procedure that achieves good microbiological test results for cages with average levels of soil first requires removal (or emptying) of feeders, waste pans, and water bottles (if present). Then the racks with the suspended wire cages still in place are passed through a commercial rack washer that operates much like an automatic dishwasher. All cycle times are variable, but an effective combination for average soil is a wash cycle of about 10 min, followed by initial and final rinse cycles of about 3 min each. The wash cycle includes an effective disinfectant detergent (e.g., PRL-18, manufactured by Pharmacal Research Laboratories, Inc., Naugatuck, Connecticut). All water temperatures (wash, initial, and final rinse) are maintained at or above 82°C (180°F), but this temperature is most important for the final rinse.

Solid-Bottom Cages. Solid-bottom cages, often referred to as shoebox cages, can be suspended in a rack, like wire-bottom cages, or can be supplied with tops, and arranged on shelves, which are typically movable as a rack. Shoebox cages are typically constructed of polycarbonate, which allows convenient observation of the animals; polypropylene, which is a translucent plastic; or sheet metal, such as stainless steel. Shoebox cages provide a secure base for animals, and are essential if animals are to be allowed to deliver and suckle live litters. Solid-bottom cages can be provided with filter tops, which can significantly reduce airborne contamination of the environment within the cage. Filter tops do have a negative impact on ventilation within the cage, and levels of CO_2 and ammonia have been found to be substantially higher in cages with filter tops than in those with stainless steel rod tops (Serrano 1971). There are a number of disadvantages associated with solid-bottom cages. These cages must be provided with some form of low-dust or dust-free absorptive bedding. This bedding must be changed regularly, which is labor intensive. If matings are conducted, vaginal plugs are often difficult or impossible to find in the bedding. As mice engage in coprophagy, a toxic substance or metabolite that is eliminated in the feces may be "recycled," thereby leading to an overestimate of the true toxicity of the substance. In addition, mice commonly ingest various types of bedding, such as wood chips, which renders impossible any serious estimate

of food consumption. If an automatic watering system is in use, solid-bottom cages have the potential to fill with water if there is a malfunction, drowning the inhabitant(s).

In routine toxicology studies, solid-bottom cages, covers, feeders, and so on, should be rotated out of use and washed once or twice a week, and their supporting racks should washed at least once per month. This cage-cleaning schedule should not be followed if reproductive procedures (mating, delivery, and suckling of young) are being conducted, as the constancy of home-cage odor is critical to reproductive efficiency and reducing the likelihood of cannibalization of young. Cage washing might need to be suspended completely during pregnancy and lactation. (See "Bedding" section for special practices used with reproduction procedures.) The cage and accessory washing procedure should be monitored regularly, and should be adequate to produce negative results on microbiological swab testing. A procedure that achieves good micrcobiological test results in our facility for cages with average levels of soil employs passage through a tunnel washer on a steel mesh belt. The total transit or cycle time is typically about 3 min, with about 15 sec for prewash, and just under 1 min each for main wash, rinse, and drying cycles. The wash cycle includes an effective disinfectant detergent (e.g., Clout, manufactured by Pharmacal Research Laboratories, Inc., Naugatuck, Connecticut). The rinse water is heated to at least 820°C (1,800°F), and the drying cycle consists of exposure to high-temperature forced air. The belt speed in a machine such as this can be reduced (total transit time lengthened) for heavily soiled cages or increased (transit time shortened) for lightly soiled cages, but the single criteria that governs the minimum length of the transit time is the maintenance of negative results on the microbiologic monitoring of the clean cages.

Cage Size

Mouse caging must be of adequate size to allow free movement and to avoid overcrowding. Minimum space requirements for mice are provided in the *Guide for the Care and Use of Laboratory Animals* (ILAR 1985) on the basis of body weight. Those requirements are listed in table 2.6. As a practical matter, to avoid the need for multiple-sized caging and frequent (e.g., perhaps weekly) changes in the size of caging occupied, the minimun specified for adults can be used for all ages.

Population

Mice can be housed singly (one to a cage) or in groups (several animals of the same treatment group caged together) for toxicity testing. It has long been recognized, however, that group housing can substantially alter the toxicity of some substances. Chance (1946) demonstrated that the acute toxicity of a group of sympathomimetic amines was increased by two- to tenfold in mice that were group housed compared to mice housed singly. In addition, increased population in solid-bottom cages has been shown to lead to substantially higher levels of CO_2 and ammonia within the cage, even when open stainless steel rod tops were used, but especially if filter tops were in place (Serrano 1971). Increased levels of ammonia have been associated with hepatic microsomal enzyme induction, which can alter expected metabolism in a toxicity study.

Table 2.6 Minimum Cage Space Requirements for Mice

	Floor Area per Mouse		Cage Height	
Body Weight (g)	**in²**	**cm²**	**in**	**cm**
< 10	6.0	38.7	5	12.7
10–15	8.0	51.6	5	12.7
15–25	12.0	77.4	5	12.7
> 25	15.0	96.8	5	12.7

Single Occupancy. Single housing of mice used in toxicity studies offers many advantages. Of paramount importance is the reduced likelihood of mistaking the identity of individuals when conducting various study procedures (weighing, dosing, collecting various observational or other data). Singly housed mice are more quickly and easily identified and captured throughout the study. In addition, the risk of injury or cannibalism is eliminated in the event that one member of a group becomes debilitated. The biggest disadvantage to single housing is cost. Purchase price for individual caging for large numbers of mice is much higher than the cost for group housing. Individual caging requires much more floor space in the animal rooms than group caging for an equivalent number of animals, and the cost to provide animal care (food, water, cage cleaning, and sanitization) is much higher for single caging.

Group Housing. The biggest advantage to group housing is cost savings. Disadvantages include increased probability of mistaken identity, increased stress on animals as a result of establishment and testing of dominance hierarchy, and difficulty with individual animal identification systems. Group-housed mice tend to tear out each other's ear tags, and tattooed markings might be obliterated as a result of repeated fighting for dominance. Measurement of food or water consumption is generally not useful for group-housed mice, as the distribution of food and water among animals of different hierarchical positions tends to be quite uneven. Group housing in solid-bottom cages effects microenvironmental parameters within the cage such as temperature, humidity, various gas concentrations (CO_2, ammonia), and others.

Bedding

Some form of bedding material is required in solid-bottom cages to allow mice to remain clean, dry, and free of urine and feces. The Good Laboratory Practice (GLP) regulations (CFR 1988, para. 58.90) states that bedding should "not interfere with the purpose or conduct of the study" and should "be changed as often as necessary to keep the animals dry and clean." Appropriate bedding should absorb urine effectively, be as free of dust as possible to minimize pulmonary complications, be free of contaminating chemicals, and have no unacceptable effect on the normal physiology or metabolism of the mice. Inhalation of aromatic hydrocarbons from cedar and pine bedding has been shown to cause induction of hepatic microsomal enzymes, which could seriously compromise the results of a toxicity study (Vesell 1967; Wade et al. 1968). Opinions differ on whether the use of cedar wood shavings as bedding material contributes to the increased incidence of mammary gland tumors and hepatomas (Heston 1975; Sabine et al. 1973). One of the most commonly used bedding materials for toxicity studies is hardwood chips derived from woods such as maple, birch, and beech (e.g., Absorb-Dri hardwood chips, Maywood, New Jersey). This selection is probably based on the properties of high absorption and low dust, coupled with an absence of proof of harmful interactions.

Bedding material should be changed at least once or twice weekly when cages are washed. More frequent bedding changes might be required if mice are group housed**.** An exception to this practice for bedding change occurs when reproductive procedures (mating, delivery, and suckling of young) are involved in the study. A continuity of home-cage odor reduces maternal stress, is critical to reproductive efficiency, and reduces the likelihood of cannibalization of young. Therefore, during reproductive procedures, a portion (but not all) of the soiled bedding should be removed at regular intervals and replaced with clean bedding.

Animal Identification

Reliable identification of each animal used in a toxicity study is essential to the integrity of the study data and to the accurate interpretation of study results. The GLP regulations (CFR 1988, para. 58.90) state that

> animals, excluding suckling rodents, used in laboratory procedures that require manipulations and observations over an extended period of time or in studies that require the animals to be removed from and returned to their home cages for any reason (e.g., cage cleaning, treatment, etc.), shall receive appropriate identification (e.g., tattoo, toe clip, color code, ear tag, ear punch, etc.).

And further, that "All information needed to specifically identify each animal within an animal-housing unit shall appear on the outside of that unit." The GLP regulation was amended (Federal Register 1989) to eliminate toe clipping from the methods listed (previously), and to discourage the use of toe clipping in nonclinical laboratory studies, as other, more humane methods are available. One new method not listed in the GLP regulation, but which appears to offer some advantages, is the implantable electronic microchip.

Cage Cards

In practice, the comprehensive information already specified for the outside of an animal housing unit typically appears on a cage card. This information is relatively straightforward as long as animals are housed singly. The cage card should include complete identification of the study (study number is adequate if a unique coding system is used)and identification of or references to the species, strain, sex, source, age, treatment group (e.g., substance tested and dose group), and individual animal number of the mouse (or mice) housed. The identification that appears on the mouse itself is limited to a few code letters and or numbers that can be cross-referenced to the cage card and raw data record for the study to obtain all of the information available about that individual mouse.

Ear Tags

Ear tags are durable, typically made of a noncorroding metal such as Monel, with letters or numbers stamped into the surface. The tags are quick and easy to apply, using a special pliers-like applicator, and are easily read over a long period of time. One disadvantage to ear tags is that they can be torn out of the ear if they get caught on something in the cage, or especially if mice are group housed. Group-housed mice seem to find each other's ear tags especially vulnerable to pulling and hierarchical conflict, and the incidence of tag loss in group-housed mice has proven unacceptably high in our facility. Conversely, the incidence of tag loss among singly-housed mice (the norm for toxicity testing at our facility) is remarkably low, especially after the first few days of adaptation. When tags are lost from singly-housed mice, they are typically found in or under the cage, so the tag number can be confirmed and a replacement tag installed. One technique to increase tag retention is to avoid the edge of the ear, installing the piercing portion of the tag as close to the center of the ear as possible. A useful tag for mice is a small (5/16 in. long) self-piercing Monel tag (V shaped prior to application, crimped to a flattened O by installation) such as Style 4-1005, Size I from National Band and Tag (Newport, Kentucky). These tags can be ordered custom stamped with at least three numbers or letters on one surface, and four on the other surface (i.e., up to 10 million different numbers, and more combinations if letters are used as well).

Ear Notching or Punching

Ear notching or punching is a system of holes punched or notches cut into the edges of the ears based on a predetermined numeric code. Figure 2.1 is an example of an ear-marking code (from Leard 1984). Notching has the advantage of not offering a hard object that can be caught or pulled from the ear, such as an ear tag. Disadvantages include the requirement for substantially more time and care in initial identification of the animals. Clearly it takes longer and inflicts more pain on the mouse to punch and clip a precise pattern of from one to five holes and notches into the two

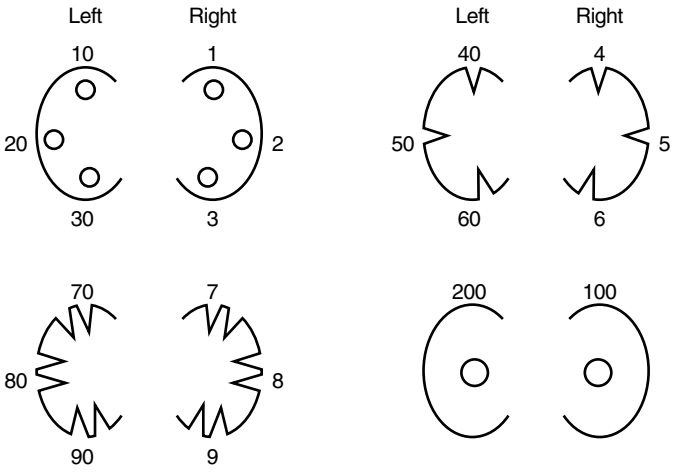

Figure 2.1 Example of an ear marking code which can be used for animal identification. (From Leard 1984, used with permission.)

ears of a mouse than to crimp on a single ear tag. Imprecisely positioned punches or notches may be difficult to read for the duration of the study. Group-housed mice might chew at each other's ears, especially when the notches are fresh, rendering the code more difficult to read. Finally, most codes allow at most a few hundred nonrepeating numbers, meaning that patterns will have to be repeated within most facilities in a short time.

Tattoos

Mice can be tattooed on the tail or feet as a means of identification. Tattoos are permanent unless they are obliterated by chewing or injury. The process of applying alphanumeric tattoos to the tails of mice is more time-consuming than ear tagging or notching, but the tails are more accessible than the feet. Another practical consideration is the size of a mouse's tail. Very small letters are both more difficult and time-consuming to apply to a mouse tail, and more difficult and time-consuming to read at each subsequent identity check throughout the study. The size of the mouse tail and practical considerations of legibility limit the tattoo to four or five alphanumeric characters on the dorsal (easily readable) surface of the tail. It is possible to tattoo an additional four to five alphanumeric characters on the ventral surface of the tail if necessary, but this surface is less easily read.

Although tattooing is a viable method of identification for longer toxicity studies because of its permanence, the amount of time required to both apply and read the tattoos makes this method impractical for shorter studies.

Color Coding of Skin or Hair

The skin or fur of a mouse can be quickly color coded using a variety of indelible felt-tip markers. A variety of combinations of colored markings on the tail or on the fur of the back are easily read without disturbing the animal. This procedure is particularly useful for short toxicity studies. Owing to the fastidious grooming habits of mice, there is a tendency for colored marks to be groomed off quite quickly. This problem is exacerbated if the mice are group housed. A number of different brands and types of markers should be tested, as there is wide variability in their durability or resistance to grooming. This problem can be reduced somewhat by locating the color code in a place that is more difficult to groom, such as the top or back of the head. One substance that confers a durable stain on

the fur is Bouin's fixative, but the color is limited to yellow, so the identity code must be based on the location of the stain rather than the color. The number of possible easily distinguished colors and combinations of colors available for a color code and the maximum number of different sites that can be easily coded on a mouse limit the number of unique codes to a few hundred.

In general, color coding is a useful procedure for identifying mice in short studies. The toxicologist should evaluate each mouse and be prepared to "touch up" the color code on a daily basis.

Toe Clipping

Toe clipping for purposes of identifying mice involves amputation of various combinations of toes using a surgical scissors, nail clipper, or other suitable device to establish an identity code. Toe clipping has the advantage of permanence, but an individual mouse might become "unreadable" through accident or injury that compromises unclipped toes. Toe clipping has many disadvantages, and no clear advantages over other methods such as ear tagging or notching. Most important, toe clipping is more traumatic than other methods, and has an increased likelihood of resulting in infection because it involves the feet. The method is time-consuming to perform and the codes are difficult to read through the duration of the study because the mouse must be picked up and often the toes of each foot must be spread to facilitate accurate reading. As stipulated at the beginning of this section on animal identification, the Food and Drug Administration (FDA) has amended the GLP regulations to eliminate toe clipping from the list of procedures recommended for identifying animals. Toe clipping might have some utility in neonatal (or very young) mice, in which the nervous system is not as well developed, and more conventional methods are impractical.

Implantable Electronic Microchips

The current preferred approach to animal identification is the implantable electronic microchip. The microchip is a transponder that has been sealed into a glass capsule about 1.0 cm in length and about 1.5 mm in diameter. This device is suitable for subcutaneous implantation in mice. The device is "energized" by one of a variety of portable or stationary readers that emits a low-power radiofrequency signal. The transponder is stimulated to transmit its unique identification number back to the reader, where it can be both displayed and linked directly to a computer system. The microchips are easy to install, resistant to loss, and should perform well over the course of a longer term study. The device has a capacity for up to 34 billion different numbers. Preliminary data from several laboratories suggest that the devices are well tolerated, and do not produce problematic histological changes at the implantation site. One disadvantage is that the electronic reading equipment must read the devices. The implication is that in the event of equipment failure, there is no means for manual decoding or reading of the chips. Possession of multiple readers should provide reasonable redundancy for most facilities.

Food

Nutritional Requirements

The explicit nutrient requirements for mice have not been extensively studied nor defined. What is known of these requirements has been estimated on the basis of a number of studies that have had other objectives. Some studies have focused on the effects of specific dietary deficiencies, and others have looked at acceptable performance in growth and reproductive parameters as evidence of nutrient adequacy. Mice have different nutrient requirements for growth, reproduction, and maintenance, and the many diverse genetic strains differ in their minimal requirements as well. Estimated minimum nutritional requirements for proper growth and reproduction of conventional mice have been compiled in table 2.7 from *Nutrient Requirements of Laboratory Animals* (National Research Council [NRC] 1978).

Table 2.7 Estimated Nutrient Requirements of Mice: National Research Council (1978)

Nutrient	Unit	Requirement
Linoleic acid	%	0.3
Protein (growth)	%	12.5
Protein (reproductive)	%	18.0
L-Amino acids		
Arginine	%	0.3
Histidine	%	0.2
Isoleucine	%	0.4
Leucine	%	0.7
Lysine	%	0.4
Methionine	%	0.5
Phenylalanine	%	0.4
Threonine	%	0.4
Tryptophan	%	0.1
Valine	%	0.5
Minerals		
Calcium	%	0.4
Chloride	(required, but not quantified)	
Magnesium	%	0.05
Phosphorus	%	0.4
Potassium	%	0.2
Sodium	(required, but not quantified)	
Chromium	mg/kg	2.0
Copper	mg/kg	4.5
Fluoride	(status uncertain for mice)	
Iodine	mg/kg	0.25
Iron	mg/kg	25.0
Manganese	mg/kg	45.0
Selenium	(required, but not quantified)	
Vanadium	(status uncertain for mice)	
Zinc	mg/kg	30.0
Vitamins		
A	IU/kg	500.0
D	IU/kg	150.0
E	IU/kg	20.0
K_1 equivalent	mg/kg	3.0
Biotin	mg/kg	0.2
Choline	mg/kg	600.0
Folacin	mg/kg	0.5
Inositol(myo-)	(bacterial synth. usually adequate)	
Niacin	mg/kg	10.0
Pantothenate (Ca)	mg/kg	10.0
Riboflavin	mg/kg	7.0
Thiamine	mg/kg	5.0
Vitamin B_6	mg/kg	1.0
Vitamin B_{12}	mg/kg	0.01

Selection

The choice of a specific diet to be used in a mouse toxicity study should take into account all of the objectives and requirements of the study as well as the convenience, efficiency, cost, and availability of a particular diet. In general, diets obtained from well-known commercial suppliers with established procedures for quality control and documentation will be worth any added cost. One of the basic choices is whether to use pelleted diet or meal. Pelleted diet is generally easier and neater to handle, both for the people and the mice involved. The meal form of a diet is usually easier to use if the study design requires mixing of a test chemical with the diet or if food consumption will be measured.

Mice can obtain adequate nutrition from a variety of types of diets. Diets are classified on the basis of the amount of refinement of the ingredients (NRC 1978). Three types of diets are described here. The types are natural ingredient diets, purified diets, and chemically defined diets.

Natural Ingredient Diets. These types of diets contain grains such as corn, wheat, oats, beet pulp, and other ingredients that have been subjected to minimal processing such as fish meal, soybean meal, wheat bran, and a variety of vitamin and mineral supplements. They also have been referred to as a cereal-based, unrefined, nonpurified, or stock diets. Natural ingredient diets are the most widely used and are relatively economical. The principal objection to such diets is that they have had a tendency to vary widely in terms of nutrient and contaminant content (Newberne 1975; Rao and Knapka 1987; Wise 1982; Wise and Gilburt 1980). Data were compiled on key nutrient and contaminant concentrations for all of the lots of a commercially available natural ingredient rodent diet (Purina Certified Rodent Chow 5002, Purina. Mills, Inc., St. Louis, Missouri) received at our facility over a period of 1 year. The variability of these concentrations from lot to lot is relatively low, as seen in table 2.8 and table 2.9.

Table 2.8 Content of Several Key Nutrients in Different Lots of a Closed Formula, Natural Ingredient Rodent Diet (Purina Certified Rodent Chow 5002)

	Protein (%)	Fat (%)	Fiber (%)	Calcium (%)	Phosphorus
Pellets	20.80	5.75	4.46	0.781	0.680
	20.30	4.64	4.20	0.708	0.650
	20.30	5.08	4.14	0.684	0.633
	21.60	5.45	4.76	0.834	0.666
	20.30	5.40	4.28	0.893	0.661
	21.90	4.93	4.11	0.758	0.695
	20.80	5.00	4.60	0.774	0.588
	20.20	5.73	4.09	0.710	0.629
	20.10	5.82	3.77	0.760	0.607
	21.00	6.07	3.97	0.786	0.683
	20.20	5.94	4.36	0.768	0.599
	20.80	5.89	4.39	0.831	0.745
	20.60	5.80	3.95	0.841	0.592
	20.50	5.60	4.08	0.682	0.592
	20.30	5.74	3.75	0.763	0.606
	20.70	5.68	3.86	0.853	0.622
	20.30	5.90	3.87	0.726	0.536
	20.80	5.50	3.74	0.754	0.588
	20.80	6.17	3.81	0.860	0.600
Means	20.65	5.58	4.12	0.777	0.630
Meal	21.30	5.76	4.47	0.850	0.589
	21.90	5.45	4.45	0.924	0.716
	21.50	5.40	4.26	0.715	0.542
	21.70	4.82	3.84	0.877	0.696
	21.40	4.92	4.07	0.718	0.647
	20.30	5.74	4.00	0.632	0.561
	20.30	5.91	4.31	0.903	0.566
	20.70	6.43	3.68	0.741	0.676
	21.10	5.83	4.13	0.809	0.701
	21.10	6.02	4.03	0.681	0.587
	21.20	5.76	4.45	0.817	0.732
	21.20	6.15	3.99	0.880	0.576
	21.10	5.75	3.67	0.849	0.581
	21.50	6.33	3.93	0.832	0.605
	20.50	6.16	4.20	0.732	0.561
	20.40	5.81	3.97	0.790	0.602
Means	21.08	5.77	4.09	0.797	0.621
Combined means	20.84	5.67	4.10	0.786	0.626
Range	20.1–21.9	4.64–6.43	3.67–4.76	0.632–0.924	0.536–0.745

Table 2.9 Content of Several Key Contaminents in Different Lots of a Closed Formula, Natural Ingredient, Rodent Diet (Purina Certified Rodent Chow 5002) (in ppm)

Arsenic	Cadmium	Lead	Mercury	Selenium	Lead
Pellets	0.334	0.0632	0.247	<0.05	0.119
	<0.200	0.0499	0.188	<0.05	0.190
	<0.200	0.0362	0.142	<0.05	0.221
	0.206	0.0711	0.139	<0.05	0.214
	<0.200	0.0647	0.136	<0.05	0.231
	<0.200	0.0620	0.138	<0.05	0.171
	<0.200	0.0779	0.181	<0.05	0.267
	<0.200	0.0551	0.132	<0.05	0.199
	<0.200	0.0838	0.146	<0.05	0.223
	0.210	0.0761	0.273	<0.05	0.267
	<0.200	0.0747	0.136	<0.05	0.243
	<0.200	0.0755	0.132	<0.05	0.176
	<0.200	0.0907	0.156	<0.05	0.243
	0.269	0.1070	0.246	<0.05	0.178
	0.263	0.1070	0.266	<0.05	0.214
	<0.200	0.1110	0.147	<0.05	0.250
	<0.200	0.1090	0.144	<0.05	0.161
	0.224	0.1040	0.202	<0.05	0.205
	<0.200	0.1050	0.147	<0.05	0.260
Means		0.0821	0.174	<0.05	0.212
Meal	0.255	0.0514	0.348	<0.05	0.258
	<0.200	0.0677	0.233	<0.05	0.280
	<0.200	0.0919	0.155	<0.05	0.283
	0.403	0.0498	0.145	<0.05	0.236
	<0.200	0.0427	0.214	<0.05	0.156
	<0.200	0.0520	0.147	<0.05	0.217
	<0.200	0.0804	0.117	<0.05	0.253
	0.321	0.0700	0.296	<0.05	0.208
	<0.200	0.0677	0.150	<0.05	0.212
	<0.200	0.0831	0.105	<0.05	0.224
	<0.200	0.0718	0.150	<0.05	0.228
	<0.200	0.0824	0.164	<0.05	0.216
	<0.200	0.1220	0.131	<0.05	0.197
	<0.200	0.1090	0.268	<0.05	0.214
	<0.200	0.1200	0.153	<0.05	0.193
	<0.200	0.1200	0.146	<0.05	0.199
Means		0.0801	0.183	<0.05	0.223
Combined means		0.0802	0.178	<0.05	0.217
Range	<0.200–0.403	0.0362–0.1220	0.105–0.348	N/A	0.119–0.283

Purified Diets. These types of diets are formulated exclusively with refined ingredients. Dietary protein can be derived from sources such as casein or isolated soy protein, carbohydrate can be derived from sugar or starch, fat can be derived from vegetable oil or animal fat, and dietary fiber can be derived from some form of cellulose. Inorganic salts and pure vitamins are added to provide essential vitamins and minerals. Purified diets also have been referred to as semipurified, synthetic, or semisynthetic. They offer consistent nutrient concentrations and the ability to modify those concentrations to achieve specific nutritional deficiencies or excesses. Purified diets are less palatable for animals, and food consumption should be monitored prior to study initiation and during the study to assure that adequate nutrition is being maintained.

Chemically Defined Diets. These types of diets are formulated entirely with chemically pure compounds. Amino acids, sugars, triglycerides, essential fatty acids, inorganic salts, and vitamins are

blended to provide appropriate nutrition. These diets offer strict control of specific nutrient concentrations at the time of manufacture, but the bioavailability of those nutrients can be altered by oxidation or interaction among nutrients. The availability of specific nutrients to the mice, then, might not be what the toxicologist believes is being provided. Chemically defined diets have the further disadvantage of being difficult to formulate, and they have a very narrow appeal in toxicity testing.

In addition to the degree of refinement of ingredients, mouse diets are classified as closed formula or open formula on the basis of the availability of the quantitative as well as the qualitative aspects of the feed blend.

Closed Formula Diets. Manufacturers and vendors of closed formula diets typically provide a list of ingredients used to manufacture the diet, but not the actual proportions of each ingredient in the final blend. They also typically provide target or mean nutrient analyses from a number of batches. A reputable commercial vendor will analyze feed in production and make minor adjustments to the quantitative aspects of the blend on a frequent basis to maintain nearly constant concentrations of key nutrients such as protein, fat, fiber, and minimal concentrations of potentially important contaminants (D. Ettle, Purina Mills, Inc., personal communication).

Open Formula Diets. These types of diets are typically based on a published formulation for the quantity of each nutrient to be included; for example, ground wheat, 230 g/kg; ground corn, 245 g/kg; and soybean meal, 120 g/kg. An example of an open formula, natural ingredient diet (Knapka et al. 1974) is given in table 2.10. It is often assumed that open formula diets provide more consistent nutrient and contaminant concentrations than equivalent closed formula diets because the relative proportions of ingredients stay fixed among lots and across time. The opposite might be true. Although it is true that the diet described in table 2.10 will always contain 230 g/kg of ground wheat, it is also true that the actual protein and contaminant contents of that wheat (and of many other constituents) could vary widely during the year, depending on climatic conditions during growth, geographical location of the source, and storage conditions since harvest (Greenman et al. 1980). These variables also affect lots from year to year.

Key nutrient and contaminant concentrations of an open formula natural ingredient diet (34 different lots of NIH-07; Rao and Knapka 1987) are compared to those of a commercially available closed formula diet (35 lots of Purina Certified Rodent Chow, 5002) in table 2.9. Based on the relative ranges of values for each of the constituents compared in this example, the closed formula diet was substantially less variable than the open formula diet in every constituent compared.

Provision of Feed and Feeders

Feed. Feed is typically provided on an ad libitum basis for toxicity studies. Healthy adult mice will consume about 4 to 5 g of feed per day, and ordinarily they should be given a quantity adequate for at least 3 to 4 days. Typically, quantities sufficient to last more than a week are provided, with the balance discarded and replaced about weekly to maintain freshness. Two notable exceptions to ad libitum feeding are immediately prior to oral dosing in acute toxicity studies, and prior to blood collection for clinical laboratory analysis in studies of any duration.

The process of fasting mice presents a dilemma. Mice are nocturnal and, therefore, when food is available ad libitum, most of what they consume during a 24-hr period will be consumed during the dark phase of the light–dark cycle. Their stomachs are normally quite full in the morning, and empty slowly through the day. Any fasting procedure that begins in the morning and continues for up to about 12 hr will result in a nutritional state (based on average stomach contents) that does not differ appreciably from fed mice. The next longer convenient interval is to fast animals overnight, from the end of one working day until the morning of the next (a period of about 15–16 hr, which includes the normal feeding time). Overnight fasts are probably the norm for toxicity studies in mice. If food is available, young adult outbred albino mice (25–30 g) will gain about 7% to 10%

Table 2.10 Open Formula Natural Ingredient Mouse Diet

Ingredient	Amount per Ton
Major ingredients (lb)	
Dried skim milk	100
Fish meal (60% protein)	200
Soybean meal (49% protein)	240
Dehydrated alfalfa meal (17% protein)	80
Corn gluten meal (60% protein)	60
Ground #2 yellow shelled corn	490
Ground hard winter wheat	460
Wheat middlings	200
Brewer's dried yeast	40
Dry molasses	30
Soybean oil	50
Dicalcium phosphate	25
Salt	10
Ground limestone	10
Mineral and vitamin premixes	5
Mineral premix (g)	
Cobalt (cobalt carbonate)	0.4
Copper (copper sulfate)	4.0
Iron (iron sulfate)	120.0
Manganese (manganese oxide)	60.0
Zinc (zinc oxide)	16.0
Iodine (potassium iodate)	1.4
Vitamin premix	
Vitamin A (IU)	5,500,000.
Vitamin D3 (IU)	4,600,000.
a-Tocopheryl acetate (IU)	20,000.
Vitamin K (menadione sodium bisulfite) (g)	2.8
Choline (choline chloride) (g)	560.0
Folic acid (g)	2.2
Niacin (g)	30.0
d-Pantothenic acid (calcium pantothenate) (g)	18.0
Riboflavin (g)	3.4
Thiamine (g)	10.0
Vitamin B12 (g)	4000.0
Pyridoxine (g)	1.7

Source: Knapka et al. (1974).

of their "afternoon" body weight owing to feed consumption during that 16-hr period. If food has been removed, those same mice will lose about 8% to 10% of their "afternoon" body weight as a result of the fast (data from our laboratory). Fasting mice for a period of 24 hr results in about a 30% reduction in absolute liver weight (liver–body weight ratios stay constant), a reduction of more than 90% in hepatic glycogen content, and about a 50% reduction in reduced hepatic glutathione, but an increase of about 150% in total hepatic triglycerides (Strubelt et al. 1981). These changes were associated with increased hepatotoxicity from a variety of xenobiotics, and at least one study (Strubelt et al. 1981) believes that overnight fasting of mice should be avoided. The dilemma, then, is whether they should be fasted for a few hours during the day, which will be unlikely to achieve the needed effect, or overnight, which might have considerable effect on hepatic parameters.

Feeders. Feed is provided to mice in one of a very wide variety of feeders. One manufacturer alone (Lab Products, Inc.) lists over 25 different feeders suitable for mice. The mere existence of such a variety of designs testifies to the fact that no single design exists that solves all of the problems encountered in providing clean, dry feed to mice. Some of the basic problems include the fact that mice will, if given the opportunity, urinate, defecate, and sleep in their feeders as well

as dig in them, play with them, and in almost any other conceivable way distribute feed outside of their feeders and even their cages. The challenge in feeder design, then, is to keep the mice out of (and off the top of) the feeder, minimize spillage (especially of meal), and still provide free access to the feed at all times. This challenge is more easily met for pelleted diet, especially when feed consumption is not being measured. In that situation, a feeder that is enclosed at the top and provides access to the feed through some form of slots of wire mesh at the bottom and sides is effective.

The more common situation in toxicity testing is that feed consumption will be measured. Although this can be done with pelleted feed, and an inexpensive feeder has been reported (Dunn and Stem 1978), meal is the more common dietary form when feed consumption will be measured. Feeders containing meal must be easy to install and remove from cages without spillage to facilitate weighing. They must be heavy enough and fit securely enough into the cage to prevent tipping or movement. A common form consists of a small glass jar or stainless steel bowl with a restrictive top plate to limit ease of entry. Various forms of feed followers (heavy washers with several holes, wire mesh, or even marbles) can be placed on the surface of the feed to discourage digging or other means of expulsion of the feed.

Analysis

If toxicity studies are being conducted to support a safety claim to a regulatory agency (such as the U.S. FDA), the GLP regulations should be considered. The GLP document (CFR 1988, para. 58.90) stipulates that feed be analyzed periodically for levels of contaminants that might interfere with the toxicity study, and that documentation of such analyses be available. This consideration is particularly important for natural ingredient diets, which will almost certainly contain at least trace amounts of a variety of heavy metals, pesticides, and other environmental pollutants. The chemistry of the test article being studied should be evaluated for any special sensitivities to common contaminants, and if those sensitivities appear likely, special care should be taken to avoid those contaminants. In the absence of any suspected special sensitivities, diet should be screened on a regular basis for well-known environmental contaminants such as heavy metals, aflatoxins, and various pesticides. One of the most practical (and common) solutions to the requirement for routine feed analysis is to purchase what is referred to as “certified” feed from one of the major commercial suppliers that offer such products. Although more expensive than ordinary feed, certified feeds are analyzed for common contaminants by the supplier, and each lot is typically supplied with documentation of the results of those analyses, which should meet regulatory requirements.

Water

Mice should be provided with ad libitum access to a source of clean, fresh water. For most toxicity studies, ordinary potable water available from a typical municipal water supply is appropriate. Some animal facilities are equipped to treat drinking water to alter pH, or to reduce chemical or microbiological contaminant levels. Treatment can include chlorination, reverse osmosis, distillation, or ion exchange.

Little is known about the mean quantitative water requirements for mice, but quantities of 6 to 7 ml/day are thought to be representative for adults (Jacoby and Fox 1984). It is known that environmental temperature is the primary factor influencing water requirements (Knapka 1983), with higher temperature and lower humidity leading to increased water consumption. The type of diet can also affect water consumption. The mouse has a biological half-time for turnover of water of 1.1 days, which is shorter than in larger animals (Jacoby and Fox 1984). Owing to this short half-time, mice are particularly sensitive to water deprivation, and if they are maintained on a dry diet, they can die within as little as 1 day of water deprivation. Any restriction in availability of water results in an immediate and dramatic reduction in food consumption and commensurate fecal

output. This relationship is so reliable that a toxicology technician trained to be aware of reductions in fecal output on a regular basis will almost never miss a malfunction or deficiency in a watering system or feed source.

Water is typically distributed to individual caging by one of two methods: water bottles with sipper tubes attached to each cage or an automatic watering system,

Water Bottles with Sipper Tubes

This type of watering device has the advantages of lower initial purchase cost and the ability to estimate water consumption on a per-cage basis. Bottles have several disadvantages, including higher maintenance or use cost (bottles must be removed, disassembled, washed, refilled, and reinstalled at least once or twice per week to minimize bacterial contamination), configurations that make access to suspended caging more difficult, stoppers that must be carefully installed to prevent leakage with resultant water deprivation, and some that require very careful installation of the bottle on the cage to assure that sipper tubes have an adequate downward slope to preclude air lock and resultant water deprivation. Some cage designs allow mice to chew on water bottle stoppers, with the potential for ingestion of the debris.

Automatic Watering Systems

Such systems carry high initial purchase (and installation) costs, and generally lack the capacity to indicate water consumption per cage. Automatic watering systems for both the room and the cage rack distribution systems are usually constructed of stainless steel or PVC piping with stainless steel fittings. Stainless steel is more expensive, but also more durable. Automatic systems are supplied by some type of pressure-reduction station connected to the facility water supply, such that the water pressure at the cage is in the range of 1.5 to 4.0 lb/in^2 (psi). The pressure-reduction station can be fitted for automatic system monitoring (e.g., for leaks, overly high or low distribution pressure), and can provide connections for local water treatment such as chlorination. Racks are connected to the room distribution system by means of flexible hosing, and might include an air-gap isolator to prevent microbial contamination from traveling from a rack into the distribution system. Automatic watering systems require less labor while in use than water bottles, limited to periodic checks that individual sipper fittings are functioning properly and that air has not been trapped in the rack distribution system. Whenever a rack is connected to the system, whether new to a room or reconnected after relocation, the rack should have all air bled from its distribution system and representative sippers should be checked to confirm the absence of air in the lines. Room distribution systems, particularly those in rooms with a small number of resident animals, have the potential to foster bacterial growth. This problem seems least severe in rooms with the highest daily water flow rates. Automatic watering systems can be fitted with a central flushing system that provides a programmed flushing of the room distribution system at increased system pressure and flow rate. A program of daily flushing for about 5 min has been found to produce an acceptable level of microbiological control.

Water Quality

This should be monitored by a regular system of chemical and microbiological analyses. Samples should be collected from water bottles that have been in use for nearly the maximum allowable period, from clean bottles, and from the water source. Samples from automatic watering systems should be collected at sippers that have been in use in individual cages, at clean sippers, at one or more sources on the animal room distribution system, and at both the high- and low-pressure side of the pressure reduction station. Water samples should be analyzed for the routine contaminants (e.g., heavy metals, pesticides) and for microbiological content (both type and plate

count or concentration). Any special requirements of the toxicity study should be considered when selecting parameters for analysis. In general, water that is of acceptable quality for human consumption will be acceptable for mice.

Prevention of Infectious Diseases

The occurrence of an infectious disease during the conduct of a toxicity study will at best confer uncertainty about the interpretation of the study results, and at worst require the repetition of the entire study. The effect of even relatively benign infections on the health of mice that have been compromised by the administration of high doses of a potentially toxic test article can rarely be accurately predicted.

This section discusses some of the fundamental considerations in the prevention of infectious disease in a mouse colony. A comprehensive discussion of infectious, neoplastic, and noninfectious diseases in mice is beyond the scope of this chapter, but has been addressed elsewhere (Foster et al. 1982; Jacoby and Fox 1984).

Simplistically, infectious diseases can be prevented by obtaining high-quality, healthy mice from a reputable supplier, protecting those mice from exposure to infectious disease, and maintaining those animals in a clean, well-controlled environment with good-quality food and water.

It is fair to assume that the health status of mice will be no better than it is at the time they leave the breeding colony. Some commercial breeders are now offering mice that are free of antibodies to specific lists of mouse viruses as well as most important bacteria and parasites. These animals could be especially susceptible to infectious disease precisely because they lack antibodies to most common mouse diseases.

Periods when healthy mice are especially subject to exposure to infectious disease include transit from the breeder to the user and introduction into the new home colony. Risk during transit is increased if the animals are transported by common carriers, such as commercial airlines and trucking companies. Often common carriers will be less cautious with animals than trained staff. These risks can be reduced if mice are shipped in filtered containers that exclude much of the biological contamination to which the container is exposed, and if they are shipped in a clean, environmentally controlled vehicle without intermediate transfer between shipper and receiver.

The largest risk on receipt of mice at the new home colony is that the mice will be transported through or into an area that is inhabited by animals that are already infected with a contagious disease. This risk can be reduced by maintaining the integrity of the filtered shipping carton until the mice reach the room in which they will be housed. The room should have been previously sanitized to reduce the probability of infection from previous residents. It is generally poor practice to introduce new mice into an animal room that already contains animals, especially if those animals have been housed there for any period of time or were received from another supplier. Different species should never be mixed in the same room. Environmental conditions, including temperature, humidity, and ventilation, and their contribution to animal health were discussed earlier in this chapter.

Once clean healthy animals have been introduced into a clean room stocked with clean caging, food, and water, their health status becomes a function of the quality and training of the animal care staff. A good understanding of the biology of mice, infectious disease, and the kinds of things that can act as vectors for potential infection are invaluable assets for animal care staff. Handlers should be encouraged to wear gloves and laboratory coats or uniforms when working with mice, and to change those garments regularly. Proper hygiene is especially important when moving from one animal to another. In the event that an infectious disease is diagnosed in the colony, every reasonable effort should be made to isolate (or even eliminate) the infected animals to prevent spread. This might include maintenance by a handler or handlers who do not come into contact with important study animals.

Study Design

Most toxicity and teratology studies conducted in mice are designed to provide information on potential human toxicity. Test substances are typically administered by the expected route of human exposure. A pharmaceutical product that is intended for oral administration (tablet, capsule, solution, or suspension) or a food additive would generally be administered by the oral route. Oral administration to mice is usually accomplished by administration of a solution or suspension by oral gavage, by mixture of the test substance with the diet, or less commonly by adding it to the drinking water.

The specific design of toxicity studies should be tailored to the objective to be achieved and to any specific characteristics of the test substance. Many features of study design will be predicated on guidelines and practices of regulatory agencies such as the FDA or Environmental Protection Agency (EPA) in the United States or their counterparts in other countries to which the results of the *4902MIN* submitted in support of a safety claim. Recommendations for study length (duration of dosing) fall in this category.

Toxicity studies are usually conducted in order of increasing duration of dosing, beginning with acute toxicity studies. When this regimen is followed, each study provides progressively more useful information for the selection of doses for the next longer study.

Acute Toxicity Studies

Acute toxicity studies are conducted to evaluate the effects of a single substance. Usually each animal receives a single dose of the test substance in this study design. On rare occasion, repeated doses might be administered, but in any event, all doses are administered within 24 hr or less. Historically, a primary objective of acute toxicity testing was to determine an LD50 dose, or that dose which would be lethal to 50% of the animals treated. To achieve this objective, groups of mice, often numbering 10 or more per sex, are treated with a single dose of the test substance. Depending on the rate of survival in the initial groups, additional groups are added to the study at higher and lower doses such that most animals die that receive the highest doses and most survive that receive the lowest doses. Survival is assessed at some predetermined interval after dosing, usually 7 or 14 days, but occasionally as early as 24 hr. The resultant dose-response data can be analyzed by a statistical method such as probit analysis (Finney 1971) to provide an estimate of the median lethal dose (LD50) and some measure of the precision of that estimate, such as the 95% fiducial limits. There are very few scientifically valid reasons to include determination of the LD50 as a significant objective of acute toxicity testing. Most regulatory agencies have dropped their requirements for a specific value for the LD50, and animal welfare considerations preclude the use of the large numbers of animals previously required.

A more contemporary design for acute toxicity testing attempts to derive a maximum amount of information from a minimum number of animals. Study objectives include determination of the most important clinical signs attributable to high doses of the test substance, time of onset and remission of those signs, possible determination of a minimum lethal dosage, and in the event of lethality, the sequence and timing of effects leading to death or recovery. These objectives are achieved by means of a comprehensive schedule of animal observations following dosing. These objectives can usually be achieved by treating from one to three groups of three to five mice of each sex per group at different doses.

Traditionally, acute toxicity testing of potential new pharmaceutical products is conducted in at least three species, with one being a nonrodent, and by at least two routes of administration, one of which is the intended clinical route. Mice are the most frequently selected rodent species for acute toxicity testing. The choice of administration routes depends on the intended clinical route and on how much is already known about the oral bioavailability of the test substance. If the intended clinical route is oral, acute testing by oral gavage with a solution or suspension is of primary importance. If other clinical routes are anticipated (e.g., IV or dermal), they represent good

secondary routes for acute testing. Ordinarily, at least one parenteral route is used for acute testing, and that route might be IV if the product is soluble in a fairly innocuous vehicle (e.g., water or saline) or intraperitoneal (IP) as a suspension if the product is insoluble in an aqueous (or other innocuous) vehicle. If the intended clinical route is not oral, the oral route is usually selected as a secondary route for acute toxicity testing to provide information relevant to accidental oral ingestion. A rough estimate of oral bioavailability can be based on a comparison of the acute toxicity associated with various doses administered by the oral and parenteral routes. Acute toxicity testing conducted for other purposes is usually more limited in scope. Most regulatory agencies no longer require a full complement of species and routes of administration to render decisions on acute toxicity.

There are a few characteristics of acute toxicity testing that are not common in other toxicity protocols. In a typical repeated dose toxicity study, several groups of animals are treated concurrently with predetermined doses of the test substance and a control substance. To reduce animal use in acute toxicity testing, studies that include more than one dose group are usually dosed sequentially, with an interval of at least 24 hr between dosing of subsequent groups. This allows the effects of the previous dose to be fully manifested, and allows selection of the subsequent dose to provide the highest probability of contributing more useful information. Another unusual aspect of acute toxicity studies is the nutritional status of the animals at dosing. Because some schools believe that the results of acute toxicity testing are more reliable if all animals are in a uniform nutritional state, mice to be dosed orally are often fasted overnight prior to dosing. Fasting allows dose volumes to be higher than in repeated dose studies, and because dosing only occurs on 1 day, dietary, stress is considered tolerable. The scientific merits of this practice are debatable, but fasting is traditional in oral acute toxicity studies. Although the practice of conducting gross necropsies at the end of acute toxicity studies is growing in popularity, this practice rarely yields useful information. The toxicity resulting from acute exposure is usually associated with a biochemical or functional imbalance rather than with a change in the gross or microscopic architecture of an organ system. Changes observable at gross necropsy are more often associated with repeated dosing at sublethal levels. For similar reasons, microscopic examination of tissue is rarely conducted in acute toxicity studies unless there is some scientific reason to expect it would be useful.

The results of a well-designed acute toxicity study can help to predict likely target organ systems or possible outcome in the event of massive human overexposure, can help in establishing risk categories for EPA or Department of Transportation (DOT) classification, and can help in dose selection for the initial repeated dose toxicity tests to be conducted. An example of an acute toxicity study design is shown in table 2.11.

Table 2.11 Typical Acute Toxicity Study Design for Mice

Number of mice/sex/dose group	3–5
Number of dose groups	1–3
Number of control groups	None
Dosing frequency	Single dose
Dosing days	1 day
Survival checks	Not done (part of clinical observations)
Clinical observations	4 or more on day of treatment, then 1–2 daily
Physical examinations	Not done
Body weights	Prior to dosing
Feed consumption	Not done
Number of reversal mice	None
Duration of reversal period	Not applicable
Blood collection	Not done
Hematology parameters	Not done
Clinical chemistry parameters	Not done
Urine collection	Not done
Necropsy	Gross (increasingly, but rarely useful)
Tissue collection	Rarely (specific cause only)

Short-Term Toxicity Studies

The objective of short-term or subchronic toxicity studies is to describe and define the toxicity associated with repeated administration of high, but generally survivable doses of a test substance. This might include identification of target organs and systems, definition of the maximum survivable repeated dose, and the highest "clean" or no-effect dose. Short-term repeated dose studies also serve as dose range-finding studies for longer term repeated dose studies.

Short-term toxicity studies range in duration of dosing from about 7 to 90 days. Mice typically receive a single daily dose of the test substance, 7 days per week, by the expected clinical route of administration. If the test substance is administered in the diet (or rarely, the drinking water), that admixture is available continuously. Short-term studies usually include three to four groups of mice exposed to different dose levels of the test substance, and an additional group exposed to the carrier to serve as a control for the effects of treatment. Group sizes for these studies are on the order of 5 to 10 mice/sex/dose. Ideally, dose levels should be selected for these studies such that a few animals die at the highest dose prior to the completion of dosing (to assure exposure to the maximum survivable dose), and all survive at the lowest dose with minimal evidence of toxic effects. The middle dose or doses should be set at approximately equal log increments between the high and low doses. It is important to begin to identify the highest dose level that is free of serious toxic effects to determine whether the test substance is likely to be toxic to humans at the expected therapeutic dose or exposure level.

Parameters monitored in a typical short-term repeated dose study might include daily observations for clinical signs of toxicity and mortality; weekly physical examinations, body weight, and feed consumption; and terminal measurement of serum glucose and urea concentrations, serum aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase activity. Animals found dead or killed by design are typically submitted for gross necropsy, and selected tissues, such as adrenal gland, bone (sternum, including marrow), brain, heart, kidney, liver, lung, testis, and thymus are collected, weighed (except for bone and lung), and processed for routine microscopic examination by a qualified veterinary pathologist. An example of a short-term toxicity study design is shown in table 2.12.

Table 2.12 Typical Short-Term Toxicity Study Design for Mice

Number of mice/sex/dose group	5–40
Number of dose groups	3–4
Number of control groups	1
Dosing frequency	Once, daily
Dosing days	Daily for 7–90 days
Survival checks	1–2 daily
Clinical observations	Daily
Physical examinations	Weekly
Body weights	Weekly
Feed consumption	Weekly
Number of reversal mice	None
Duration of reversal period	None
Blood collection	Terminal, all animals
Hematology parameters	None
Clinical chemistry parameters	Limited
Urine collection	Not done
Necropsy	Gross, all animals
Tissue collection	Limited list, all animals

Chronic Toxicity Studies (26 Weeks to 2 Years)

The objective of chronic, or long-term, toxicity studies is to refine the description of the toxicity associated with long-term administration of high, survivable doses of a test substance. Chronic toxicity studies are more commonly conducted in rats than in mice, but such studies can be conducted in mice, and this discussion describes objectives and practices for conducting such studies in mice. Target organs and systems have usually been identified prior to the conduct of chronic studies, but it is chronic studies that provide the best opportunities to understand the subtle changes associated with long-term administration of high doses, and to focus more closely on the highest clean or no-effect dose. Chronic toxicity studies also serve to refine the doses to be administered in the carcinogenicity studies that typically follow them.

Chronic, or long-term, toxicity studies range in duration of dosing from about 26 weeks to as long as 2 years, but most do not exceed 1 year. Single daily doses of the test substance are administered by the expected clinical route of administration. If the substance is intended for oral administration, the convenience and economy of administration in the diet (or rarely the water) becomes important. Diet admixtures are made available ad libitum unless they must be removed for a specific procedure during the study. Chronic studies usually include three groups of mice exposed to different dose levels of the test substance, and an additional group exposed to the carrier to serve as a control for the effects of treatment. Chronic toxicity studies often include *reversal groups,* or subsets of each dose group that are not sacrificed immediately on completion of treatment. The purpose of the reversal groups is to determine whether any toxic effects associated with treatment are permanent or subject to recovery or reversal. Mice in the reversal groups might be allowed from 2 to 4 weeks of recovery time from the end of treatment until necropsy. Group sizes for chronic studies are on the order of 20 to 50 mice/sex/dose. Sizes of the reversal groups, if they are included, might be about 25% to 35% of the original dose groups. Dose levels should be selected for these studies such that there is substantial toxicity at the highest dose, but few if any treatment-related deaths. The low dose in chronic studies should confirm, or if necessary, refine previous estimates of the highest dose level that is free of serious toxic effects, and thereby reinforce previous estimates of the relative safety of the test substance at the expected human dose or exposure level. The middle dose should be the approximate geometric mean of the high and low doses.

Parameters monitored in a typical chronic toxicity study might include daily observation for moribundity and mortality; weekly physical examinations, body weight, and feed consumption; and terminal measurement of serum glucose and urea concentrations, serum aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase activity. Hematological parameters for mice are often limited to differential smear evaluations. Although red blood cell counts, white blood cell counts, and hemoglobin concentrations can be determined, the values for mice are somewhat variable from many of the commonly used laboratory instruments, so these parameters are not always evaluated. Animals found dead or killed by design are typically submitted for gross necropsy, and a list of 30 to 50 selected tissues are collected, weighed (except for bone and lung), and processed for routine microscopic examination by a qualified veterinary pathologist. An example of a chronic toxicity study design is shown in table 2.13.

Carcinogenicity Studies (18–24 Months)

The objective of carcinogenicity studies is to determine whether the test substance is a carcinogen when administered at maximum tolerable doses for a period of time approaching the life expectancy of the mouse. This objective is simpler in many respects than the objective of the longer toxicity studies. It is assumed that by the time carcinogenicity studies are undertaken, the chronic

Table 2.13 Typical Chronic Toxicity Study Design for Mice

Number of mice/sex/dose group	20–50
Number of dose groups	3
Number of control groups	1
Dosing frequency	Once daily
Dosing days	Daily 26–52 weeks
Survival checks	Daily
Clinical observations	Not done
Physical examinations	Weekly
Body weights	Weekly
Feed consumption	Weekly
Number of reversal mice	25%–35% of main groups
Duration of reversal period	2–4 weeks
Blood collection	Terminal, all animals
Hematology parameters	Dif. smear, red blood count, white blood count
Clinical chemistry parameters	Limited list
Urine collection	Not done
Necropsy	Gross, all animals
Tissue collection	Comprehensive list, all animals

toxicity studies have been essentially completed, and the actual toxicity of the substance is about as well understood as it can be based on animal studies. Carcinogenicity studies are not usually encumbered by tests to further the understanding of toxicity, but rather are focused to maximize the ability to answer the single question of carcinogenicity.

Carcinogenicity studies in mice are generally designed to expose the animals for a period of 18 months to 2 years. Improvements in animal husbandry during the past decade have increased the life expectancy of most strains of mice, so there has been a tendency to extend carcinogenicity studies in mice to 2 years. The study design normally contains provisions to allow for termination of the study prior to the intended endpoint if excessive mortality is encountered. This provision is intended to ensure that an adequate number of survivors are sacrificed with successful collection of all necessary tissues for a meaningful, statistical analysis of tumor incidence. Carcinogenicity studies usually include three groups of mice exposed to different dose levels of the test substance and an additional group exposed to the carrier to serve as a control for the effects of treatment. Group sizes for these studies are on the order of 50 to 70 mice/sex/dose.

The high dose in carcinogenicity studies should be the maximum tolerated dose. This dose should produce evident toxicity by the end of dosing. A commonly held minimum criterion for evident toxicity is a decrement in body weight or body weight gain of at least 10% from the control to the high-dose group. If the test substance is not very toxic, most regulatory agencies will accept a carcinogenicity study in which the high dose is the highest dose that can be practically administered, even though that dose does not produce evident toxicity. In this example, the animal has, in effect, tolerated the highest dose that could be administered.

The selection of lower doses is not as critical as in chronic studies because the concept of a clean dose of a known carcinogen is not widely accepted, and will be of little value in attempting to commercialize a product.

The middle dose might become important if the high dose has been inadvertently set too high, resulting in excessive toxicity and early mortality. In that case a middle dose that elicits evident toxicity without excessive mortality might become an acceptable maximum tolerated dose and effectively "salvage" the study. As in chronic studies, the middle dose should be at the approximate geometric mean of the high and low doses.

The most important data generated in a carcinogenicity study are the histopathology data. Of particular importance are the data on the incidence of various types of malignancies in the different

Table 2.14 Typical Carcinogenicity Study Design for Mice

Number of control groups	2
Dosing frequency	Once daily
Dosing days	Daily for 18–24 months
Survival checks	Daily
Clinical observations	Not done
Physical examinations	Monthly during first year, 2 times/month thereafter
Bodv weights	Weekly during first 2 months, 2 times/month thereafter
Feed consumption	Weekly during first 2 months, 2 times/month thereafter
Blood collection	Optional periodic, terminal
Hematology parameters	Periodic peripheral smears, terminal smears, red blood count, white blood count
Clinical chemistry parameters	Not done
Necropsy	Gross, all animals (including those found dead during study.)

treatment groups. Control or even untreated mice normally will have some background incidence of various types of malignancies over the course of a carcinogenicity study. The key question then is whether the treated groups have a significantly higher incidence of normally expected tumor types than the control group, whether they have occurrences of nonnormal tumor types that are not seen (concurrently or historically) in control animals, and, most important, whether such incidence is attributable to the test substance.

Other parameters monitored in a typical carcinogenicity study might include daily observation for survival and moribundity; periodic physical examinations; periodic examinations for palpable masses, body weight, and feed consumption (especially important in dietary admix studies); and for some studies periodic peripheral blood smears and terminal red and white blood cell counts. Animals found dead or killed by design are submitted for gross necropsy, and a comprehensive list of 40 to 50 prescribed tissues plus any tissue masses, suspected tumors, and an identifiable regional lymph tissue are collected from each animal to be processed and examined histologically by a qualified veterinary pathologist for evidence of carcinogenicity. An example of a carcinogenicity study design is shown in table 2.14.

Teratology Studies

Mice are occasionally used in teratology studies to assess the effects of test substances on congenital defects in the young when administered to pregnant females. Mice have a regular, short estrus cycle, a short gestation period, high fertility, and typically produce relatively large litters of young. Mice rank behind rats and rabbits as the species of choice for assessing teratology, but there might be good scientific reasons to use mice in some instances. The conduct of Segment II teratology studies in mice and rabbits with Segment I and III studies in rats provides an opportunity to evaluate teratogenicity in three species. Although there is an advantage in conducting teratology studies in at least two species, preferably one of which is a nonrodent, some substances (e.g., certain antibiotics) are especially toxic to rabbits, making teratology testing impractical in that species. In that case, mice become the second best species available (behind rats) for teratology testing, and are usually the choice as the second species. A significant disadvantage in using mice for teratology studies is that they are much more cannibalistic than rats or rabbits. This characteristic renders the species unusable for Segment I and III studies, in which pregnant females are allowed to deliver their young, and limits them to use in Segment II studies, in which the young are delivered by cesarean section on gestation day 18, prior to delivery. Mating can be confirmed in mice by daily inspection of cohabitating females for the presence of a vaginal plug. The copulatory plug in mice is much more persistent than in rats, in which mating must be confirmed by vaginal lavage and microscopic examination.

Segment II Teratology Studies

A Segment II teratology study is conducted to assess the effects of a test substance on fetal survival and congenital malformations (teratology). Females are mated and monitored daily for the presence of copulatory plugs. The presence of a plug confirms that mating has occurred, and the day of discovery is defined as gestation day 1. Mated females are dosed with the test substance from gestation days 6 through 15, a period that begins soon after implantation (day 5), and continues through completion of organogenesis (day 13). This dose period exposes the young throughout the period of organogenesis, but tends to minimize preimplantation embryotoxicity and postorganogenesis maternal and fetal toxicity. Young are delivered by cesarean section on gestation day 18, prior to normal parturition on day 19, to avoid cannibalism. The maternal reproductive organs are examined for numbers of corpora lutea, implantations, resorptions, and live and dead fetuses. The fetuses are weighed, sexed, and examined for gross, visceral, and skeletal abnormalities. An example of a Segment II teratology study design is shown in table 2.15.

Genetic Toxicity Studies

The objective of genetic toxicity testing is to identify and describe the effects of agents that specifically produce genetic alterations at subtoxic doses. Mice are used in a variety of genetic toxicity study designs in an effort to achieve this objective. Neither a comprehensive listing of genetic toxicity procedures using mice nor a comprehensive description of any number of those procedures is within the scope of this chapter. Rather, a few of the most commonly employed procedures are summarized. The reader is referred to other sources such as Brusick (1980), Thorgeirsson (1982), and Dean (1983, 1984) for more detailed discussions of genetic toxicity and more comprehensive descriptions of some of the specific tests used in that field.

Mouse Micronucleus Assay

The objective of the mouse micronucleus assay is to determine whether a test article causes disruption and separation or breakage of chromosomes. The mouse micronucleus assay is one of the most commonly conducted *in vivo* tests for genetic toxicity. Comparison of the incidence of micronuclei in proliferating cells from treated versus control mice provides an indirect measurement of chromosome damage in somatic cells. Micronuclei can only be formed as a result of disruption and separation or breakage of chromosomes, followed by cell division. The preferred cells for evaluation are newly formed erythrocytes in mouse bone marrow because the micronuclei formed

Table 2.15 Typical Segment II Teratology Study Design for Mice

Number of female mice/dose group	20
Number of dose groups	3
Number of control groups	1
Dosing frequency	Once daily
Dosing days	Days 6–15 of gestation
Survival checks	Daily
Clinical observations	Daily
Physical examinations	Not done
Body weights	1–3 times/week
Feed consumption	Not done
Number of reversal mice	None
Duration of reversal period	Not done
Blood collection	Not done
Caesarean section	Day 18 of gestation
Necropsy	All dams gross, all fetuses external, and one-third of fetuses visceral exam at caesarean section

Table 2.16 Typical Mouse Micronucleus Assay Study Design

Number of mice/sex/dose group	5 or more
Number of dose groups	1 or more
Number of control groups	2: one positive, one negative
Dosing frequency	Single dose
Dosing days	One day
Survival checks	Daily
Clinical observations	Not done
Body weights	Prior to dosing
Feed consumption	Not done
Bone marrow collection	3 or more intervals, from 12–72 hr after dosing
Polychromatic erythrocytes evaluated/mouse	1,000 minimum

in these cells are not expelled during the last division in which the nucleus is extruded from the normoblast.

One or more dose levels of test article and a control treatment are administered to separate groups of at least five mice per sex. The highest dose should be the maximum tolerated dose, or one that produces some evidence of cytotoxicity. It is important that high-quality (e.g., specific pathogen-free) mice of known genetic stability and consistent species, strain, source, age, weight, and clinical condition be used to assure comparability with historical controls. Each animal typically receives a single dose of test or control article. Bone marrow samples are collected at a minimum of three different intervals after dosing, ranging from 12 to 72 hr. At least 1,000 polychromatic erythroyctes are evaluated for the presence of micronuclei for each test and control mouse. An example of a mouse micronucleus assay study design is shown in table 2.16.

Heritable Translocation Assay

The objective of the heritable translocation assay (HTA) is to assess the potential of a test article to induce reciprocal translocations between chromosomes in germ cells of treated male mice. This assay has the advantage of detecting transmissible genetic alterations, which are potentially more damaging to the gene pool than nontransmissible or lethal changes. Induced translocations can be detected by mating the F1 progeny of treated males with untreated, unrelated females. Translocations will be evidenced by a reduction in the number of viable fetuses sired by affected males. The presence of translocation figures in meiotic metaphase serves as cytogenetic verification of the presence of reciprocal translocations.

The HTA typically consists of three groups of 30 male mice each, treated with different dose levels of the test article, and a negative control group treated with the dosing vehicle. A positive control group is optional. As the period of spermatogenesis in the mouse is about 7 weeks, all treated and control animals are dosed on a daily basis for 7 weeks. The dosing route is usually oral gavage, and the dose levels are selected on the basis of the oral LD50. The high dose is typically one-eighth of the LD50, the medium and low doses about one-third and one-tenth of the high dose, respectively. On completion of dosing, each male is mated to two females. Two hundred healthy males are selected from the offspring of each of these groups of matings and allowed to reach sexual maturity, whereupon each male is mated to three virgin females. The females from this second mating are sacrificed about 3 weeks after cohabitation with the males was initiated, and the number of living fetuses and resorbing embryos present in the uteri are counted. Any male that produces 10 or more living fetuses in any one female is considered fertile, and no further matings are needed. Males falling below that criterion are mated to an additional set of three females, and the evaluation is repeated. Failing males can be remated up to three times. Males that never succeed in producing at least one litter of 10 or more living fetuses are considered sterile (or semisterile), are sacrificed, and their gonadal cells are examined for cytogenetic evidence of translocations. An example of an HTA study design is shown in table 2.17.

Table 2.17 Typical Mouse HTA Study Design

Number of male mice/dose group	30
Number of dose groups	3
Number of control groups	1–2 (negative; pos. optional)
Dosing frequency	Once daily
Dosing days	Daily, 7 weeks
Survival checks	Daily
Clinical observations	Not done
Mating of dosed males	On completion of dosing
Number of F1 males mated/group	200
Number of F1s examined cytogenetically for translocations	All sterile and semisterile
Tissue collection	Testes

Microbial Host-Mediated Assay

The objective of the microbial host-mediated assay is to determine the ability of a mammalian system (e.g., the mouse) to metabolically activate or detoxify a test article with respect to its mutagenic potential. The mutagenic potential is measured by means of one of a variety of microorganisms, depending on the specific types of mutations being investigated (e.g., base–pair substitution, frame shift). Some of the microorganisms used for this type of testing include various strains of *Salmonella typhimurium, Escherichia coli,* and *Neurospora crassa.* The results of the host-mediated assay can be compared with the direct effect of the test article on the same tester strain of microorganisms to determine whether the host (e.g., mouse) is metabolically activating, detoxifying, or having no effect on the mutagenic potential of the test article. The microbial host-mediated assay, then, is an attempt to combine the convenience of microorganisms for detecting hereditary changes with the metabolism of the test article gained by administration to a whole animal.

The microbial host-mediated assay consists of three groups of about 10 mice each treated with different dose levels of the test article, a negative control group treated with the dosing vehicle, and a positive control group. Doses are administered daily for up to about 5 days. The high dose is usually about one-half of the LD50, with the medium and low doses about one-third and one-tenth of the high dose, respectively. The preferred route of administration is oral, but intramuscular or IP injection can be used if necessary. At the end of the period of test article administration, the tester strain of microorganism is administered, usually intravenously. Following appropriate incubation periods (e.g., 1, 2, and 4 hr), mice are sacrificed and the microorganisms are collected, frequently from liver tissue. The collected microorganisms are grown on minimal agar plates to assess mutation rate. An example of a microbial host-mediated assay study design is shown in table 2.18.

Table 2.18 Typical Mouse Microbial Host-Mediated Assay

Number of mice/dose group 10	
Number of dose groups	3
Number of control groups	2: one positive, one negative
Dosing frequency	Once daily
Dosing days	Daily for 5 days
Survival checks	Daily
Clinical observations	Not done
Body weights	Prior to dosing
Tester organism administered	After last dose
Tester organism recovered	For example, 1, 2, and 4 hr after administration
Necropsy	Not done
Tissue collection	Liver or other appropriate tissue for organism recovery

Special Studies

The diversity of toxicity study designs using the mouse as a test system to examine a specific hypothesis defies description. Many of these designs do not strictly fit within the major section headings chosen for this chapter, and might be referred to as special studies. This chapter is intended to focus on the more commonly conducted types of toxicity studies conducted in mice, but it might be useful to describe two special study designs here as examples of some of the interesting endpoints that can be evaluated in this species.

Mouse Ear Swelling Test

The concept of a mouse ear swelling test (MEST) is that sensitization can be detected by measuring edema in the ear of a mouse, which results from topical application of a test article to an animal that has been previously sensitized by means of dermal application to the abdomen.

The objective of the MEST is to provide an alternative test for dermal sensitization potential that makes more efficient use of animals, labor, and other resources than traditional study designs conducted in guinea pigs. A MEST is often preceded by a dose-finding activity to identify the highest concentration of test article that is no more than minimally irritating to the abdominal skin, and the highest concentration that is nonirritating to the ear. These concentrations are then employed in the sensitization assay.

The sensitization assay is carried out in three phases: an induction phase, a challenge phase, and, if necessary, a rechallenge phase. The sensitization assay requires 15 mice treated with the test article and 10 treated with a control substance, typically the vehicle used for the test article. During the induction phase (study day 1), the abdomen is shaved using a small animal clipper, and 20 µl of 1:1 emulsion of Freund's complete adjuvant in distilled water is injected intradermally at each of two abdominal sites on opposite sides of the ventral midline. Once daily on study days 1, 2, 3, and 4, the stratum corneum is stripped from the abdominal skin using adhesive tape, and the appropriate concentration of test article is applied to the abdomen at a volume of 100 µl.

On study day 11, the challenge phase is initiated. The appropriate concentration of test article is applied to the skin of the left ears of all treated and 5 of the 10 control mice at a volume of 10 µl, and an equal volume of the control (vehicle) is applied to the right ears of those same animals. Thicknesses of both ears of all treated and the five selected control mice are measured on days 12 and 13. Any mouse with a left ear thickness greater than 120% of its right ear thickness is considered to be a positive responder. If one or more mice are judged positive in the absence of primary irritation (any control mice with left ear thickness greater than 110% of right ear thickness) on study day 12 or 13, the test article is considered to be a sensitizer. Evidence of primary irritation requires that the test be repeated using a lower concentration for the challenge dose.

If results are negative (all mice with a left ear thickness increases of less than 10%) or equivocal (some mice with increases of 10 to 19%, but none greater than 20%), a rechallenge is conducted on study days 17 through 20. Baseline ear thicknesses are measured on study day 17. The test article is applied to the right ears of all test mice and the control mice that were not used during the initial challenge on study day 18. Ear thicknesses of the right ears of all test mice and the new control mice are measured on study days 19 and 20, and are compared to the baseline thicknesses taken on day 17. The criteria for positive response are the same as for the initial challenge. An example of a MEST study design is shown in table 2.19.

Dermal Carcinogenicity (Skin Painting) Study

The concept of the dermal carcinogenicity (or skin painting) study is that carcinogens, or of more recent interest, cocarcinogens and tumor promoters can be evaluated or their potencies compared in as little as a few months of testing.

Table 2.19 Typical MEST Study Design

Number of male mice/dose group	15/treated group, 5/control group
Number of dose groups	1
Number of control groups	2
Dosing frequency	Once daily
Dosing days	Days 1, 2, 3, and 4 (induction) Day 11 (challenge) Day 18 (optional rechallenge)
Survival checks	Daily
Clinical observations	Not done
Body weights	Not done
Ear thickness measured	Day 10, 12, and 13 (challenge) Day 17, 19, and 20 (rechallenge)
Necropsy	Not done
Tissue collection	None

The carcinogenicity of some chemicals (e.g., polycyclic aromatic hydrocarbons) can be detected easily by the production of papillomas or carcinomas. Mouse skin apparently functions in this system because it contains enzymes necessary to produce the active intermediates that lead to initiation. Tars from tobacco, coal, and various petroleum products show active carcinogenic potential in this system, although many of the same products are not carcinogenic when administered systemically. Hepatic detoxification of systemically administered doses probably accounts for this difference.

This study design has been especially useful in recent years in the study of cocarcinogens and tumor promoters. In a typical study design, groups of 25 to 50 mice might receive from one to a few systemic doses of a known tumor initiator. Following receipt of the initiator, the fur over the anterior portion of the back is shaved, and the suspected tumor initiators are applied to the skin of the back at a frequency of two to three times per week. Shaving will need to be repeated approximately weekly. Development of papillomas or carcinomas of the skin is readily visible in the shaved area. Active chemicals are often detected within a few months' treatment. This study is generally intended to continue treatment for a period of about 30 to 40 weeks, but can be continued for up to 2 years if necessary.

Obvious advantages of this study design include its relative efficiency in terms of animal numbers and labor, and its relative brevity compared to a conventional 18- to 24-month carcinogenicity bioassay. It is reasonable to conclude that positive findings of carcinogenicity in this test would make a conventional carcinogenicity bioassay unnecessary. Negative findings in a dermal carcinogenicity test, however, would not assure the absence of carcinogenic potential, and a conventional bioassay would still be necessary.

Disadvantages of this procedure include difficulty in accurate quantification of dose, as the topically applied dose can run off the animal, be scratched or licked off, or can accumulate as a crust, effectively reducing absorption. Another criticism centers around the fact that if treatment with a suspected promoter is interrupted after a period of 60 days or so, evident papillomas often regress, raising the question of whether they represented sites of true carcinogenicity.

Dosing Techniques

Techniques are available to administer test substances to mice by most routes of potential human exposure. The choice of a route of administration for a toxicity study should consider the expected route of human exposure and any other scientific objectives that need to be achieved to facilitate safe use of the test substance. Of the various routes available, most test articles will have the most rapid onset of effects and the greatest potency when administered by the IV route, followed in approximately descending order by the inhalation, IP, subcutaneous, intramuscular, intradermal,

oral, and topical routes (Klaassen and Doull 1980). The expected route of human exposure is probably the most important single determinant of route for toxicity testing. In the following discussion, the most commonly employed routes for toxicity testing in mice are discussed first, followed by the less commonly employed routes.

Oral Administration

Oral administration is probably the most frequently used route of exposure for toxicity testing in mice. Many products are intended for oral administration to humans, and many others are subject to accidental ingestion. Oral administration subjects the test substance to limitations of absorption and metabolism that are similar but not necessarily identical to those in humans. Mice, like rats, differ from many other species in that they do not have an emetic response. For this reason, large doses of substances that would cause emesis in dogs or primates will be retained in the stomachs of mice. Although this characteristic facilitates testing at high doses and maximizes potential exposure to toxic effects, it could lead to an overestimate of potential human toxicity because the animal lacks the protective aspect of the emetic response. Another area in which mice differ from dogs and primates is that mice are nocturnal. This characteristic adds some pharmacokinetic variables to the equation for extrapolating toxicity findings from the mouse to the human. Doses that are administered during the day are administered to animals that are in the lower phase of their circadian metabolic cycles. This might mean slower absorption, slower metabolism to either more or less toxic metabolites, or slower elimination of the test substance. Conversely, test substances administered in the diet or drinking water will be largely consumed at night, as that is when mice consume most of their daily intake of food and water. Although this regimen more closely approximates human consumption during the active part of the day, it makes observation of the animals during the period of peak exposure and metabolism difficult. Three means of oral administration are oral gavage, dietary admixture, and mixture with the drinking water.

Gavage

Oral gavage offers the advantages of precisely measured doses that can be administered at precise times. Doses can be administered during the day so animals can be conveniently observed for toxic effects during the first few hours after dosing. Volatile substances and those that lack stability over longer periods in the presence of diet, air, or water can be effectively administered by this method. Gavage allows administration of unpalatable substances that might not be accepted in the diet or water. There are disadvantages associated with gavage administration. The test substance must either be a liquid or must be soluble or suspendable in a liquid vehicle system. The method is relatively labor intensive compared to diet admixture. The processes of daily handling and intubation of all animals engenders the risk of injury during the intubation process, including esophageal puncture and aspiration of test article. In addition, the process of frequent handling causes stress to the animals. Although it is convenient to administer doses during the day, daytime is the period of lowestmetabolic activity for nocturnal species such as mice. This circadian effect might not be most representative of diurnal species such as humans

Description of Technique. Gavage administration entails intubation of the mouse with an intubation needle attached to a graduated hypodermic syringe. The dose is administered into the esophagus. Intubation needles for mice are typically constructed of stainless steel tubing with a stainless steel ball tip to reduce the probability of esophageal perforation and reflux and aspiration of the dose. Acceptable tubing sizes range from 22 to 18 gauge, with the larger bore reserved for older mice (e.g., $\geq$ 25g). Tubing length is not critical but can range from 1 to 3 in. The ball tip is typically 1.25 mm to 2.25 mm in diameter. Intubation needles are available commercially (e.g., Popper & Sons, Inc., New Hyde Park, New York) in straight and curved configurations. The choice

of shape is a question of personal preference of the dosing technician. Prior to dosing, the test substance must be prepared in a liquid form at an appropriate concentration. Liquid test substances might require dilution. Solid substances will require either dissolution or suspension in an innocuous vehicle. The preferred vehicle is water. If the substance is insoluble in water, various agents can be added to improve wetting (e.g., 0.1% w/v polysorbate 80) and to reduce settling (e.g., 0.5% w/v methylcellulose). Suspensions should be analyzed prior to administration to assure proper concentration, homogeneity, and stability of the substance in the suspending vehicle. Appropriate dose volumes for gavage administration are in the range of 5 to 10 ml/kg of body weight, but volumes as high as 20 ml/kg can be administered carefully, particularly in acute studies in which the mice have been fasted prior to dosing.

For the actual process of dose administration, the mouse should be weighed, and the individual dose calculated. The appropriate dose volume should be drawn into the dosing syringe and any air bubbles should be expelled. The mouse is then picked up by the skin of the back and neck, and the head tipped back to form a straight line from the nose through the back of the throat and to the stomach.

The intubation needle is inserted into the back of the mouth, then gently tipped back, if necessary, to enter the esophagus. The mouse generally facilitates entry into the esophagus by swallowing the ball of the needle. One successful approach is to envision the tip of the sternum as a target for the tip of the intubation needle. When properly positioned, the tube can easily be inserted to a reasonable depth, but it need not reach the stomach. When in position, the dose should be administered slowly to avoid reflux, but promptly to reduce the likelihood that the mouse will struggle and injure itself.

Dietary Admixture

Oral administration by dietary admixture offers several advantages, including ease of administration, minimal handling of animals for dosing, and elimination of the risk of injury associated with intubation. The method offers relatively precise dose administration for the group (better than water mix; not as good as gavage), as both mean food consumption and mean body weight for periods of a week or longer are easily measured. Dry, insoluble substances can be administered easily, and administration of test substances to mice during the awake phase of their circadian cycle is an advantage. There are disadvantagcs associated with dietary admixture. Accuracy of individual doses is lower than with gavage, and there is not a single identifiable time of dosing. Volatile substances and those that lack stability over periods of at least 4 to 7 days in the presence of diet, air, or water are precluded from this method. Diet admixtures must be sampled and the samples analyzed periodically to assure proper concentration and homogeneity of the mixture during the course of the study. Unpalatable test substances typically result in reduced dietary intake, which leads to an increase in the concentration of test substance in diet during subsequent weeks in an attempt to reachieve the desired doses. The increased concentrations might be even less palatable, leading to further reduction in dietary intake, and in some cases, eventually to malnutrition. In any dietary study, results should be evaluated carefully to discriminate between changes associated with altered nutritional status and true test substance toxicity.

Description of Technique. Oral administration by dietary admixture entails presentation of a mixture of the test substance in diet in place of the normal diet received by the animals. The concentration of test article in diet is adjusted, based on the most recently collected data on mean body weight and food consumption for each sex and dose group, to provide the desired doses of test article during the period in question. Early in a study, when body weight and food consumption are changing due to rapid growth of the animals, projections of the mean body weight and food consumption for the coming period should be based on both the most recent measurements and the rate of change (slope of the plot) of those parameters over several recent periods. If test article

stability permits, a convenient period for measurement of body weight and food consumption is about 1 week.

Test substances are usually mixed with the meal form of rodent diet in one or more of a variety of mechanical blenders. Common types of blenders include the Turbula, paddle style, and twin shell or "Y" blenders. When large quantities of diet admixture are required, it might be advantageous to blend the test substance with the diet in two steps, preparing a premix of 1 to 2 kg in a blender such as a Turbula, then adding the premix to a larger scale blender, such as a paddle blender. This procedure often produces a more homogeneous mix in a shorter blending period.

Following mixing, the appropriate concentration of blend is dispensed into animal feeders for presentation to the mice. Feeders are weighed when they are placed in the cages and when they are removed to determine the average amount of feed consumed per day. Mice are weighed at the same times, both to determine body weight gain over the period and to calculate the food consumption in grams per kilogram of body weight per day. Based on this calculation, the concentration of test substance in diet can be varied up or down to more closely approach the intended doses of test substance in milligrams per kilograms per day.

Among potential problems that might be encountered in conducting dietary admixture studies, two could lead to inappropriate calculations of feed consumption, leading to incorrect calculation of concentrations for future periods. These problems are excessive feed spillage, which might result from mice digging or playing in their feeders, and contamination of the feeder with urine and feces, which might result from mice living in their feeders. Feeders should be checked daily for excessive spillage at the time survival checks or observations are conducted, and excessive spillage should be documented. Contamination with urine and feces will lead to an incorrectly high feeder weight at the end of the feed consumption period, and an underestimate of true feed consumption.

Excessive contamination should be documented. Animals with excessive spillage and those with excessive contamination of feeders should be excluded from the calculation of mean body weight and food consumption used to prepare future concentrations of diet admixture.

Drinking Water

Administration of a water-soluble test substance in the drinking water has many of the same advantages as administration of a dietary admixture. This method is rarely used for toxicity studies, however, because of the difficulty in accurately measuring the quantity of water actually consumed. Although graduated water bottles can be used, spillage due to mice rubbing against sipper tubes, inefficient drinking, evaporation, vibration, and leaking bottles makes these measurements imprecise. If that practical problem could be solved, administration in the drinking water would bear little conceptual difference from dietary admixture. Stability of environmental temperature and humidity is essential to the conduct of a study in which the test article is mixed in the drinking water, as increased temperature and lowered humidity lead to increased water consumption. Water consumption must remain relatively stable to allow calculation of appropriate concentrations of test article in water to achieve study objectives.

Description of Technique. From a practical standpoint, administration of the test article in the drinking water has many similarities to administration by dietary admixture. It is essential that the test article be both soluble and chemically stable in water for the period of presentation. That period should be in the range of about 2 days to a maximum of about 1 week. Water remaining in a water bottle for periods in excess of a week can become heavily contaminated with bacteria. Analogous to the situation with diet admixture, the precision of dose administration is directly related to the accuracy with which average daily water consumption and body weight can be measured. Concentrations of the test article in water should be adjusted, if necessary, after each measurement of average daily water consumption and body weight to assure precise dose administration.

As solubility in water is a prerequisite for this method, mixing procedures are usually simpler than for diet admixtures. Homogeneity analysis should not be required for a true solution, but samples should be analyzed regularly to confirm that concentrations are what they were intended to be.

Water can be provided in graduated bottles. Contents of the bottles should be recorded at the beginning of the consumption period (but after the bottles are placed on the cages to accommodate spillage during that operation) and again at the end of the consumption period to determine average daily water consumption. Evidence should be documented. Difficulty in accurately measuring the amount of water actually consumed by the mice is the largest disadvantage to this administration method.

Intravenous Injection

IV injection offers the advantages of immediate, complete systemic availability of a precise dose at a known point in time. The process of absorption is eliminated, as is the possibility that some or all of the test article can be metabolized by the liver prior to distribution to the systemic circulation and target organs. Most substances exhibit the biggest potency and rapidity of onset of activity of all routes of administration when administered intravenously. IV administration provides a useful benchmark against which absorption and bioavailability from administration by other routes can be compared.

It is essential that test articles administered intravenously be in solution at the time of administration, and remain in solution after injection. Solutions that are subject to precipitation by changes in pH, temperature, or osmolarity should be confirmed at physiological conditions to assure that they will not precipitate after injection. Introduction of insoluble particles, such as those in a suspension, introduces a high probability of embolism, particularly in the pulmonary capillary bed, which produces severe moribundity or death. The toxicologist is left with the problem of differentiating such moribundity or mortality from the true toxicity of the test article.

Other characteristics of an IV solution that should be evaluated prior to undertaking an IV toxicity study involving repeated dosing include the potential for the solution to cause hemolysis or vascular or subcutaneous irritation. Hemolysis could be a result of the administration of solutions of inappropriate osmolarity (hypotonic solutions are particularly damaging), in which case the problem can be resolved by adjusting the osmolarity of the solution.

Ideally, solutions for IV administration should be isotonic to blood, and have a pH of about 7. Usually a pH in the range of about 5 to 9 will be acceptable. Solutions that cause appreciable vascular or subcutaneous irritation could result in sufficient injury to the veins and surrounding tissue to preclude repeated administration.

The rate of IV injection is an important variable that must be controlled fairly precisely to achieve reproducible results within a study. If the IV toxicity of two or more test articles is to be compared, it is essential that each be administered at the same rate. IV injections can be administered as a bolus over a period as short as a few seconds, as a continuous, 24-hr-a-day infusion, or over just about any interval in between.

There is nothing particularly magical about any specific dosing period, but it is critical that it remain constant. As a practical matter, an injection period of about 2 min is a reasonable upper limit for hand-held injections into caudal veins of reasonable numbers of mice. Longer periods lead to very slow injections, increase the risk of extravasation, are time consuming, and are difficult and tiring for the toxicologist. Injection periods much shorter than 1 min increase the likelihood that an inordinately high peak plasma concentration that might precede mixing with the total blood volume could compromise the survival of the animal. A corollary to the artificially high peak plasma concentration associated with a short injection period can occur if the injection is administered at an uneven rate. It is particularly critical that the rate of injection not be increased during the last half of the injection period, as this is a time when the animal has already received an initial loading, and the deleterious effect of increasing the rate of administration will be amplified. In our laboratory,

we have found that administration of volumes of 5 to 10 ml/kg body weight (0.15–0.30 ml for a 30-g mouse) administered evenly over a period of 2 min represents a good compromise. One of the biggest disadvantages of IV administration is that it is a very labor-intensive procedure, requiring more time per animal than any other route. In addition, repeated daily administration to caudal veins of mice for periods longer than 2 to 4 weeks becomes technically difficult owing to the accumulation of scar tissue and occasional trauma.

Long duration or continuous, 24-hr-per-day infusions, although possible, are technically difficult, and are not discussed in detail here. As a practical matter, long-duration injections or continuous infusions are typically administered through a surgically implanted catheter using an infusion pump. The catheter is usually placed in a large, superficial vein such as the jugular or femoral vein, then exteriorized at a site such as between the scapulae, which is difficult for the animal to chew or scratch. The surgical procedure is relatively simple. The difficulty lies in keeping the cannula patent and secure in the vein during recovery after surgery, then through the period of dosing. The maximum volume of infusion, even over a continuous 24-hr period should not exceed about 20 to 30 ml/kg/day, which is typically less than 1 ml administered over a 24-hr period. This infusion rate is so slow as to be difficult to administer, even with a high-quality infusion pump. The catheter must be attached to the mouse in a way that prevents mutilation of the catheter by the animal without undue limitation of mobility.

Description of Technique

IV administration entails injection of the desired dose into an appropriate vein. In a typical study, mice receive a single injection daily over a period of about 2 min or less. Such injections are usually administered into a lateral intra medial (IMM) (tail) vein, using a hypodermic needle attached to a graduated syringe. Hypodermic needles used for caudal vein injections in mice are usually no more than 1 in. long, and in the range of about 23 to 25 gauge. The smallest hypodermic syringe that will contain full dose volume will provide the greatest precision in dose measurement, but 1-cc disposable syringes are often used.

The needle should be installed on the syringe such that the bevel of the needle faces the markings on the syringe that will be used to measure the dose. This will allow the graduations to be read easily when the needle has been inserted into the vein in an "up" configuration. A stopwatch that is easy to read is useful for timing dose administration, and an electronic timer that is activated by a foot pedal is most convenient. A convenient restrainer should hold the mouse securely, but without undue risk of suffocation or injury, while allowing free access to the tail. A source of warm water or a tourniquet device are useful, as is a supply of small gauze sponges. A tourniquet can be constructed from a disposable plastic syringe and a piece of suture. The larger the syringe, the more pressure it is able to exert on the suture loop. Relatively large (e.g., size 0) braided silk suture should be used for the loop to minimize the risk of cutting the skin of the tail. The suture loop is attached to the plunger inside the barrel of the syringe, then threaded out through the tip of the syringe.

The dosing solutions should be prepared prior to dosing. Solutions should be analyzed periodically to assure that proper concentrations are being attained. Each mouse should be weighed, and its dose calculated. As previously noted, doses in the range of 5 to 10 ml/kg are acceptable for injection over periods of about 30 sec to 2 min. The mouse is placed into the restrainer, and the appropriate dose is drawn into the syringe. Any air bubbles should be expelled from the syringe. This is most important for IV injections. A tourniquet device can be applied at this point, but should not be applied too tightly. The objective is to block venous return, but not arterial supply, thus dilating the veins. As an alternative to a tourniquet, some toxicologists prefer to warm the tail with a gauze sponge wetted in warm (not hot) water to enhance vasodilation. The tail is now held in one hand while the needle is inserted with the other. The needle should be inserted with the bevel up to minimize the chance of puncturing through both sides of the vein. Successful venipuncture

will result in the reflux of a small amount of blood into the hub of the needle. Owing to the small volume of blood that usually refluxes, this phenomenon will be most easily visualized if needles are used that have transparent "flashback" hubs. The initial attempt at venipuncture should be made toward the tip of the tail, such that if the vein is missed, a subsequent attempt can be made closer to the base of the tail without risk that the dose will leak out of the initial hole. When the needle is securely in the vein, it can be held with the tail-holding hand while the plunger of the tourniquet is depressed to open the vein with the dosing hand.

Now that the needle is in the vein and the vein is open, the timer can be started, and the dose can be administered. One convenient method to assure even dose administration is to divide the dosing period and the dose volume into a convenient number of parts. A 2-min dosing period might be divided into eight 15-sec intervals, and the dose volume divided by eight. The doser can then administer one-eighth of the total dose volume over each 15-sec interval for 2 min to assure a relatively even rate of injection. When the full dose has been administered a clean, dry gauze sponge should be pinched over the injection site and the needle should be withdrawn. Maintaining pressure on the site of the injection for 10-30 sec after withdrawal of the needle is usually adequate to prevent bleeding. The mouse can then be removed from the restrainer. As IV injections typically result in a rapid onset of activity, it is often appropriate to observe a mouse for the first few minutes after dosing for clinical signs of toxicity.

Intraperitoneal Injection

The IP route of administration generally offers the second most rapid absorption of a test article among the parenteral routes, with systemic availability second only to IV injection. Rapid absorption is conferred by the large surface area of the lining of the peritoneal cavity, and by the rich blood supply to that area.

IP administration leads to absorption primarily through the portal circulation. As a result, test articles that are metabolized by the liver are subjected to extensive (or even complete) metabolism prior to reaching systemic circulation and target organs, unless, of course, the target organ is the liver, in which case toxicity might even be amplified. Test articles that are excreted in the bile are similarly subject to elimination prior to reaching the systemic circulation and target organs. Water-insoluble mixtures, such as aqueous suspensions, can be administered by the IP route. This might provide the opportunity for rapid systemic absorption of lipid-soluble or certain other test articles. Solutions or suspensions for IP injection should be adjusted to a pH in the range of about 5 to 9 to reduce the potential for irritation. Osmolarity of the dosing formulation is not critical, as it is for IV injection. Dose volumes for IP administration are in the range of 5 to 10 ml/kg/day, but volumes as high as 20 ml/kg/day are acceptable, particularly if the study is of limited duration, or if it is known that the test article will be absorbed by the IP route.

One of the most significant disadvantages of IP administration is the risk of peritonitis. Peritonitis can result from any of three primary causes: physical irritation caused by accumulation of a truly insoluble or irritating test article in the peritoneal cavity, introduction of exogenous microbiological contamination, or microbiological contamination resulting from injury to the GI tract or urinary bladder. The potential for a test article to produce physical irritation or chemical peritonitis can be assessed in studies of 1 to a few days in duration. Although physical or chemical peritonitis is the most frequently seen form of peritonitis in toxicity studies, it is still found with only a small percentage of test articles. Mice are relatively resistant to microbiological infection, so microbiological peritonitis is even less common than physical or chemical peritonitis. Peritonitis resulting from injury during the injection process is extremely rare when qualified toxicologists administer the injections. There is a slight risk to the animals of physical injury to a major organ or vessel during the injection process, but again this is extremely rare in the hands of qualified dosers.

Description of Technique

IP injections are administered into the peritoneal cavity using a hypodermic needle attached to a graduated syringe. Each mouse receives a single daily dose, administered as a bolus, for the duration of the toxicity study. The injection should be administered into the animal's lower right abdominal quadrant to minimize the risk of injury to the liver, spleen, and bladder. For initial training purposes in dosing by the IP route, it is useful to sacrifice a mouse, then open the abdominal cavity to expose the internal organs that might be susceptible to injury during the injection procedure. This allows a novice to hold the animal in a dosing position and clearly visualize where the lobes of the liver, the spleen, and the urinary bladder will be, and the area of less vulnerability between these organs. Hypodermic needles used for IP injections to mice need be no longer than about 5/8 in., and should be of the smallest diameter that will allow easy injection of the dose volume to minimize the trauma to the abdominal wall and the commensurate potential for leakage. Needles in the range of 23 to 25 gauge are appropriate for use with solutions and suspensions of low viscosity. Suspensions of high viscosity might require the use of needles with a larger bore. Needles as large as 19 to 20 gauge can be used, but require great care to avoid injury and leakage of the test article from the injection site.

Prior to initiation of a toxicity study, dosing formulations should be prepared, and samples analyzed for concentration and homogeneity of suspensions, if appropriate. Each mouse should be weighed and its dose calculated. The appropriate dose is then drawn into the syringe, and air bubbles are expelled. The mouse is picked up with one hand, and held with the ventral surface toward the doser. Movement of the animal's right hind leg should be restricted to limit interference with the needle during dosing. The needle should be inserted at an angle of about 15° to 30°. into the abdominal cavity to facilitate penetration of the abdominal wall.

The location should be to the right of the midline (to avoid the spleen) at a position about midway between the lower edge of the liver and the urinary bladder to a depth of about 1 cm (3/8 in.). Following insertion, the needle is withdrawn slightly, moved about, and the angle of insertion is reduced to assure that the tip has not penetrated or snagged any internal organs. The dose is now administered as a bolus and the needle withdrawn. If a large-bore needle has been used, it might be necessary to apply gentle finger pressure over the injection site for a few seconds to prevent leakage of the dose.

Intramuscular Injection

The intramuscular (IM) route of administration is less commonly used in toxicity testing, but it might be appropriate if the test article is intended for IM administration to humans. The IM route generally results in slower absorption of a test article, with lower peak plasma levels, but more sustained effects than IV or IP injection. The rate of absorption can be influenced by the amount of vascular perfusion of the tissue surrounding the injection, the vehicle, and the injected volume, which indirectly might alter the surface area of tissue available for absorption. Coadministration of a vasodilator generally increases the rate of absorption, whereas coadministration of a vasoconstrictor generally decreases that rate. Administration of the test article as a solution or suspension in a viscous, poorly absorbed vehicle generally retards absorption. The ability to control the rate of absorption can be a significant advantage in some cases, as it allows the toxicologist to administer a dose of a test article that can be absorbed over a period of many hours or even days. This can be especially useful in the case of test articles that have short half-lives after absorption, as a result of rapid metabolism or elimination. Limitations of IM dosing include the limited number of muscle groups in the mouse that are large enough to accept dosing (e.g., the muscles of the posterior aspect of the femoral region) and the small dose volume that can be administered. If possible, the same injection sites should not be treated every day to allow time for absorption and recovery from the

trauma of dosing. This means that whereas a single acute dose might be divided into the hind limbs, repeated daily doses should be administered into alternate limbs. The dose volume should not exceed 1.0 ml/kg per injection site, or about 0.03 ml for a 30-g mouse; smaller volumes are preferable. An acute study in which each animal is dosed once would allow 1 ml/kg to be administered into each hind limb, for a total dose volume of 2 ml/kg. This dose volume coupled with the limit of solubility or suspendability of the test article in the vehicle selected might restrict the maximum dose of test article below toxic levels. A further limitation on toxicity testing by the IM route is that the formulation to be injected must not cause significant local irritation, particularly if repeated doses will be administered. This limitation might require that a separate study be conducted to assess IM irritation potential prior to initiation of a repeated dose study by this route. IM injection is more labor intensive than most other routes with the exception of IV injection.

Description of Technique

IM injections are administered into the large muscle groups of the posterior aspect of the femoral region using a hypodermic needle attached to a graduated syringe. Each mouse receives a single daily dose, administered as a bolus into alternate hind limbs for the duration of the study. Hypodermic needles used for IM injection should be the smallest diameter that will allow injection, but in the range of 27 gauge up to a maximum of about 23 gauge. Prior to dosing, the same procedures for formulation, analysis, weighing of mice, and calculation of doses should be followed as those recommended for IP dosing. The dose is drawn into a syringe, and air bubbles are expelled. The mouse can be held by an assistant, and the needle is inserted to the approximate center of the muscle mass. The dose is injected as a bolus, and the needle is withdrawn. The muscle can be massaged gently to distribute the dose prior to returning the mouse to its cage.

Subcutaneous Injection

The subcutaneous (SC) route of administration is not commonly used in toxicity testing, but might be appropriate if the test article is intended for SC administration to humans, or as a more practical substitute for IM testing in mice. The SC route is similar in many characteristics of absorption to the IM route, and generally results in slower absorption of a test article, with lower peak plasma levels, but more sustained effects than IP administration. The rate of SC absorption can also be influenced by the amount of vascular perfusion of the tissue surrounding the injection, the vehicle, and the injected volume, which indirectly might alter the surface area of tissue available for absorption. Coadministration of a vasodilator generally increases the rate of absorption, whereas coadministration of a vasoconstrictor generally decreases that rate. Administration of the test article as a solution or suspension in a viscous, poorly absorbed vehicle generally retards absorption. The ability to control the rate of absorption is similar to that seen with IM injection, and can offer the same advantages, as it might allow the toxicologist to administer a dose of a test article that can be absorbed over a period of many hours or even days. Some limitations to IM dosing do not apply to SC dosing. SC doses can be injected at a wide variety of sites, if necessary. In addition, dose volumes of up to 10 to 20 ml/kg per day can be administered repeatedly if they are well absorbed and do not cause excessive local irritation. These large dose volumes allow administration of much higher total doses than can be administered IM. It might still be necessary to conduct a separate study to assess SC irritation potential prior to initiation of a repeated dose toxicity study. SC injections can easily be administered to mice without assistance.

Description of Technique

SC injections are administered into the region beneath the skin using a hypodermic needle attached to a graduated syringe. Each mouse receives a single daily dose, administered as a bolus.

Daily doses can be administered at the same site if absorption is complete and irritation is minimal, but SC trauma might be reduced if the injection site can be changed from day to day. Hypodermic needles used for SC injection should be the smallest diameter that will allow injection, but in the range of about 26 gauge up to a maximum of about 20 gauge. Although larger volumes can be administered, dose volumes of about 10 ml/kg per day are preferable.

Prior to dosing, the same procedures for formulation, analysis, weighing of mice, and calculation of doses should be followed as those recommended for IP dosing. The dose is drawn into a syringe, and air bubbles are expelled. The mouse is grasped by a fold of skin. One of the most convenient injection sites is the skin in the midscapular region, which allows the restraint and dosing of the mouse with minimal risk of being bitten. The needle is inserted through the skin into the SC region. The dose is injected as a bolus, and the needle is withdrawn. The injection site can be pinched for a few seconds to prevent leakage, and the area around the injection site can be massaged gently to distribute the dose prior to returning the mouse on its way.

Intradermal Injection

Intradermal (ID) injection is not a commonly used route for toxicity studies. It might be appropriate to test products intended for ID administration to humans by that same route in mice, and studies of limited duration are technically feasible. The ID route offers the advantage of slow absorption owing to the poor vascular perfusion of the skin relative to tissues in other areas of potential administration. This slow absorption is typically associated with longer time to onset of effects and lower peak plasma levels, but more sustained effects than routes that result in faster absorption. To the extent that the test article could be metabolized by the skin, the ID route would be expected to offer greater opportunity for such metabolism than SC injection, but less than topical administration. Injected volume for ID dosing should be limited to about 1 ml/kg per injection site or less, with smaller volumes preferred if repeated doses will be administered.

It is acceptable to administer IS doses at multiple sites simultaneously if higher total doses are required. Irritating formulations of the test article must be avoided, especially if multiple doses will be administered, as ulceration and necrosis of the skin can result.

Description of Technique

ID injections can be administered at a variety of accessible sites, but the skin of the abdomen or back is often used. The area in which the injections will be administered should be shaved with a small animal clipper to allow good visualization during and after dosing. Doses can be administered using a small hypodermic needle attached to a graduated tuberculin syringe. Needle diameter should be limited to 27 gauge or smaller, and 30 gauge is preferable. The use of a needle with an ID level is not necessary. Prior to dosing, the same procedures for test article preparation and analysis of formulations, weighing of mice, and calculation of doses should be followed as recommended for IP dosing. The dose is drawn into the syringe and air bubbles are expelled. The mouse is held in one hand, and the needle is inserted into the skin at a shallow angle with the bevel of the needle up to avoid penetration into the SC space. With practice, the toxicologist can feel the needle penetrate into the SC space, if that happens by accident, and can relocate the needle prior to injection. A properly administered ID dose will appear as a small bleb on the surface of the skin. A dose administered into the SC space will not appear as a bleb, as the dose will be distributed over a larger area.

Topical Administration

The topical route of administration is occasionally used for toxicity testing. This route might be appropriate for testing the systemic and local toxic effects of substances intended for human

topical administration or those that are likely to come into accidental contact with human skin. Data suggest that the mouse is one of the less appropriate species for extrapolation of percutaneous toxicity to the human, as the permeability of mouse skin (as well as rat and rabbit skin) is substantially higher than the permeability of human skin (Maibach and Wester 1989). Nevertheless, topical application to mice might be appropriate in special cases, such as the conduct of the MEST for dermal sensitization potential (Gad et al. 1986).

Historically, the skin was perceived as a barrier to absorption. It is now clear that lipophilic compounds are readily absorbed into and across the skin, and further that the skin might be a source of significant metabolism of some chemicals (Maibach and Wester 1989). Variables in addition to lipophilicity that are likely to effect dermal absorption include the integrity of the skin at the treatment site; the vehicle employed for dosing, occlusion, and/or restraint of the mouse after treatment; and whether the test article is washed off after some prescribed period. Variations on the integrity of the skin include totally intact skin, skin from which the outer epidermal layers have been tape stripped using a surgical adhesive tape (e.g., Dermiclear), and skin that has been abraded. The presumption is that nonlipophilic test articles will penetrate stripped (thinned) or abraded (interrupted) skin more extensively than they would intact skin. The proper choice of a vehicle might enhance the permeability of the skin to a nonlipophilic chemical. Occlusion of the treatment site and restraint of the mouse after application of a topical dose improves retention of the dose in contact with the skin and reduces the probability that the animal will orally ingest the topical dose. Washing excess test article from the treatment site after a prescribed time limits the exposure period to a known interval. The appropriate choices for these (and other) variables in topical toxicity study design are a function of the specific objectives of the study, and the physical and chemical characteristics of the test article.

Description of Technique

As the number of procedural variables for topical dosing is so great, the procedures described for topical dosing in the MEST (Gad et al. 1986) are described as a representative technique. In that procedure, the hair is clipped from the treatment site (e.g., abdomen or back) on the first day of treatment and the epidermal layer is tape stripped until the site has a slightly shiny appearance, typically 10 to 20 applications and removals of a surgical adhesive tape such as Dermiclear (Johnson & Johnson Co., New Brunswick, New Jersey). Next, a fixed volume (e.g., 100 μ) of the test article in a volatile solvent such as ethanol is applied to the treatment site. The solvent is allowed to dry using a warn air blower if necessary, and the animal is returned to its cage. On subsequent treatment days the tape-stripping operation can be reduced to about 5 to 10 applications of adhesive tape to achieve the shiny appearance.

Inhalation

The inhalation route of administration offers the most rapid absorption of most test articles, with systemic availability second only to IV injection. Efficiency of absorption by the inhalation route is conferred by the large surface area of the respiratory system, the close proximity of the inner alveolar surface to the blood circulating through the lungs, and the fact that the entire cardiac output passes through the lungs with each circuit of the blood through the body. Absorption of inhaled agents proceeds via one or more of the following mechanisms depending on specific characteristics of the agent: direct absorption into the blood stream, absorption from the GI tract following deposit in the nasopharynx or transport by mucociliary escalation and swallowing, or lymphatic uptake following deposit in the alveoli.

Inhalation studies are particularly useful in estimating the risk of accidental or occupational exposure to a gas, vapor, dust, fume, or mist as well as in evaluating the toxicity of agents intended to be administered by inhalation. Administration by inhalation is the most technologically complex

means of routine exposure, and a comprehensive description of the procedures is beyond the scope of this chapter. The reader is referred to other works (e.g., Kennedy and Trochimowicz 1982; McClellan and Henderson 1989; Menzel and McClellan 1980) for further description. Rather this discussion is limited to some of the advantages, disadvantages, and variables to be considered in inhalation testing. The primary advantage of inhalation is rapid, effective absorption. The primary disadvantage is the technological complexity of the method, with the associated risk of technical error and disregard of an important variable.

For a mouse to inhale a test article, the mouse must be placed in an environment that contains the test article in the form of a gas, vapor, dust, fume, or mist. The test article must exist in a particle size that is inspirable, generally having an aerodynamic diameter from 1 to about 10 μm. Particle size dictates where in the respiratory tract the test article will be deposited and absorbed. Larger particles are deposited in the nasopharyngeal region, with successively smaller particles deposited in the trachea, bronchial, bronchiolar, and finally the alveolar region for particles of about 1 μ or less. The technology of particle generation and uniform distribution through the exposure apparatus is complex in itself. In addition to generating and uniformly distributing the test atmosphere, care must be exercised to capture the exhaust from the exposure apparatus, such that the test article can be contained without contamination of the laboratory or environment. Exposure periods can range from a few minutes, appropriate for test articles that might pose only an acute exposure risk, to continuous exposure over a prolonged period, appropriate for test articles that might pose a risk of long-term environmental or occupational exposure. Exposure apparatus generally takes the form of a chamber that contains the whole animal or groups of animals, or a device that exposes only the head or nose of the animal(s) to the experimental atmosphere.

Chamber (Whole Body)

Inhalation chambers allow relatively large numbers of mice to be exposed simultaneously without restraint. The aerodynamic considerations are complex, but simpler than for a head-only or nose-only exposure system. Flow rate through a chamber must be adequate to provide temperature and humidity control. Disadvantages of whole body chambers include the tendency for test article to accumulate in the fur, from which it can be ingested; on the skin and eyes, which could interfere with the intended route of exposure; and the difficulty in monitoring respiratory volume and rate of individual animals.

Head/Nose Exposure (Head Only/Nose Only)

Head- or nose-only exposure apparatus limits exposure of the mouse to the test article by routes, other than inhalation, as only a small amount of skin and fur are exposed to the test environment. In addition, it is possible to monitor respiratory volume and rate of individual animals with some of the head- or nose-only equipment. Disadvantages to this equipment include the fact that a relatively small number of animals can be simultaneously exposed, and those animals must be restrained in a position that keeps their heads or noses in close contact with the exposure apparatus. This restraint imposes stress on the animals, and virtually precludes continuous exposure, as the processes of eating and drinking are not possible with most of this equipment. The restrainer might limit the animals' ability to dissipate excess body heat.

Data Collection Techniques

Types of data that are routinely collected during the conduct of toxicity studies in mice fall into three broad categories: clinical observations and physical examinations, clinical laboratory evaluations, and postmortem procedures. Cardiovascular parameters are not measured in routine toxicology studies. Heart rates in awake mice have been measured in the range of 300 to more than

800 beats/min (Kaplan et al. 1983). Reliable blood pressure measurements are best made by cannulation of a major artery, such as the carotid. Such procedures require anesthesia and surgery, neither of which is especially desirable during the course of a study that could be of long duration and involve many animals.

Clinical Observations and Physical Examinations

Clinical observations entail the recording of effects that can be detected by direct observation, such as abnormal gait and body weight. For the sake of this discussion, a variety of parameters that can be observed or measured directly are discussed in this section. Clinical observations often provide the first indication of which physiological systems are being affected by the test article.

Mice should be observed regularly throughout the in-life portion of a toxicity study. The type and frequency of these observations should be tailored to meet the scientific objectives of the specific study. Most effects observed following administration of acute (single) doses occur within a relatively short time after dosing. As acute IV doses are often associated with almost immediate effects, it might be appropriate to observe treated mice within 5 min, at about 15, 30, and 60 min, and again at 2 and 4 hr after dosing. Observations should be repeated at least once daily on all subsequent study days throughout the postdosing observation period. This schedule should provide information on the times of onset, peak activity, and remission from toxic effects, as well as information on the sequence and severity of effects observed. The high intensity of data collection on the day of dosing in acute studies requires that the system for conducting and recording observations be simple and time efficient. Typically, a system of exception reporting is used, in which observations of exceptions from the norm are recorded, and the absence of comment on a system (e.g., respiration) implies that parameter is normal. Clinical observations in repeated dose studies should be conducted at approximately the same time each day to assure that changes in findings over the course of the study can be attributed to the accumulation of or adaptation to toxic effects rather than incidental changes attributable to circadian rhythm or time after dosing. Minimally, all animals should be observed early in the day, prior to daily dosing, and it is highly desirable to conduct at least one additional daily observation at 2 to 4 hr after dosing (or late in the day) to be aware of effects that might be associated with higher blood levels of test article usually found from a few minutes to a few hours after dosing.

The simplest form of clinical observation is an observation for survival and moribundity. This or a higher level of observation must be conducted at least once daily in all toxicity studies. The next level of observation is an observation for clinical signs of toxicity, such as abnormal level of spontaneous motor activity, abnormal gait, abnormal respiration, and abnormal quantity or quality of fecal output.

The next level of observation is more structured, and is typically conducted about once weekly during studies of a few weeks' duration and as infrequently as about once monthly during the later phases of 26-week to 2-year studies. During the conduct of a physical examination, specific parameters are evaluated, such as quality of coat, body orifices (for excessive or unusual discharges), eyes, and respiratory sounds. In studies longer than about 26 weeks, animals are examined carefully for evidence of visible or palpable masses. Body weight and feed consumption are typically monitored in studies longer than a few days. An appropriate interval for measuring body weight and feed consumption is about a week. These two parameters should be measured concurrently, such that changes in one can be compared directly to changes in the other. In longer studies, in which the mice have reached maturity and body weight gain has approached zero, the frequency at which body weight and feed consumption are measured can be reduced to as infrequently as once per month. The interval over which they are measured would remain at about a week, however.

Clinical Laboratory Evaluations

Clinical laboratory evaluations of mice refer to evaluations of blood and urine. Blood is routinely collected at sacrifice in repeated dose studies, and small quantities (e.g., about 0.10 ml) of blood can be collected at interim periods during the course of the study for the purpose of evaluating differential smears or other limited objectives. Interim (nonterminal) blood samples can be collected by retro-orbital venous plexus puncture, cardiac puncture, and tail snip, among other techniques. Each of these techniques has certain disadvantages. Retro-orbital puncture is technically difficult, and might require anesthesia or immobilization of the animal. Cardiac puncture typically requires anesthesia, and cardiac injury could compromise the histological evaluation of cardiac tissue. Tail snip often yields samples that are contaminated with extravascular, extracellular fluids. Any administration of anesthetic agents during the study of a test article that is not thoroughly understood engenders some risk to the interpretation of the study, as potential interactions of the anesthetic with the metabolism or direct effect of the test article are nearly impossible to predict. Blood collected at the time of sacrifice is typically drawn from the inferior vena cava or the abdominal aorta while the mouse is under anesthesia. In the case of terminal blood collection, potential interaction of the anesthetic agent with the test article, induction of liver enzymes, and so on, is not an issue.

Parameters evaluated in blood samples drawn from mice include evaluation of differential smears for morphological abnormalities and differential white counts, measurement of serum glucose and urea concentrations, serum aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase activity. Red blood cell counts, white blood cell counts, and hemoglobin concentrations can be measured, but these parameters are quite variable for mice on many of the commonly used laboratory instruments, so they are often omitted. In addition, bone marrow smears can be prepared, but are usually only prepared at sacrifice in mice. Bone marrow smears might help in understanding hematological changes. Caution should be exercised in comparing experimental data with results obtained from the literature or with results obtained on different instrumentation or by different procedures. For greatest utility, a set of normal values should be compiled for the laboratory procedures and equipment used to produce the data in the toxicity study.

As a practical matter, urine is not usually collected in routine toxicity studies. The primary difficulty in conducting urinalysis is that the mouse produces a very small volume of urine during a reasonable collection period (e.g., 16–24 hr), and of that volume, considerable and variable quantities are lost to evaporation and on the surfaces of the collection apparatus. As a result, attempts to evaluate urinary concentrations of practically anything can be very misleading.

Postmortem Procedures

Postmortem procedures, literally those procedures performed after the death of the animal, include confirmation of the identification number and sex of the animal, an external examination, examination of the significant internal organs in place prior to removal, then removal, weighing of appropriate organs, and collection of tissue specimens for histological processing and microscopic examination. The microscopic examination of tissue specimens by a qualified veterinary pathologist might be the single most important source of information in understanding the toxicity of a test article. The pathologist's findings should be carefully integrated with the other study data (e.g., clinical observations, body weights, feed consumption, and clinical laboratory findings) to fully comprehend the effects of the test article on the mouse under the conditions of study.

Ordinarily the list of tissues to be routinely weighed, collected, and processed for histological examination is specified in the study protocol. In addition to the tissues specified in the protocol, specimens are usually collected of all lesions or target organs that have been identified during the

course of the study or at gross necropsy. It is important to provide the necropsy staff with a current list of abnormal clinical observations, especially any evidence of visible or palpable masses, as this is the time when the visible and palpable lesions can be linked to the histopathological evaluation of those lesions. Every effort should be made to locate all lesions described and collect representative tissue from those sites.

A detailed description of necropsy procedures is beyond the scope of this discussion. It should be emphasized that the necropsy process, particularly when conducted on a large number of animals at the scheduled termination of a study, is a process in which a large number of tissue samples can be collected, and a similarly large quantity of data might be gathered during a short period of time. As such, this process presents many opportunities for loss or misidentification of samples and data. A rigorous system of accounting for which tissues have been collected from each animal and for tracking the samples and data collected is critical to the accurate interpretation of the toxicity study.

Summary

In summary, the mouse is one of the most useful species for toxicity testing. Mice have been used in biomedical research for hundreds of years. As a result of this long usage, many techniques have been developed to dose and evaluate mice, and a wealth of historical data has been accumulated in the literature. A wide variety of genetic strains have been developed for specific purposes. It is often possible to select a strain for testing that is particularly vulnerable or resistant to either the test article or a particular type of lesion that might be expected to be associated with that test article. The small size of the mouse confers economy in acquisition, husbandry, handling, and test article consumption. The relatively short gestation period and life span of the mouse are useful in conducting reproductive studies, or studies in which the test article will be administered for a high percentage of the lifetime of the animal.

The small size of the mouse is responsible for most of the disadvantages of the species as well. The species is relatively susceptible to environmental stress. Small size and blood volume make it difficult or impossible to collect multiple samples of blood and urine over short periods of time. Assays that might require large volumes of blood or urine are precluded. Certain physiological evaluations, such as electrocardiograms, are difficult owing to the small size and high activity level of the species.

PATHOLOGY

As they age, animals of all species, including humans, develop a variety of lesions, both neoplastic and nonneoplastic. Such lesions are usually referred to as spontaneous or age-associated lesions. These lesions are most frequently proliferative, degenerative, or inflammatory in nature. Each species and strain of animal has certain characteristic lesions, which are commonly observed with aging. The purpose of this chapter is to discuss those age-associated lesions frequently observed in various strains and stocks of mice and those that might interfere with interpretation of toxicology studies. The B6C3F1 hybrid mouse strain and the CD-1 mouse stock are the two strains or stocks of mice most commonly used in toxicology studies in the United States. The National Toxicology Program (NTP) utilizes the B6C3F1 mouse, whereas private industry utilizes primarily the CD-1 strain for toxicology studies. Both strains have their advantages and disadvantages. Data are available regarding the time of onset and incidences of age-associated lesions found in these strains or stocks. This chapter primarily discusses lesions found in B6C3F1 and CD-1 mice. There is a section on transgenic animals that are becoming increasingly important in toxicology studies.

The B6C3F1 mouse is the F_1 hybrid of two inbred strains, C57BL/6 females and C3H/HeN males, and is designated (C57BL/6 × C3H/HeN)F_1. The C57BL/6 strain was originated by Little in 1921. The C3H strain was originated by Strong in 1920 (National Institutes of Health 1981).

When the C3H strain was obtained by Heston in 1941, it was designated as the C3H/HeN. Subsequently, the strain was established behind the specific pathogen-free (SPF) barrier. The mouse mammary tumor virus (MTV), which is transmitted through the milk, was not transmitted to the offspring, which were caesarean-derived and maintained behind the barrier. The strain was then designated as C3H/HeN-MTV-. This strain is used to provide the male parent for the B6C3F1 mouse used by the NTP and, therefore, does not carry the MTV (Goodman et al. 1985). The B6C3F1 mouse from the Charles River Laboratories is likewise MTV negative (Anonymous 1982) However, it is possible that B6C3F1 mice from other sources might be derived from C3H mice carrying the MTV.

The CD-1 mouse is an outbred stock maintained at the Charles River Laboratories. The full designation for this stock is Crl: CD-1(ICR)BR. The CD-1 mouse was derived from Swiss stock (Anonymous 1982). The so-called Swiss mouse was imported to the United States in 1926 by C. J. Lynch of the Rockefeller Institute for Medical Research, and was derived from a noninbred stock maintained in Europe. Subsequently, the stock was given to other researchers and commercial laboratories (Lynch 1969) The Swiss stock used by the Charles River Laboratories to establish the CD-1 mouse was obtained from Hauschka and Mirand of Roswell Park Memorial Institute (HaM/ICR) in 1959. The CD-1 mouse was caesarean derived at that time and, subsequently, maintained behind an SPF barrier (Anonymous 1982).

In evaluating toxicology studies, it is important to be aware of the types of age-associated lesions observed in untreated animals of the strain or stock of mouse used in the study, at what age such lesions are generally observed, the incidences observed, and the degree of severity. Generally, few if any lesions are encountered in CD-1 and B6C3F1 mice less than 6 months of age. Those that are encountered are usually sporadic in occurrence and minimal to mild in severity. Neoplasms of any type are rarely seen in animals of this age, although they can occur. Most acute and subchronic toxicity studies in mice are conducted in animals less than 6 months of age. Thus, spontaneous lesions rarely complicate interpretation of data from these types of studies. After 1 year of age, spontaneous lesions start to appear, increasing in frequency and severity with advancing age. Most long-term toxicology studies are terminated at 18 months or 2 years with CD-1 or B6C3F1 mice, respectively. B6C3F1 mice have good longevity with 75% to 80% surviving to the end of 2-year studies (Goodman et al. 1985). At 2 years only 50% of CD-1 mice are still alive (Anonymous 1982).

For the B6C3F1 mouse, extensive data have been published on the incidences of spontaneous neoplasms (Anonymous 1989; Goodman et al. 1985; Haseman et al. 1998; Haseman et al. 1984; Haseman et al. 1985; Ward, Bernal, et al. 1979).

When using databases such as the one compiled by the NTP it is important to apply the correct scientific principles in the evaluation of these data (Haseman 2000). Although the CD-1 mouse is used in many laboratories that have developed their own databases for disease incidences, relatively few data have been published in a concise form (Homburger et al. 1975; Lang 1987; Percy and Jonas 1971). For both the CD-1 and the B6C3F1 mice, there are few published data on the incidences of nonneoplastic diseases.

It is well known that factors such as nutrition, husbandry (e.g., individual vs. group housing), and genetic background can affect the development of many spontaneous lesions and alter the tumor profile (Haseman et al. 1994). Necropsy protocols, tissue sampling procedures, and diagnostic criteria can also have an impact on the reported incidences of lesions; thus, it is important that each laboratory develop its own historical incidence data. Primary emphasis should be given to concurrent rather than historical control data when interpreting experimental results (Haseman et al. 1997). Nomenclature and diagnostic criteria have been published for neoplastic lesions, providing some degree of standardization. For nonneoplastic lesions, nomenclature and diagnostic criteria are not well established. In addition, some pathologists might use a single diagnosis for a specific disease entity (e.g., nephropathy), whereas others might diagnose each of the components of the disease process. Some pathologists do not diagnose common spontaneous lesions, particularly if the lesions are minimal to mild in severity. All of these factors make it difficult to compare incidences of

spontaneous nonneoplastic lesions between laboratories and even between studies evaluated by different pathologists.

Because there is some standardization of neoplastic lesions, incidence data derived from other laboratories for specific neoplasms for untreated animals of the same strain and sex can be relevant. Data from different laboratories can be used to determine whether the in-house laboratory controls really do reflect the incidences of specific neoplasms in the overall population. Such controls are also useful when few in-house laboratory data are available. With this in mind, historical control data for neoplasms with an incidence of greater than 0.5% are presented for CD-1 mice (table 2.20). Historical control data for B6C3F1 mice includes tumors of incidences as low as 0.1%

The historical control data presented in table 2.21 and table 2.22 for B6C3Fl mice are based on data from the NTP (Anonymous 2000; Haseman et al. 1999). These data are derived from studies conducted at several contract laboratories. Table 2.22 reflects data from animals fed the NTP 2000 diet whereas table 2.21 reflects data from animals on the NIH07 diet. Although the data are collected from multiple laboratories, some variables have been minimized. The derivation and source of the animals as well as the diet used are controlled by the NTP. The data include only untreated controls. The data are based on all animals placed on study regardless of date of death. The NTP conducts an extensive review of the pathology portion of their studies for neoplastic lesions. Thus, the terminology for neoplastic lesions is generally comparable between studies and laboratories (Goodman et al. 1985).

The historical control data presented in table 2.20 for CD-1 mice have been obtained from Hazleton Washington, Vienna, Virginia (courtesy of S. Jones and S. Weymouth). All studies were conducted between 1984 and 1989 and were dietary studies lasting approximately 78 weeks. The data are based only on animals killed at terminal sacrifice and do not include animals dying on study.

As mentioned earlier, there are few published data on the incidences of spontaneous nonneoplastic lesions in mice. Some of the more common lesions observed in CD-1 and B6C3F1 mice as well as some of the more common lesions in other strains of mice are described in the text, although incidences are generally not given. With current husbandry practices, infectious diseases are uncommon in laboratory mice. Important infectious diseases are briefly described where applicable.

At necropsy, organ weights are usually taken for a number of organs. Historical control data on this information are sadly lacking in the literature. These data are included for B6C3F1 and CD-1 mice in this chapter. There can be problems in interpreting such data, and these are discussed in the section on organ weights.

Cardiovascular System

Vessels

Nonneoplastic Lesions

Vascular Ectasia (Angiectasis). Vascular ectasia can occur in a variety of organs and consists of dilatation of the capillaries in a focal area. Microscopically, angiectasis consists of dilated vascular spaces or sinusoids lined by flattened or slightly plump endothelial cells. This lesion is frequently seen in the ovary of aged mice. Vascular ectasia is also often seen in the mesenteric lymph node. The affected lymph nodes are usually enlarged and dark red. Microscopically, there are dilated vascular channels filled with red blood cells and lined by well-differentiated endothelium. The lining of the vascular channels is sometimes incomplete. The lesion is more prominent in the medulla but can affect the cortex and, occasionally, the perimesenteric fat as well. This lesion has been referred to as mesenteric disease (Dunn 1954). Vascular ectasia in any organ must be distinguished from vascular tumors (i.e., hemangioma or hemangiosarcoma). The absence of endothelial cell proliferation or nuclear atypia differentiates angiectasis from neoplastic vascular lesions (hemangioma and hemangiosarcoma).

Table 2.20 Incidences of Primary Tumors in Untreated Control CD-Mice[a] 18-Month Studies

	Male		Female	
	Number of Tumors (%)	Range (%)	Number of Tumors (%)	Range (%)
Circulatory system	(363)[a]		(391)[a]	
Hemangioma	5 (1.4)	0–8	5 (1.3)	0–3
Hemangiosarcoma	9 (2.5)	0–6	10 (2.6)	0–12
Digestive tract				
Liver	(362)[a]		(391)[a]	
Hepatocellular adenoma	35 (9.7)	0–16	5 (1.3)	0–5
Hepatocellular carcinoma	19 (5.2)	0–16	2 (0.5)	0–3
Endocrine system				
Adrenal	(362)[a]		(391)[a]	
Cortical adenoma	3 (0.8)	0–5	0 (0.0)	—
Pituitary	(362)[a]		(391)[a]	
Adenoma	0 (0.0)	—	3 (0.8)	0–3
Thyroid	(362)[a]		(391)[a]	
Follicular cell adenoma	2 (0.6)	0–4	0 (0.0)	—
Follicular cell carcinoma				
Hematopoietic system	(362)[a]		(391)[a]	
Mixed cell lymphoma	2 (0.6)	0–5	6 (1.5)	0–6
Histiocytic sarcoma (lymphoma)	0 (0.0)	—	4 (1.0)	0–2
Lymphocytic lymphoma	8 (2.2)	0–11	15 (3.8)	0–17
Reproductive system				
Testis	(362)[a]			
Interstitial cell tumor	3 (0.8)	0–5		
Ovary			(391)[a]	
Cystadenoma			3 (0.8)	0–5
Luteoma			4 (1.0)	0–3
Uterus			(391)[a]	
Endometrial stromal polyp			14 (3.6)	0–10
Endometrial stromal sarcoma			2 (0.5)	0–3
Leiomyoma			7 (1.8)	0–4
Leiomyosarcoma			5 (1.5)	0–5
Mammary gland	(362)[a]		(391)[a]	
Fibroadenoma	0 (0.0)	—	1 (0.3)	0–4
Adenocarcinoma	0 (0.0)	—	4 (1.0)	0–3
Respiratory system	(362)[a]		(391)[a]	
Alveolar bronchiolar adenoma	47 (13.0)	0–30	33 (8.4)	0–19
Alveolar bronchiolar carcinoma	10 (2.8)	0–8	8 (2.0)	0–8
Special sense organs				
Harderian glands	(335)[a]		(359)[a]	
Adenoma	7 (2.1)	0–6	3 (0.8)	0–5
Carcinoma	0 (0.0)	—	1 (0.3)	0–2

Note. Tumors with an incidence of 0.5% or greater in one or both sexes. Data includes only terminal sacrifice animals. Data supplied by Ms. S. Weymouth and Dr. S. Jones of Hazelton Washington, Vienna, Virgina (1990).

[a] Number of animals examined.

Table 2.21 Incidences of Tumors in Untreated Control B6C3F1 Mice Fed NTP 2000 Diet in 2-Year Studies

	Male		Female	
NTP diet 2000	**Number of Tumors (%)**	**Range (%)**	**Number of Tumors (%)**	**Range (%)**
Circulatory system	659[a]		659 [a]	
Hemangioma	5 (0.8)	0–4	8	0–5
Hemangiosarcoma	37 (5.6)	2–14	15	0–8
Digestive tract				
Liver	659 [a]		655 [a]	
Hepatocellular adenoma	195 (29.6)	12–46	101 (15.4)	8–28
Hepatocellular carcinoma	145 (22)	13–46	49 (7.5)	3–16
Forestomach	659[a]		659[a]	
Squamous cell papilloma	11 (1.7)	0–6	10 (1.5)	0–6
Small intestine	659[a]		659[a]	
Adenoma	3 (0.5)	0–4	1 (0.2)	0–2
Carcinoma	15 (2.3)	0–10	1 (0.2)	0–2
Endocrine system				
Adrenal cortex	655[a]		649[a]	
Cortical adenoma	24 (3.7)	0–10	1 (0.2)	0–2
Adrenal medulla	653[a]		644[a]	
Pheochromocytoma benign, complex, malignant, not otherwise specified	3 (0.5)	0–2	6 (0.9)	0–2
Pituitary site unspecified	622[a]		623[a]	
Adenoma	3 (0.5)	0–2	59 (9.5)	0–20
Carcinoma	1 (0.2)	0–2	0	0
Thyroid	651[a]		646[a]	
Adenoma	1 (0.2)	0–1	2 (0.3)	0–2
Carcinoma	1 (0.2)	0–2	0	0
Hematopoietic system	659[a]		659[a]	
Lymphoma/leukemia	33 (5)	2–8	98 (14.9)	6–32
Integumentary system	659[a]		659[a]	
Epithelial neoplasms	2 (0.3)	0–2	1 (0.2)	0–1
Fibroma, sarcoma, or fibrous histiocytoma	1 (0.2)	0–1	21 (3.2)	0–6
Musculoskeletal system	659[a]		659[a]	
Rhabdomyosarcoma	0	0	3 (0.5)	0–3
Osteosarcoma	0	0	2 (0.3)	0–2
Reproductive system				
Ovary			626[a]	
Cystadenoma			24 (3.8)	0–16
Granulose cell tumor			5 (0.8)	0–3
Luteoma			4 (0.6)	0–3
Uterus			659[a]	
Endometrial stromal polyp			11 (1.7)	0–6
Mammary gland	659[a]		659[a]	
Fibroma, fibroadenoma, carcinoma, or adenoma	0	0	2 (0.3)	0–2
Respiratory system	659[a]		654[a]	
Alveolar/bronchiolar adenoma	119 (18.1)	4–26	37 (5.7)	0–12
Alveolar/bronchiolar carcinoma	68 (10.3)	8–24	17 (2.6)	0–6
Harderian glands	659[a]		659[a]	
Adenoma	53 (8)	2–16	41 (6.2)	0–20
Carcinoma	4 (0.6)	0–4	9 (1.4)	0–4

[a] Number of tissues examined.

Table 2.22 Incidences of Tumors in Untreated Control B6C3F1 Mice Fed NIH07 Diet in 2-Year Studies

	Male		Female	
NIH07 diet	**Number of Tumors (%)**	**Range (%)**	**Number of Tumors (%)**	**Range (%)**
Circulatory system	1,355[a]		1,353[a]	
Hemangioma	7 (0.5)	0–4	22 (1.6)	0–8
Hemangiosarcoma	73 (5.4)	0–12	37 (2.7)	0–8
Digestive tract				
Liver	1,350[a]		1,350[a]	
Hepatocellular adenoma	397 (29.4)	4–60	234 (17.3)	2–50
Hepatocellular carcinoma	241 (17.9)	6–29	113 (8.4)	0–20
Forestomach	1,355[a]		1,353[a]	
Squamous cell papilloma	18 (1.3)	0–6	20 (1.5)	0–6
Small intestine	1,355[a]		1,353[a]	
Adenoma	5 (0.4)	0–4	2 (0.1)	0–2
Carcinoma	12 (0.9)	0–6	8 (0.6)	0–6
Endocrine system				
Adrenal cortex	1,335[a]		1,347[a]	
Cortical adenoma	46 (3.4)	0–22	9 (0.7)	0–6
Adrenal medulla	1,330[a]		1,330[a]	
Phaeochromocytoma	11 (0.8)	0–5	14 (1.1)	0–4
Pituitary (pars distalis)	1,265[a]		1,290[a]	
Adenoma	5 (0.4)	0–6	185 (14.3)	0–36
Carcinoma	0	0	6 (0.5)	0–4
Thyroid	1,343[a]		1,340[a]	
Follicular cell adenoma	20 (1.5)	0–4	24 (1.8)	0–8
Follicular cell carcinoma	6 (0.4)	0–2	2 (0.1)	0–2
Hematopoietic system	**1,355**[a]		**1,353**[a]	
Lymphoma/leukemia	113 (8.3)	2–20	284 (21)	0–42
Integumentary system	1,355[a]		1,353[a]	
Epithelial neoplasms	6 (0.5)	0–2	2 (0.2)	0–2
Fibroma, fibrosarcoma, sarcoma, myxoma, myxosarcoma, or fibrous histiocytoma	83 (6.1)	0–24	33 (2.4)	0–8
Musculoskeletal system	1,355[a]		1,353[a]	
Osteosarcoma	1 (0.1)	0–2	8 (0.6)	0–4
Reproductive system				
Ovary			1,323[a]	
Adenoma			7 (0.5)	0–4
Cystadenoma			19 (1.4)	0–4
Granulose cell tumor			10 (0.8)	0–4
Luteoma			3 (0.2)	0–2
Teratoma			6 (0.5)	0–2
Uterus			1,353[a]	
Endometrial stromal polyp			44 (3.3)	0–16
Mammary gland	1,355[a]		1,353[a]	
Carcinoma or adenoma	0	0	9 (0.6)	0–2
Respiratory system	1,354[a]		1,352[a]	
Alveolar bronchiolar adenoma	217 (16)	4–30	80 (5.9)	0–24
Alveolar bronchiolar carcinoma	69 (5.1)	0–14	32 (2.4)	0–8
Harderian glands	1,355[a]		1,353[a]	
Adenoma	64 (4.7)	0–18	45 (3.3)	0–10
Carcinoma	9 (0.7)	0–4	9 (0.7)	0–4

[a] Number of tissues examined.

Polyarteritis. Polyarteritis in the mouse has been compared to periarteritis nodosa in man. The etiology of both is unknown, but an immune origin is suspected. In mice, the lesion involves small muscular arteries and is usually evident in multiple sites. Organs commonly involved include the heart, tongue, uterus, testis, kidney, and urinary bladder. The media of the affected vessels is homogeneous and intensely eosinophilic with hematoxylin and eosin (H&E) stain. Both fibrosis and an infiltration of mononuclear cells occur around the affected vessels. This disease is uncommon in the B6C3F1 and CD-1 strains, but it has been reported in BALB/c mice (Frith and Ward 1988).

Neoplastic Lesions

General. Tumors of endothelial cells (hemangioma, hemangiosarcoma) can be found at any site in the body. The most common sites are the spleen and liver. The subcutis, skeletal muscle, and female reproductive tract are also other common sites (Strandberg and Goodman 1982). Lymphangiomas and lymphangiosarcomas, tumors of lymphatics, are rare.

Hemangioma. Hemangiomas are benign tumors arising from endothelial cells. Microscopically, they consist of dilated vascular spaces or thin-walled capillaries lined by a single layer of endothelial cells. The endothelial cells are well differentiated and usually slightly plump. Mitotic figures are scant. The connective tissue stroma is often delicate but can consist of dense collagenous tissue, particularly around the dilated spaces.

Hemangiosarcoma. Hemangiosarcomas are malignant neoplasms arising from endothelial cells. These neoplasms may be pleomorphic. They consist of vascular spaces of varying sizes that might or might not be filled with red blood cells. The cells lining the vascular spaces are plump with oval basophilic nuclei and indistinct cell borders. There is often piling up of the lining cells. In areas of the tumor, there might be solid sheets of cells. Particularly in the spleen, the tumors can be predominately solid. Necrosis, hemorrhage, and thrombosis are frequent. Hemangiosarcomas can metastasize, although it is usually difficult to determine the primary site. Often, there might be multiple primary sites. This is one of the reasons that the incidence of vascular tumors is calculated using the number of animals with vascular tumors regardless of site.

Heart

Nonneoplastic

General. Spontaneous nonneoplastic lesions of the heart are relatively uncommon in CD-1 and B6C3F1 mice.

Cardiomyopathy. This is a spontaneous, age-related degeneration frequently evident in the myocardium (Elwell and Mahler 1999). Early in this process there is myocyte degeneration or necrosis. The inflammatory component of this lesion varies and this is not solely a consequence of the age of the lesion. There is frequently an increase in the interstitial fibrous connective tissue of the myocardium. Cardiomyopathy is most common in the ventricles. Cardiomyopathy can be induced by chemicals such as methyl bromide and diethanolamine (Melnick et al. 1994).

Epicardial and Myocardial Mineralization. Mineralization of the myocardium and epicardium occurs in some strains of mice as early as 1 month of age, and is more common in males than in females; it occurs most often on the right ventricle. Microscopically, the mineralized foci are characterized by distinctly basophilic areas after H&E staining, by black areas when stained with von Kossa, and red areas when stained with Alizarin Red. The mineralization can be minimal or quite extensive, covering most of the epicardial surface of the right ventricle. The mineralized areas

might be surrounded by fibrosis, but an inflammatory component is minimal. The exact etiology is unknown, but the lesion appears to have a genetic predisposition (Brownstein. 1983; DiPaola et al. 1964).

Mineralization of the myocardium is also a common lesion in a number of strains of mice (Rings and Wagner 1972). The mineralized areas are focal and can involve the myocardium of both ventricles and the interventricular septum. The atria are usually spared. The lesion appears to be a distinct entity from the epicardial mineralization. Microscopically, the mineralized areas stain distinctly basophilic with H&E. The focal areas of mineralization are often surrounded by fibrous connective tissue and a minimal infiltrate of mononuclear inflammatory cells is present.

Atrial Thrombosis. Atrial thrombosis is not a common lesion, but occurs as both a spontaneous and an induced lesion in mice (Schieferstein et al. 1985). The thrombus more commonly involves the left atrium. Grossly, the involved atrium is enlarged and red. Microscopically, the distended atrium contains an organizing mural thrombus. The degree of organization depends on the age of the thrombus. Some of the thrombi might contain focal areas of cartilaginous metaplasia. If the thrombi are large, they might lead to a secondary chronic passive congestion of the lungs.

Dietary factors are known to influence atrial thrombosis. A high-fat and low-protein diet and copper deficiency have increased the incidence of atrial thrombosis. There is a high incidence of atrial thrombosis in the BALB/c strain of mice. Chemicals such as doxorubicin that induce myocardial damage can also induce atrial thrombosis (Fujihira et al. 1993).

Proliferative Lesions

Hyperplastic Lesions: Heart. Proliferation of the capillary endothelium has occurred in females in association with mammary gland adenocarcinomas (Iwata et al. 1994). This endothelial proliferation also occurs as a precursor to chemically induced hemangiosarcomas that are induced by 1,3 butadiene (Melnick et al. 1990).

Neoplasia: Heart. Primary tumors of the heart are extremely rare in mice (Strandberg and Goodman 1982). Neoplasms that have been found in association with ionizing irradiation are mesenchymoma and mesenchymal cell sarcoma (Hoch-Ligeti and Stewart 1984). Systemic, metastatic, or locally invasive neoplasms that can be found in the heart are lymphoma, histiocytic sarcoma, and pulmonary alveolar/bronchiolar carcinomas.

Digestive System

Salivary Glands

General

The mouse as well as many other species has three paired salivary glands: submaxillary (submandibular), parotid, and sublingual (Frith and Townsend 1985). All three major salivary glands are closely associated and located in the subcutaneous tissue of the ventral neck area.

Sexual Dimorphism

The submandibular or submaxillary salivary gland is distinctly different morphologically in adult male and female mice. Immature mice of both sexes present a pattern similar to that in adult female mice, suggesting that the difference in adult males is due to the production of testosterone. Castration of males before puberty results in loss of the male characteristics of the gland (Botts et al. 1999). In the female mouse, the acini are small and epithelial cells have centrally located nuclei

and only a few cytoplasmic granules. In the adult male, the acini are much larger and the tall columnar lining epithelial cells have basally located nuclei and abundant eosinophilic cytoplasmic granules. This sexual dimorphism is not present in the rat.

Nonneoplastic Lesions

Lobular Hyperplasia and Atrophy. Lobular hyperplasia and atrophy are seen in both the submaxillary and parotid salivary glands, but are rare in the sublingual. The lesion commonly involves a single lobule in which some acini are atrophied and replaced by hyperplastic ducts.

Basophilic Foci. Chiu and Chen (1986) described the occurrence of basophilic hypertrophic foci in the parotid glands in both Sprague-Dawley rats and Swiss mice. They were characterized by focal hypertrophy and hyperchromasia of acinar cells. The incidence in the Swiss mice was 4.8%. The lesions were considered to be spontaneous, and their morphology and growth pattern suggested that they were not degenerative, necrotic, hyperplastic, preneoplastic, or neoplastic. The investigators considered them to be a distinct pathological entity of an unknown nature.

Salivary Mucocoele, Duct Dilatation, or Cyst. These might be seen occasionally and are likely to be the consequence of duct obstruction by calculi or foreign bodies. Inflammatory cell infiltration occurs frequently in B6C3F1 mice. This consists typically of lymphocytes with occasional plasma cells. Suppurative inflammation occurs infrequently.

Neoplastic Lesions

Spontaneous neoplasms of the major salivary glands of mice occur relatively infrequently (Frith and Townsend 1985). Because mammary tumors often arise in the neck area immediately adjacent to the salivary glands, they have sometimes been mistakenly identified as salivary gland tumors. Careful dissection of a tumor and the contiguous normal tissues in the neck and head region, followed by histological demonstration of actual involvement of a salivary gland by the tumor is, therefore, essential if one is to have reasonably sound evidence of salivary gland origin. Any spontaneous tumor in the neck region of a female mouse of strain BALB/c, A, C58, CF-1, or any hybrid of the C and A strains is most likely to be a salivary gland tumor of myoepithelial type.

Spontaneous neoplasms of the salivary gland are extremely rare in all strains of mice. The NTP Carcinogenesis Bioassay Data System reported only 30 epithelial tumors from 40 chronic studies using the B6C3F1 mouse, representing approximately 8,000 animals.

Myoepithelioma. Myoepithelioma of the salivary glands is rare in mice except in certain strains (Delaney 1977; Peters et al. 1972). It is extremely rare in the B6C3F1 and the CD-1 strains. The neoplasm can occur at a low incidence in the BALB/c mouse and is more common in the female. It develops most frequently in the submaxillary or parotid salivary glands and is rare in the sublingual gland. Myoepitheliomas have also been reported in the human parotid gland (Leifer et al. 1974). The myoepithelioma is believed to be derived from the myoepithelial cells in the salivary glands. The tumors become quite large and grossly are often cystic. Microscopically, they are composed of large pleomorphic cells, suggesting both an epithelial and a mesenchymal origin. The larger tumors commonly metastasize to the lung.

Other Tumors. Spontaneous tumors of the mouse salivary glands other than myoepitheliomas are extremely rare. Mouse polyoma virus causes salivary gland tumors, including a mesenchymal, an epithelial, and a mixed epithelial/mesenchymal type (Dawe 1979). Polyoma virus infection does not occur as a spontaneous disease.

Adenomas can arise from the serous or mucous acinar cells. The absence of ducts is a feature that distinguishes adenomas from hyperplasia (Betton et al. 2001). Adenocarcinomas can have solid, papillary, or mixed patterns of growth with local invasion and possibly metastases in the lung.

Pancreas

Nonneoplastic Lesions

Acinar Atrophy and Fatty Replacement. Atrophy of the acinar pancreas with fatty replacement occurs at a low incidence in mice and can be lobular or diffuse. The existing islets appear normal and are embedded in a stroma of adipose tissue. Occasionally, the adipose tissue contains a chronic inflammatory cell infiltrate. The occurrence of the lesion only in aged mice suggests that it is a true atrophy and not hypoplasia. Atrophy occurs at a rate of 1% to 2% in 2-year studies in B6C3F1 mice (Boorman and Sills 1999).

Cysts. These occur rarely and are usually solitary. They are lined by cuboidal to flattened epithelium. Foci of cellular alteration are found occasionally in mouse 2-year studies. They are composed of adjacent acini that vary in staining intensity from the surrounding parenchyma.

Neoplastic Lesions

Acinar Cell Tumors. Acinar cell tumors have been described in the rat (Boorman and Eustis 1985), but they are extremely rare in the mouse (Cavaliere et al. 1981; Prejean et al. 1973).

Acinar cell hyperplasia is differentiated from acinar cell adenoma by the lack of compression of the adjacent tissue (Deschl et al. 2001). *Acinar cell carcinoma* is differentiated from acinar cell adenoma by cellular pleomorphism and anaplasia and local invasion or distant meatastases.

Esophagus

Nonneoplastic

Esophageal Rupture. Rupture of the esophagus is seen in mice as a result of faulty oral intubation and gavage. If the animal survives, an associated inflammatory reaction is present. The lesion is usually fatal.

Neoplastic Lesions

Papilloma and Squamous Cell Carcinoma. Papillomas and squamous cell carcinomas have been reported in the rat (Cardesa and Ovelar 1985; Ovelar and Cardesa 1985), but appear to be rare in the mouse. The experimental production of papillomas has been reported in the mouse (Horie et al. 1965).

Stomach

Normal Anatomy

The stomach of both the mouse and the rat is divided into a glandular stomach and a nonglandular forestomach. The two regions are separated by a ridge around the entrance of the esophagus. The ridge is formed by the thickened lamina propria of the nonglandular stomach. The nonglandular stomach is lined by stratified squamous epithelium, and the glandular stomach is lined by glandular epithelium. The glandular region contains three types of glands: cardiac, pyloric, and fundic.

Nonneoplastic Lesions

Glandular Hyperplasia. Hyperplasia of the glandular gastric mucosa can occasionally be seen in mice. The lesion can be either focal or diffuse in nature.

Squamous Cell Hyperplasia of the Forestomach. The forestomach (nonglandular stomach) of the mouse is lined by stratified squamous epithelium that might occasionally become hyperplastic. This lesion could result from the oral administration of toxic irritants. Erosions and ulcers of the forestomach can occur in association with treatment and are more common in mice treated by gavage, inhalation, or with irritating agents via dosed food and water than in control groups (Leininger et al. 1999).

Cysts. These occur in the esophagus and forestomach and consist of squamous epithelial lining and multiple layers of keratin filling the lumen. Cystic gastric glands, with calcium deposits and inflammation, are a frequent finding in mice in the cardia.

Neoplastic Lesions

Adenoma and Adenocarcinoma of the Glandular Stomach. Adenoma and adenocarcinoma of the gastric mucosa are rare in mice. Adenomas are usually well circumscribed and delineated from the normal mucosa. The cells within the adenomas are well differentiated. Gastric adenocarcinomas are invasive and infiltrate into the lamina propria and muscularis.

Neuroendocrine neoplasms (carcinoids or enterochromaffin cell-like tumor) can be induced in the mouse and also have occurred spontaneously in B6C3F1 mice in NTP studies.

Squamous Cell Papilloma and Carcinoma of the Forestomach. Hyperplasia and neoplasia of the squamous epithelium are part of a continuum that ranges from focal hyperplasia to papilloma to localized squamous cell carcinoma to invasive squamous cell carcinoma. Papillomas are composed of a stalk with a vascular connective tissue core covered by neoplastic squamous epithelium. Squamous cell carcinomas are usually relatively well differentiated and produce keratin. They are locally invasive and might occasionally metastasize to the lungs.

Intestine

Noneoplastic Lesions

Epidermal Inclusion Cysts. Epidermal inclusion cysts are an infrequent finding in the B6C3F1 mouse and are present in the muscularis of the colon and rectum (Shackelford and Elwell 1999). *Diverticuli* of the caecum and colon are infrequently detected and must be differentiated from adenocarcinomas. *Fat necrosis* of mesentery is thought to be associated with torsion.

Intussusception and Rectal Prolapse. Intussusception can occur in both the large and small intestines and can lead to intestinal obstruction, inflammation, necrosis, and death. Prolapse of the rectum can be associated with a pinworm infestion or Citrobacter infection (Barthold 1978); it is characterized by an eversion of the mucosal surface of the rectum through the anus.

Pinworms. Pinworms in mice commonly involve two species, *Syphacia obvelata* and *Apicularis tetraptera*. They are usually found in the colon and sections of the parasite can be seen microscopically in the colonic lumen of infected mice. Pinworms are not usually associated with any pathological changes in the large intestine, but occasionally intussusception or rectal prolapse can occur.

Inflammatory Lesions. The current high standard of husbandry practices for laboratory mice means that infectious diseases are extremely rarely encountered in toxicology studies. Citrobacter freundii causes a disease entity known as colonic hyperplasia or transmissible murine colonic hyperplasia. Although this infection is rare it might complicate carcinogenicity studies as the transitory hyperplasia of the colonic mucous membrane has been found to increase the incidence of tumors and decrease the dose of carcinogen required for tumor induction. Salmonella infection of the small and large intestine is characterized by multifocal necrosis and thrombosis. Tyzzers disease (*Bacillus piliformis*) produces necrotizing inflammatory lesions especially in the terminal ileum and cecum.

Several viruses cause intestinal lesions in mice: mouse hepatitis virus, EDIM (a group A rotavirus), Reovirus, and mouse adenovirus infections.

Neoplastic Lesions

Adenoma and Adenocarcinoma. Adenomas of the small intestine are frequently small and might not be detected if the intestine is unopened during gross examination. They are especially common in the duodenum.

Microscopically, the adenoma appears as a polypoid epithelial growth projecting into the lumen of the intestine. The epithelium is relatively well differentiated, but usually appears more basophilic than adjacent normal epithelium. Benign neoplasms can develop dysplastic foci and transform into malignant tumors.

Adenocarcinomas are composed of more anaplastic or pleomorphic cells, which can project into the lumen as well as infiltrate into and beyond the submucosa and tunica muscularis. Adenocarcinomas frequently are cystic or papillary and microscopically show many mitotic figures.

Leiomyoma and Leiomyosarcoma. Smooth muscle tumors of the small intestine are relatively uncommon. Leiomyomas are usually well-circumscribed lesions composed of well-differentiated smooth muscle cells. Leiomyosarcomas are not well circumscribed and neoplastic smooth muscle cells can infiltrate the submucosa and serosa.

Other neoplasms that occur in the intestines are schwannoma, fibroma, fibrosarcoma, hemangioma, hemangiosarcoma, and lymphomas.

Endocrine System

Adrenal Gland

Nonneoplastic Lesions

Subcapsular Cell Hyperplasia. Many strains of mice develop proliferations of spindle cells in the subcapsular region of the adrenal cortex (Dunn 1970; Goodman 1983). The cell of origin is the glomerulosa cortical cell.

The proliferating cells can extend downward into and through the zona glomerulosa and zona fasciculata. The cells are fusiform or oval with spindle-shaped nuclei and scant basophilic cytoplasm. The spindle cells have been referred to as type A cells (Dunn 1970; Goodman 1983) and are the most common type seen. Type B cells are occasionally found. These are larger polygonal cells with abundant eosinophilic cytoplasm that is usually lipid-laden and round vesicular nuclei. Large hyperplastic foci are composed of type A and type B cells arranged in spherical nests or glandular structures. In the advanced stage the edge of this lesion reaches the corticomedullary junction (Nyska and Maronpot 1999).

This lesion is rare in young mice but has been reported to occur from 4 months old in both sexes. It has been reported to increase in incidence and severity with age and to be more common

in aging females than males. Incidence might also increase in association with stressful housing conditions (Chvedoff et al. 1980).

The pathogenesis of subcapsular hyperplasia is thought to be related to hormonal alterations because gonadectomy or gonadal atrophy often enhances development in both sexes (Yoshida et al. 1986). Subcapsular hyperplasia can, but does not inevitably, progress to adenoma (Dunn 1970).

Differential diagnosis from subcapsular adenoma depends on the extent of the lesion; hyperplastic lesions can be focal, multifocal, or circumferential. They do not extend into or beyond the capsule, and lesions are not larger than the normal width of the cortex in a young mouse. There is a lack of distinct compression; hyperplastic lesions might slightly bulge above the capsular surface and can cause minimal compression of the subjacent cortex (Capen et al. 2001).

Cortical Hyperplasia and Hypertrophy. Small focal lesions are occasionally found involving primarily the zona fasciculata of the adrenal cortex. There might be increased numbers of cells per unit area within the focus compared to the adjacent cortex (hyperplasia). These cells are smaller than normal cortical cells. Large foci of cortical hyperplasia are well circumscribed, blend with surrounding normal parenchyma and cause no compression of adjacent cortex (Hamlin and Banas 1990). It is not certain whether these represent true hyperplasia or an altered functional state resulting in a reduced cytoplasmic volume. Cortical hyperplasia is much less common than subcapsular cell hyperplasia (Nyska and Maronpot 1999).

In other foci, the cells might be enlarged with abundant cytoplasm that is eosinophilic or clear. Some foci contain both types of cells. They are usually single, focal or less commonly diffuse lesions, which occur in the zona fasciculata and can extend into the zona glomerulosa.

Adrenal cortical hypertrophy is considered to reflect a functional change, maybe associated with stress and not a preneoplastic lesion (Nyska and Maronpot 1999). Compression is minimal or nonexistent. These lesions must be distinguished from cortical adenomas, which cause distinct compression of adjacent tissue and have loss of normal architecture (Capen et al. 2001).

Medullary Hyperplasia. Single or, more rarely, multiple foci of small to normal-size pheochromocytes with basophilic cytoplasm are occasionally found in the adrenal medulla. Foci of hyperplasia can occupy up to 50% of the normal size of the medulla. Medullary hyperplasia is considered to be part of the neoplastic continuum for adrenal medullary neoplasms (Nyska and Maronpot 1999).

There is little if any compression of surrounding tissue and the edges of the focus blend in with adjacent medullary parenchyma.

The main differential diagnosis is phaeochromocytoma, which causes distinct compression at the periphery or loss of normal architecture (Capen et al. 2001). Hyperplasia and phaeochromocytoma are the most common chemically induced adrenal lesions in the NTP in B6C3F1 mice.

Lipofuscin. Pigmented macrophages are commonly observed at the corticomedullary junction in aged mice of many strains, particularly females, and the incidence increases with age. This lesion has also been referred to as ceroid deposition, or brown degeneration (Dunn 1970; Frith 1983c). At first, the pigment appears as yellow-brown, faintly granular material in the cortical cells adjacent to the medulla. As the lesion increases in severity, the cells become enlarged with abundant foamy brown to yellow cytoplasm and small pyknotic nuclei. Occasional multinucleate giant cells are found. The pigment is periodic acid Schiff (PAS) positive and acid fast.

Other Nonneoplastic Lesions. Amyloidosis can be seen in the adrenal cortex, usually in the zona fasciculata (Sass 1983b). Amyloidosis is discussed under the section on multisystem diseases. Hematopoiesis within the adrenal cortex is seen as part of a generalized response to hematopoietic stimuli in the animal. Accessory adrenal cortical tissue is observed sporadically (Sass 1983a).

Single or multiple spherical to ovoid nodules are located at one adrenal pole, within the cortex (subcapsular), or in the surrounding fat (Yarrington 1996). Accessory nodules are subject to the

same degenerative changes as the cortex. They are best differentiated from cortical adenoma by lack of a common capsule, lack of capsular invasion, and normal cellular architecture (Nyska and Maronpot 1999).

Cortical Vacuolation. Cells in the zona glomerulosa and zona fasciculata are characterized by variable cytoplasmic vacuolation, associated with their function of steroidogenesis from cholesterol. Any toxin causing disruption of the cortex or enhancement of biosynthesis (via disturbance of the pituitary/hypothalamic hormonal regulation pathways) will therefore result in the accumulation of lipid-containing vacuoles (Dunn 1970; Krinke et al. 2001).

In females, the normal regression of the x-zone (located between the cortex and medulla) appears as lipid vacuolization, and should not be confused with a pathological process (Nyska and Maronpot 1999).

Neoplastic Lesions

Subcapsular Cell Adenoma. These are benign tumors of old mice, composed of spindle-shaped (type A) cells comparable to those seen in subcapsular cell hyperplasia and causing compression of the adjacent adrenal cortex. Large polygonal type B cells are often found interspersed among the spindle cells, either singly or in nests. These tumors might be hormonally active, showing oestrogenic (B cells), androgenic, or adrenocortical hormonal effects (Dunn 1970).

Subcapsular Carcinoma. These malignant tumors tend to invade the capsule, show cellular pleomorphism and frequent mitotic figures. Metastasis to the lungs is most common (Frith and Dunn 1994).

Cortical Adenoma. Cortical B-cell adenomas consist of nodules of well-differentiated cortical cells that are demarcated from and compress the adjacent parenchyma (Frith 1983a). There is distortion and loss of the normal cord arrangement. The cells are polygonal with abundant eosinophilic or amphophilic cytoplasm and round vesicular nuclei. There might be karyomegaly and cellular atypia. The presence of mitoses is variable. The absence of invasion or distant metastases differentiates this lesion from cortical carcinoma.

Pheochromocytoma. Pheochromocytomas are tumors of the chromaffin (secretory) cells. These tumors tend to be unilateral in mice. Component cells are polyhedral with amphophilic or basophilic cytoplasm and basally located nuclei, or small and basophilic with little cytoplasm. The cells are arranged in trabeculae or nests separated by dilated vascular spaces. In the benign form, there is compression of adjacent tissue and loss of normal architecture. Cellular atypia and plentiful mitoses might be present. The size of the primary mass is reported to correlate best with the metastatic capability (Frith and Ward 1988).

Malignant pheochromocytoma is diagnosed when there is evidence of capsular invasion or distant metastases. Subendothelial growth of tumor cells is common in adrenal medullary tumors and is not indicative of vascular invasion and malignancy. Similarly, small groups of medullary cells within the cortex, particularly in the region of the hilus are a normal feature and should not be misinterpreted as evidence of malignancy.

Pheochromocytomas demonstrate a positive chromaffin reaction, staining with chromogranin A and tyrosine hydroxylase (Capen et al. 2001).

Ganglioneuroma is an infrequent tumor of the adrenal medulla, resembling nervous tissue containing large well-differentiated ganglion cells and neurofibrils. Diagnosis of malignant ganglioneuroma is based on unequivocal evidence of invasion. These tumors stain positively with antibodies against synaptophsin and neuron-specific enolase (Capen et al. 2001).

Pituitary Gland

Nonneoplastic Lesions

Pituitary Cysts and Cystic Degeneration. True pituitary cysts, lined by epithelium, have been described in mice (Carlton and Gries 1983). These cysts can be single or multiple, are usually microscopic and lined by ciliated cuboidal to columnar or infrequently by squamous epithelium and contain eosinophilic colloid-like material. Those cysts close to or connected to the cleft between the pars distalis and intermedia are considered likely to be cystic remnants of Rathke's pouch.

Cystic or cystoid degeneration (Cameron and Sheldon 1983) of the pars distalis is characterized by large spaces lacking a cyst wall but lined by viable pars distalis cells. Cystic spaces usually contain faintly granular eosinophilic material and occasionally cell debris. This lesion is seen as a spontaneous aging change or can be induced by estrogens. In addition it is often seen as a component of proliferative changes in the pars distalis.

Proliferative changes of the pituitary gland (most commonly the pars distalis) are the most frequently observed spontaneous and induced lesions of the pituitary gland in mice (Frith and Ward 1988). The incidence of spontaneous proliferative lesions increases with age. There is a biological and morphological continuum between hyperplasia, adenoma, and carcinoma of the pituitary gland.

Hyperplasia of the pars distalis can be focal multifocal or, rarely, diffuse. Focal hyperplasia is more common and more easily recognized. The cells comprising the focus are of a single cell type and can be any of the cell types found in the pars distalis. Most frequently, they resemble chromophobes; that is, large, pale cells with round nuclei. The borders of the foci blend into the adjacent parenchyma with little if any compression. Foci close to the periphery might cause slight elevation of the surface. Larger foci can contain areas of cystic degeneration or angiectasis. Differentiation from adenoma depends on the absence of compression and cellular atypia.

Neoplastic Lesions

Adenoma of the Pars Distalis. Adenomas of the pars distalis are common in female B6C3F1 and CD-1 mice and are infrequent in males. Adenoma of the pars distalis are solitary, sharply demarcated, expansile lesions causing distinct compression of the adjacent parenchyma. As with hyperplasia, they usually contain a single cell type. The cells are arranged in solid sheets or in cords and large adenomas often contain areas of cystic degeneration or angiectasis. Cellular atypia might be present.

Carcinoma of the pars distalis is rare. Microscopic criteria for malignancy include vascular invasion, local invasion of the brain, or invasion of adjacent bone. Metastases are rare and when they do occur they are usually intracranial.

Other Neoplasms. Occasional neoplasms of the pars intermedia have been reported (Goodman et al. 1981).

Thyroid Gland

Nonneoplastic Lesions

Cystic Follicles. Individual follicles can become enlarged and distended with colloid. These cystic follicles are lined by a single layer of cuboidal or flattened epithelium. Large follicles are normally found at the periphery of the thyroid.

Follicular Cell Hyperplasia. The lesion can be focal, multifocal, or diffuse. The affected follicles are of variable sizes. The epithelium is simple cuboidal to tall columnar and might form papillary

projections into the lumen or form multiple small follicles within an enlarged follicle (cystic hyperlasia). Endocytosis of colloid might proceed at a greater rate than synthesis, resulting in progressive depletion of colloid. The lack of compression of adjacent parenchyma helps to differentiate hyperplasia from follicular cell adenoma (Capen et al. 2001).

A number of goitrogenic compounds including thiourea, thiouracil, sulpha drugs, and aromatic amines exert their effect by inhibition of peroxidase-mediated incorporation of iodine into thyroglobulin. The resulting feedback mechanism causes increased secretion of TSH and follicular hyperplasia. Withdrawal of these compounds could result in regression of the proliferative lesions; however, excessive TSH stimulation has also resulted in progression of hyperplasia to neoplasia.

Other chemicals, such as phenobarbitol and chlorinated hydrocarbon insecticides, act indirectly via the thyroid-pituitary axis. The initial change is induction of hepatic cytochrome P450 enzymes. The resulting increased metabolism of thyroid hormone causes an increased rate of clearance of circulating T3 and T4, stimulating TSH secretion by the pituitary gland and thyroid gland hyperplasia (Nyska and Maronpot 1999).

Neoplastic Lesions

Follicular Cell Adenoma. Adenomas are discrete, well-circumscribed lesions that often cause compression of the adjacent parenchyma. A capsule might or might not be present. These tumors can have papillary, follicular, or solid patterns (Heath and Frith 1983). The cells are hyperchromatic, variable in size, and often have a high nuclear to cytoplasmic ratio. The cells are often multilayered, and nuclear crowding is common. The mitotic rate is usually low.

Follicular Cell Carcinoma. Carcinomas may have a papillary, solid, or follicular pattern or combinations thereof (Heath and Frith 1983). The lesion is often at least partially encapsulated with a dense scirrhous reaction and neoplastic cells or follicles within the capsule. The cells are pleomorphic with a high nuclear to cytoplasmic ratio. The mitotic rates are variable but often high. Invasion of adjacent structures is common.

Other Neoplasms. C-cell adenomas and carcinomas are extremely uncommon in mice (Frith and Heath 1983a; Russfield 1982; Squire et al. 1978; Van Zwieten et al. 1983).

Parathyroid

Spontaneous lesions, both neoplastic and nonneoplastic, are uncommon in mice (Russfield 1982; Squire et al. 1978). Diffuse, bilateral hyperplasia can occur in response to renal disease (renal secondary hyperparathyroidism). Focal hyperplasia is usually unilateral and is not associated with increased parathyroid hormone (PTH) secretion (Capen et al. 2001).

Pancreatic Islets

Nonneoplastic Lesions

Islet Cell Hyperplasia. Certain mouse strains have high incidences of this lesion (Sass et al. 1978). Hyperplasia of the pancreatic islets usually involves more than one islet (multifocal), and can involve all islets visible in a histologic section. The islets are much enlarged owing to an increased number of cells, which are morphologically similar to those in smaller normal islets. There is no encapsulation or compression of adjacent acinar tissue, and islet architecture is generally maintained. All of these features help differentiate this lesion from islet cell adenoma. The specific cell type involved is difficult to identify at the light microscopic level, and histochemistry and

electron microscopy are often needed. Immunoperoxidase staining of the hyperplastic islets has revealed that most of the cells contain insulin and some contain somatostatin.

Neoplastic Lesions

Islet Cell Adenoma. Islet cell adenomas in mice commonly involve a single islet within a histological section (Frith and Sheldon 1983), are larger than hyperplastic islets, and compress adjacent normal pancreas. The cells form ribbons along sinusoidal, thin-walled vessels, and the adenomas often appear more vascular than hyperplastic islets. The cells stain lightly eosinophilic with hematoxylin and eosin; the nuclei demonstrate a delicate chromatin pattern. The cells are well differentiated, and mitotic figures are few in number.

Islet Cell Carcinoma. Islet cell carcinomas are invariably larger than adenomas and are commonly visible grossly. The cells vary from well-differentiated cells to extremely pleomorphic and anaplastic cells (Frith and Sheldon 1983). Well-differentiated islet cell carcinomas usually invade locally and occasionally metastasize. Most tumors are probably insulinomas, but do not cause hypoglycemia. The cytoplasm of the neoplastic cells is eosinophilic and the nuclei are vesicular. Nucleoli are prominent and can be multiple. Mitotic figures are evident and pleomorphism could be prominent. Some anaplastic carcinomas might be difficult to classify with certainty as islet cell in origin.

Female Genital System

Ovary

Nonneoplastic Lesions

Ovarian Cysts. Cysts of the ovary and paraovarian tissues are extremely common in aged females of most strains of mice (Burek et al. 1982; Frith and Ward 1988; Goodman et al. 1981). They can be focal or multifocal and might become quite large, compressing and replacing most of the ovary in some instances. The character of the lining cells varies depending on the etiology of the cyst. There are often foci of chronic inflammatory cells in the capsule. Pigment-laden macrophages are also common. The cysts can be filled with clear fluid or can be hemorrhagic.

Ovarian cysts can originate from several different sites and comprise follicular and luteal cysts, epithelial inclusion cysts, epidermoid cysts, paraovarian cysts, rete cysts, and bursal cysts (Davis et al. 1999).

Follicular and luteal cysts are both derived from anovulatory Graafian follicles. Follicular cysts are lined by cuboidal granulosa cells, whereas luteinized follicular cysts are lined by luteinized granulosa cells with vacuolated cytoplasm.

Epithelial inclusion cysts are lined by columnar epithelial cells that form papillary structures. These cysts are age-related and also considered to be precursor lesions of epithelial adenomas and carcinomas.

Epidermoid cysts are lined by squamous epithelial cells and are filled with layers of keratin. They are frequently found in association with teratomas.

Paraovarian cysts originate from the mesovarium and are lined by ciliated columnar epithelial cells. Smooth muscle is present in the wall of the cyst.

Rete ovarii are composed of tubules lined by columnar epithelium. Cysts of the rete ovarii are morphologically similar to paraovarian cysts but do not contain smooth muscle.

Bursal cysts are commonly found lesions that might cause compression of the ovary.

Atrophy. This occurs in adult mice and is characterized by decreased numbers of follicles at all stages of maturation and of corpora lutea. There is an increase in the interstitial tissue.

Inflammation. Acute inflammation with abscess formation is rare in mice but does occur and Klebsiella has been cultured from some of these cases.

Lipofuscin. Lipofuscin is common in the ovarian stroma of aged female mice. Lipofuscinosis is characterized by large round cells with abundant, foamy, pale yellow, pigmented cytoplasm that are present in the ovarian stroma. The pigment is acid fast and PAS positive.

Hyperplastic Lesions

Tubular Hyperplasia/Epithelial Hyperplasia. Epithelial hyperplasia is characterized by groups of elongate tubules lined by cuboidal/columnar epithelial cells that dissect through the ovarian stroma. The cells have small round nuclei and scant cytoplasm. Epithelial hyperplasia is an age-related change and has also been chemically induced by 1,3 butadiene and 4-vinylcyclohexene. These chemicals induce both hyperplasia and neoplasia of the ovarian epithelium.

Interstitial Cell (Stromal/Luteal) Hyperplasia. The lesion consists of enlargement and increased numbers of interstitial cells. The cells contain abundant foamy cytoplasm and are arranged in nests and packets by delicate stromal fibers.

Neoplastic Lesions

Tubulostromal Adenoma. These neoplasms arise by downgrowth of the germinal epithelium into the ovarian stroma. The tubules are similar to those seen in epithelial hyperplasia. The tubular structures replace the entire ovary and occasionally invade paraovarian fat (Carter 1968; Frith and Ward 1988; Goodman et al. 1981; Goodman and Strandberg 1982; Morgan and Alison 1987b). One of the distinguishing factors from tubular hyperplasia is that the diameter of proliferative lesion is larger than a corpus luteum.

Tubulostromal Adenocarcinoma. These malignant tumors show a high degree of pleomorphism, atypia, and an infiltrative growth pattern (Davis et al. 2001).

Cystadenoma. Ovarian cystadenomas are seen sporadically in many strains of mice (Carter 1968; Frith and Ward 1988; Frith and Wiley 1981; Goodman et al. 1981; Goodman and Strandberg 1982; Morgan and Alison 1987a). Cystadenomas are the most common age-related ovarian neoplasm found in studies in the female B6C3F1 mouse (Alison et al. 1987). Microscopically, they are cystic tumors lined by cuboidal to columnar epithelium with basal nuclei. There are usually simple to complex papillary structures projecting into the lumen and lined by similar epithelium.

Cystadenocarcinoma. The cytology of these neoplasms is more atypical than cystadenomas with an increased mitotic activity.

Granulosa Cell Tumors. These are the most commonly chemically induced neoplasm in the NTP 2-year carcinogenicity studies and can be either benign or malignant (Alison et al. 1987). They are composed of variably sized follicles that can cause considerable compression of the adjacent ovary. The cells resemble granulosa cells of normal follicles. The hallmark of malignant granulosa cell tumors is invasion and metastasis. The cells of malignant tumors are more pleomorphic than those of benign tumors with high mitotic activity. Occasionally granulosa cell tumors show areas of fusiform theca-like cells.

Thecoma.Benign thecoma neoplasms are composed of densely packed fusiform cells arranged in interlacing bundles and whorls. There is a variable amount of lipid and collagen present.

Malignant thecoma differ from the benign tumors in that they have extensive areas of necrosis, pleomorphism, infiltration of adjacent tissue, or distant metastases.

Luteoma. Luteomas are composed of large polygonal cells with abundant pale cytoplasm and round central nuclei. The cells are arranged in nests and cords. The tumors are generally well circumscribed, although not encapsulated, and often involve the greater portion of the ovary (Carter 1968; Frith and Ward 1988; Goodman et al. 1981). These are the most common type of ovarian tumor seen in the CD-1 mouse.

Yolk Sac Carcinomas. These are rare tumors in the mouse. A diagnostic feature of these neoplasms is that the tumor cells produce an abundant eosinophilic PAS positive matrix in which nests and cords of neoplastic cells are embedded.

Vascular Tumors. Hemangiomas are more commonly diagnosed than hemangiosarcomas. The former are difficult to distinguish from angiectasis.

Other Neoplasms. Both benign and malignant teratomas are seen occasionally in mice. Dysgerminomas and Sertoli cell tumors are extremely rare (Alison et al. 1987; Alison and Morgan 1987a, 1987b; Carter 1968; Frith and Ward 1988).

Uterus, Uterine Cervix

Nonneoplastic Lesions

Cystic Endometrial Hyperplasia. This lesion is extremely common in aged female mice (Burek et al. 1982; Frith and Ward 1988; Goodman et al. 1981). There is both proliferation and dilatation of the endometrial glands. The glands are lined by columnar epithelium and the lumens are often filled with eosinophilic material. In severe lesions, large cysts can develop. These are lined with low cuboidal or flattened epithelium.

Hydrometra. Marked dilatation of the lumen of the uterus with fluid is termed hydrometra. The wall of the uterus is thinned and there is loss of the endometrial glands. The lumen is filled with faint fibrillar eosinophilic fluid or mucoid material.

Endometrial Stromal Hyperplasia. This is a frequent finding in the mouse cervix and uterus. It is characterized by a proliferation of stromal cells with a variable amount of collagen. The growth characteristics of these lesions are noninvasive. The lesion is generally oriented along the normal anatomic structures (e.g., circular around the cervix) and does not cause any great distortion of anatomic structures.

Neoplastic Lesions

Endometrial Stromal Polyp. These neoplasms occur frequently in many strains of mice (Goodman and Strandberg 1982). They are small masses that project into the uterine cavity. Microscopically, the mass consists of an edematous stroma composed of spindle-shaped or stellate cells and varying numbers of endometrial glands, which can be cystic. The stroma is often highly vascular and the surface is covered by a single layer of simple columnar epithelium.

Endometrial Adenoma. These tumors arise from the epithelium lining the uterine mucosa or that of the endometrial glands. The epithelial cells are arranged in papillary, glandular, or tubular structures.

Endometrial Adenocarcinoma. These malignant tumors are composed of epithelial cells arranged in acini, glandular structures, and in solid nests and cords. The neoplastic glands can show varying degrees of pleomorphism and atypia. Endometrial adenocarcinomas are invasive and can metastasize, especially to the lungs.

Endometrial Stromal Sarcoma. These are the most commonly observed uterine tumors in aged B6C3F1 mice. These neoplasms arise in the endometrium and are composed of sheets of spindle cells with scant pale eosinophilic cytoplasm and elongated, hyperchromatic, fusiform nuclei. Cell borders are usually indistinct. The cells can be organized in fasciculi that run at angles or perpendicular to one another and in which the nuclei, cut in cross-section, might appear round or oval. Occasional areas can be present in which the fusiform nuclei appear plump and contain vacuoles. Areas of necrosis are common. Cellular atypia and mitotic figures can be present but are variable. Spread is by infiltration into the myometrium, cervix, and serosa. Metastasis is infrequent. It is necessary to distinguish endometrial stromal sarcoma from mesenchymal tumors such as leiomyosarcoma or fibrosarcoma (Goodman and Strandberg 1982) as well as histiocytic sarcoma.

Leiomyoma. Leiomyomas are benign tumors of smooth muscle that are well circumscribed. They are composed of interlacing, compact bundles of fusiform smooth muscle fibers that frequently run perpendicular to each other. The nuclei are elongate with round to blunt ends and are centrally located. These tumors are seen more frequently in CD-1 mice than in B6C3F1 mice.

Leiomyosarcoma. These neoplasms are malignant tumors of smooth muscle. They infiltrate the uterine wall and invade through the serosa. The cells are less well-differentiated smooth muscle cells than those seen in leiomyomas and are arranged in interlacing or whorling patterns. Mitotic activity is variable. Smooth muscle tumors stain positively for desmin and smooth muscle actin.

Squamous Cell Carcinoma. This is a rare spontaneous tumor but can be readily induced by estrogens. This tumor is usually well differentiated and is heavily infiltrated by leukocytes.

Granular Cell Tumor, Benign. The origin of these tumors is not established but is thought to be either from Schwann cells or mesenchymal cells. These are solid masses with large epithelioid round or oval cells with large pale nuclei and abundant eosinophilic cytoplasm.

Vagina

Spontaneous lesions of the vagina are rare in mice of all strains. (Frith and Ward 1988; Goodman and Strandberg 1982). Cervical and vaginal granular cell neoplasms have been diagnosed in B6C3F1 mice. These tumors are composed of sheets of large round or polygonal PAS-positive cells with an intervening fine fibrovascular stroma.

Induced Neoplasms

Squamous cell carcinomas are induced by AZT and estrogens.

Clitoral Gland

The clitoral gland is a skin appendage composed of modified sebaceous glands.

Nonneoplastic Lesions

Cystic Ducts (Ectasia). Dilatation of the ducts of the clitoral glands occurs frequently and might be associated with inflammatory changes, either acute or chronic.

Neoplastic Lesions

Neoplasms of the clitoral gland are very uncommon but they can be chemically induced.

Acinar Cell Adenomas. These benign tumors are characterized by neoplastic acini composed of pale foamy sebaceous type cells with peripheral basophilic basal cells. The cytoplasm might contain eosinophilic granules and there might be squamous metaplasia.

Acinar Cell Adenocarcinoma. These malignant tumors are composed of variably sized nests and nodules of proliferating cells. The acinar structures are composed of pale, foamy sebaceous type cells with peripheral, small, basophilic basal cells. There can be areas of squamous differentiation, cystic areas, necrosis, and inflammation. There is evidence of local invasion.

Squamous Cell Papilloma. These benign tumors are composed of papillary structures with a central connective tissue stalk that is lined by a squamous epithelium that might or might not be keratinized. These tumors can be single or multiple and might obstruct the ducts.

Squamous Cell Carcinoma. These malignant tumors consist of irregular papillary fronds or nodules of pleomorphic squamous cells with local invasion. The amount of keratinization is variable. Mitotic figures, necrosis, and inflammation are commonly present.

Mammary Gland

The development of mammary tumors in mice is influenced by many factors. These include genetics, an oncogenic retrovirus (murine mammary tumor virus infection [MuMTV]), age, sex, diet, hormonal status, and immune status (Seely and Boorman 1999).

Strains with MuMTV have a high incidence of spontaneous mammary gland tumors (e.g., C3H and DBA/Z). Strains that are either free of MuMTV or genetically resistant such as B6C3F1, Balb/C, and C57Bl mice have a low incidence of spontaneously occurring mammary tumors.

In susceptible mice MuMTV is passed on from generation to generation by one of two ways: either via infected germ cells or through the milk during lactation. There are several distinct lesions associated with MuMTV infection such as hyperplastic alveolar nodules (HAN) and plaques. These are both considered to be preneoplastic lesions although there is no certain progression to malignancy. HAN consist of proliferating acini, are transplantable only to mammary fat pads, and are found in nonpregnant and nonlactating mice. Carcinomas might develop within HAN. Plaques are pregnancy dependent and are branching tubules radially oriented in loose connective tissue.

There is a multiplicity of endocrine influences on both mammary gland development and tumorigenesis. Hormones produced by ovaries, pituitary, adrenals, and placenta have an important permanent effect on mammary gland development.

Nonneoplastic Lesions

Spontaneous nonneoplastic lesions of the mammary gland are uncommon in B60171 and CD-1 mice. Ectasia of mammary ducts are occasionally observed.

Hyperplasia. This change can be a focal or diffuse change. Physiological hyperplasia is a feature of late gestation and during lactation. Focal hyperplasia is characterized by normal appearing alveoli and ducts that are increased in number. There is no cellular pleomorphism, compression, or encapsulation. The regular glandular architecture of the mammary gland is maintained (Bruner et al. 2001).

Neoplastic Lesions

Adenomas. These benign tumors are composed of well-demarcated proliferations of small, solid acinar structures that are uniform in size and closely packed. There is scanty intervening connective tissue. The cells are well differentiated and the epithelial lining of the acinar structures is single layered. The regular lobular architecture of the mammary gland is not maintained.

Adenocarcinomas. These malignant tumors differ from adenomas in that there is a high variability in growth pattern with papillary, tubular, cystic, solid, comedo, and undifferentiated morphologies evident. Typically mammary adenocarcinomas contain variably sized cystic structures lined by pleomorphic or frankly anaplastic cuboidal acinar epithelium. Mitoses are frequently observed. There is either local invasion of adjacent tissues or metastasis. There is frequently necrosis and hemorrhage. There is a variable amount of stroma present. These tumors are usually large. Hemangioendothelial cell hyperplasia in the heart in female B6C3F1 mice has been reported in mice bearing mammary adenocarcinomas (Iwata et al. 1994).

Fibroadenoma. These are benign tumors that are clearly demarcated with alveoli or ducts present within a prominent dense fibrous stroma. The amount of connective tissue enclosing glandular structures is variable. There is usually a fibrous capsule.

Adenoacanthoma. Despite its title this tumor is malignant. It is typically a well-circumscribed tumor comprising glandular and squamous epithelial elements that are present in variable proportions (25% or more of the tumor consists of squamous metaplasia). The squamous cells exhibit intracytoplasmic keratin and pearl formation. Metastases can exhibit either squamous or glandular tissue. These tumors have a high incidence in the BALB/C strain.

Squamous Cell Carcinomas. These are rare tumors that have been chemically induced.

Induction of Mammary Tumors in Mice

Mammary tumors in mice have been induced by many chemicals. In the NTP program chemicals that have induced (mainly malignant) mammary tumors in female mice include benzene, 1,3-Butadiene, 1,2-Dibromoethane, glycidol, sulfallate, chloroprene, 1,2-Dichloroethane, benzene, furosemide, ethylene oxide, and reserpine (Dunnick et al. 1995).

Factors such as the presence of MuMTV, the hormonal balance, and immune status influence the development of chemically induced neoplasms. In butadiene-induced adenocarcinomas several tumor suppressor genes were reported to be inactivated (Wiseman et al. 1994). The p53 pathway might be a common target for mutation in mammary gland tumor carcinogenesis (Jerry et al. 1993).

Hematopoietic System

Nonneoplastic Lesions

Thymus

Thymic Cysts. Thymic cysts have been seen in a number of strains of mice and they generally increase in incidence with age (Frith and Wiley 1981). The cysts are lined by a simple cuboidal epithelium and the lumen is usually empty. In some strains they can occur in the cranial part of the thymus at the corticomedullary junction (Wijnands et al. 1996; Yoshida et al. 1986).

Thymic Atrophy. Histologically the appearance of thymic atrophy and normal involution are similar, but whereas atrophy is seen in young mice, in response to a variety of toxic insults, and

is reversible on removal of the stimulus, involution is seen in older mice, and is irreversible (Ward et al. 1999).

Atrophy begins as a depletion of cortical lymphocytes, with loss of corticomedullary differentiation, progressing to more generalized depletion, and all cell types are decreased in number.

Apoptosis is seen in physiological involution and at an increased level in association with intoxication, infection, or injury. The primary mechanism of the induced change has been shown to be stress, mediated by glucocorticoids (Levin 1998; Levin et al. 1999). Atrophy can also be produced as a result of direct toxicity and decreased levels of growth hormone (Gopinath et al. 1987) and administration of immunosuppressive agents such as corticosteroids, cyclosporin, and cyclophosamide (Greaves 1990; Schuurman et al. 1991).

Ectopic Thymus. Ectopic thymus has been reported adjacent to or in association with the parathyroid gland. The ectopic tissue is predominately cortical thymic tissue. Conversely, ectopic parathyroid tissue can be seen close to or embedded within the thymus (Frith and Fetters 1983). This association is due to the simultaneous migration of these tissues early in gestation.

Spleen

Accessory Spleen. A small accessory spleen is occasionally noted in mice. The accessory spleen is usually near the normal spleen and can be embedded in the pancreas. Normal red and white pulp is usually present.

Splenic Pigmentation. Hemosiderin, ceroid, or lipofuscin and melanin pigment can occur in the spleens of mice. Hemosiderin is a golden-brown granular pigment derived from the breakdown of red blood cells and present within the cytoplasm of macrophages. It stains positively with iron stains such as Prussian blue. Hemosiderin is found to some extent in mice of all ages, is generally more prominent in females than males, and increases in amount with age. Background levels reflect the normal removal of effete red blood cells by the spleen but hemosiderin can be increased in cases of chemically induced hemolytic anemia or methemoglobinemia (Travlos et al. 1996).

Ceroid or lipofuscin pigment is another golden-brown pigment seen within the cytoplasm of macrophages in the spleen. It is derived from oxidation and polymerization of fatty acids and stains positively with acid-fast stains (Crichton et al. 1978).

Melanin pigment can occur in the spleen of mice with pigmented skin. It is slightly darker than hemosiderin and has a characteristic elongated or stringy appearance. It is not associated with macrophages, being present in melanocytes, and is iron negative.

Extramedullary Hematopoiesis. Hematopoiesis is normally carried out in the fetal and neonatal liver. In the adult, this function is taken over by the spleen and bone marrow (Harada et al. 1996).

Extramedullary haemopoiesis (EMH) is therefore often seen within the splenic red pulp in mice (Long et al. 1986) and can be increased in response to a number of stimuli such as inflammation, anemia, immune stimulation, cytokine effects, thrombocytopenia, and certain neoplasms. Foci of EMH are composed of variable numbers of megakaryocytes, myeloid and erythroid precursors. There might also be an associated plasma cell hyperplasia. The predominant cell type present depends on the nature of the initiating stimulus.

Hemorrhage or anemia results in erythropoiesis. Erythropoietic activity is characterized by foci of immature erythrocytic precursors with small darkly staining nuclei in the red pulp. It might or might not be associated with an increase in the granulopoietic activity.

Hyperplasia of granulocytic elements is usually accompanied by initiating lesions such as abscesses, ulcerative tumors, or other inflammatory lesions. A generalized increase in granulopoiesis

can be accompanied by granulopoietic elements in a variety of other organs, including the liver, adrenals, and lymph nodes. This is also referred to as a leukemoid reaction.

A marked increase in granulopoietic activity must be distinguished from granulocytic leukemia. In granulopoiesis, the complete series of developing cells, including the mature neutrophils, are usually present. In granulocytic leukemia, the developing stage of the neoplastic granulocytes might vary from animal to animal, but typically a single stage predominates (Dunn 1954).

Lymphoid Hyperplasia. Hyperplasia of a variety of cell types can occur in hematopoietic tissue of mice. Lymphoid hyperplasia in the spleen is more common in females than males and can have a variety of morphological patterns involving germinal centers, marginal zones, or white pulp, each of which can occur concurrently (Ward 1990b).

Differentiation from lymphoma depends largely on the maintenance of normal architecture, involvement of multiple follicles, confinement to the spleen, and demonstration of the cause for the reactive change (e.g., inflammation; Frith et al. 2001). Classification of hyperplastic lesions in the lymph nodes of rodents has been described (Ward 1990a).

Lymphoid hyperplasia of the lymph nodes is common, but it is rare in mice under 12 months of age. The incidence increases slightly with age and is slightly greater in females than in males in most strains. In mice, active hyperplasia is often seen in the mandibular and mesenteric nodes.

Hyperplasia can be seen in the B-cell areas (follicles, germinal centers), T-cell thymic-dependent areas (paracortex), and medullary cords or sinuses. Commonly in the hyperplastic lymph node, the normal corticomedullary division is not apparent (paracortical hyperplasia) and the marginal sinus is filled with lymphocytes. The lymphocytes are usually small and normal in appearance.

The lesion is difficult to distinguish from the lymphoblastic type of malignant lymphoma if only a single node is involved, but the lymphocytes are mature, few mitotic figures are observed, and the "starry sky" effect found in lymphoblastic lymphomas is not seen. Thickening of the medullary cords is often noted and is characterized by the presence of many plasma cells (plasmacytosis), occasionally resembling plasmacytoma. The plasmacytosis is a reaction to chronic inflammatory lesions or tumor antigens. It is most commonly seen in the mandibular nodes.

Lymph Nodes

Sinus Histiocytosis. Accumulations of normal histiocytes can occur in the subcapsular and medullary sinuses of lymph nodes. The histiocytes have dark basophilic nuclei and abundant distinctly eosinophilic cytoplasm. They can contain hemosiderin and other pigments, erythrocytes, and other phagocytized material.

Plasmacytosis. Plasmacytosis is an increased number of normal-appearing plasma cells in lymph nodes. The plasmacytosis could be a reaction to an adjacent chronic inflammatory lesion or to tumor antigens. The most common lymph node involved is the submandibular lymph node.

Mastocytosis. An increase in mast cells (mastocytosis) can occur in either the splenic red pulp or in lymph nodes. Differentiation of this lesion from metastatic mast cell tumor depends on identification of the primary mast cell tumor. The number of mast cells normally found in a specific organ can vary from strain to strain (Dunn 1969).

Miscellaneous Lesions. Megakaryocytosis, bone marrow atrophy, necrosis, atrophy, and mineralization of lymph nodes can occur in mice, but all are rare in both the CD-1 and B6C3F1 strains.

Neoplastic Lesions

Malignant Lymphoma

Malignant lymphomas arise from B or T lymphocytes or their precursors (Frith et al. 2001). Those tumors of B cell origin are follicular center cell, plasma cell, and immunoblastic lymphoma. Plasmacytic lymphomas originate from immunoblasts.

The cell membrane antigen CD45R is seen on the surface of normal and neoplastic B cells, and therefore can be a useful marker in differentiating the cell of origin; however, intensity of immunoreactivity can be decreased in tumor cells. Immunoglobulin stains (e.g., IgG and IgA) are useful markers for immunoglobulin-producing B cells (plasma cell and immunoblastic lymphoma).

Others such as small lymphocyte lymphomas can be of B or T cell origin. Lymphoblastic lymphoma can also be of B or T cell origin but usually arise from T cells in the thymus. T cell tumors most often arise in the thymus and metastasisze to other tissues including the spleen (Dunnick et al. 1997; Hursting et al. 1995). They are commonly CD3 immunoreactive. The immunomorphological classification illustrated in table 2.23 is adapted from Pattengale and Frith (1983).

Follicular Center Cell Lymphoma

Reticulum cell sarcoma type B as described by Dunn (1954) has been referred to as malignant lymphoma, mixed cell type (Frith and Wiley 1981) or pleomorphic cell type, and more recently has been identified as a follicular center cell lymphoma (FCC) of B-cell origin (Frederickson et al. 1985; Pattengale and Frith 1983). The B-cell nature of these cells has been confirmed using immunoperoxidase techniques with Bouin's or B-5 fixed tumors demonstrating the presence of cytoplasmic or cell surface immunoglobulin (CIg) of the various isotypes.

Most lymphomas in B6C3F1 mice are of FCC origin, based on antigenic staining and molecular studies (Della Porta et al. 1979). The disease is rare before 12 months of age and might increase dramatically in some strains after 18 months. This neoplasm is slightly more common in female than in male mice. Lesions often arise in individual mesenteric lymph nodes, intestinal Peyer's patches, or in one or more germinal centers of the follicles in the B-cell areas of the white pulp.

Microscopically, FCC lymphomas express varying degrees of pleomorphism. They might be composed of small FCCs (centrocytes), large FCCs (centroblasts), or most commonly a mixture of the two, small lymphocytes, macrophages, and immunoblasts. The proportion of each cell type varies with each lymphoma.

Table 2.23 Immunomorphological Classification of Murine Lymphomas, Related Leukemias, or Tumors

Morphological Type	Immunological Type			
	B Cell	T Cell	Non-B, Non-T	Histiocyte
Follicle center cell				
Small cell type	+	–	–	–
Large cell type	+	–	–	–
Large and small (mixed) cell type	+	–	–	–
Plasma cell	+	–	–	–
Immunoblast	+	(+)	(+)	–
Small lymphocyte	+	(+)	(+)	–
Lymphoblast	+	+	+	–
Histiocyte	–	–	–	+

Note: Parentheses indicate that the disease has been seen in humans, but to date has not been reported in the mouse.

Source. Adapted from Pattengale and Frith (1983).

In lymphomas composed of large FCCs, the cells are large and cohesive with irregularly shaped folded and notched nuclei (cleaved) and moderate amounts of cytoplasm. Small FCCs, are cohesive and nuclei are markedly irregular in size and shape with scant cytoplasm.

Immunoblastic Lymphoma

Immunoblastic lymphoma of B-cell origin is rare in the mouse (Pattengale and Frith 1983). The pattern of organ involvement in immunoblastic lymphoma is similar to that of FCC lymphoma but is usually more invasive in its behavior (Ward and others 1999). It is characterized by noncohesive, large lymphoid cells with round to oval vesicular nuclei with prominent and distinct nucleoli. In addition, the nuclei are sometimes eccentric and have clumped, peripherally marginated clock face-like chromatin, and the cytoplasm is moderately dense and amphophilic (plasmacytoid features).

Plasma Cell Lymphomas

Plasma cell lymphomas occur infrequently in control mice. The lymph nodes, spleen, and liver might be involved. The cells are large with amphophilic cytoplasm, some are binucleate, the mitotic index is high, and the cells retain a characteristic plasma cell appearance. Spontaneous plasma cell myelomas are rare, but intraperitoneal plasma cell tumors can be induced with intraperitoneal injections of mineral oil.

Lymphoblastic Lymphoma

Using immunocytochemical techniques and the newer classification of murine malignant lymphomas (Frederickson et al. 1985; Frith et al. 1985; Pattengale and Frith 1983) the lymphoblastic type is comparable to Dunn's lymphocytic leukemia. This is the most common hematopoietic tumor in the CD-1 mouse and is one of the more common hematopoietic neoplasms in many other strains. T lymphoblastic lymphoma can occur as early as 1 month of age, and can peak at 3 to 6 months in some strains such as the BALB/c and AKR mice. The incidence is generally higher in female than in male mice.

Lymphoblastic lymphoma often arises in the thymus (T lymphoblastic) but is aggressive involving a number of organs, most commonly the liver, spleen, lymph nodes, bone marrow, and lungs. In the lungs, invasion follows the vascular tree. It is often leukemic and can involve the central nervous system (CNS). The component cells are medium-sized lymphoblasts with high nuclear to cytoplasmic ratio, moderate amounts of basophilic cytoplasm, which might be vacuolated, and round vesicular nuclei, with prominent central nucleoli. Mitoses are numerous.

Small Lymphocyte Lymphomas

Component cells are well differentiated, small to medium-sized lymphocytes, which are morphologically indistinguishable from normal circulating lymphocytes. Cells are uniform, noncohesive and mitotic figures are rare. Tingible-body macrophages are not present and there is effacement of normal tissue architecture.

Other Neoplasms

Histiocytic Sarcoma. Dunn's (1954) reticulum cell neoplasm type A, or malignant lymphoma (Frith, Davis, et al. 1981), histiocytic type, or histiocytic lymphoma, has been recently classified as histiocytic sarcoma (Pattengale and Frith 1983). Other investigators have described the lesion

as endometrial sarcoma or malignant schwannoma (Stewart et al. 1974). Research findings have suggested that the neoplastic cells are derived from histiocytic cells of uncertain origin (tissue histiocytes, Kupffer's cells, and macrophages; Frith, Davis, et al. 1981).

Histiocytic sarcomas are common in both CD-1 and B6C3F1 mice over 12 months old and are slightly more common in females than in males. The liver is the most commonly involved organ in male mice; in females, the uterus and vagina as well as the liver are often involved, suggesting these tissues as sites of origin. Other organs less frequently involved include the spleen, lymph node, bone marrow, lung, kidney, and ovaries. Metastatic lesions to the lungs occur in a high percentage of cases with liver involvement. Component cells are small spindle-shaped to round cells with small dark nuclei and scant to moderate amounts of eosinophilic cytoplasm. Multinucleate forms are scattered throughout the tumor. There are frequently zones of necrosis within these tumors.

Thymoma. The term *thymoma* is usually used in the mouse to classify a lesion characterized by the presence of a neoplastic epithelial component with or without neoplastic lymphocytes. The epithelial component is positive for keratin and appears to be derived from the epithelial cells in Hassall's corpuscles or thymic reticular tissue. This type of tumor is rare in all strains of mice.

Benign thymomas are solitary lesions that can be well encapsulated. In the malignant form there is marked local invasion beyond the confines of the thymus.

Other rare nonlymphoid hematopoietic neoplasms in the mouse include mast cell tumor (Deringer and Dunn 1947; Dunn 1969; Frith and Dooley 1976), granulocytic leukemia (Dunn 1954; Frith, Davis, et al. 1981), and erythroleukemia (Frith et al. 1990).

Male Genital System

Testes

The testicular germinal epithelium is sensitive to a wide variety of internal and external influences (Faccini et al. 1990c). These include age, nutrition, hormones, temperature, lighting, stress, vascular perfusion, and spaceffects. Effects on other cell components of the testis (example.g., Sertoli cells) might have an additional effect of secondarily causing degeneration of germinal epithelium.

Nonneoplastic Lesions

Hypospermia. Hypospermia (immaturity of the seminiferous tubules) is a normal condition in very young mice (< 4 weeks of age), but it can be induced in older mice with toxicants. Maturing and mature spermatozoa are absent from both the testes and the epididymis.

Testicular Atrophy. Testicular atrophy occurs as an aging lesion in mice and can also be due to other factors such as exposure to specific toxic compounds, irradiation, and hypoxia. The lesion can be focal or diffuse. The seminiferous tubules and germinal epithelial cells are reduced in number and the testis might also be reduced grossly in size. A relative increase of Sertoli cells, interstitial cells, or multinucleated cells could be seen. The lipofuscin pigment ceroid can be associated with the atrophy. Ceroid is acid fast and PAS positive.

Testicular Mineralization. Focal dystrophic mineralization of the seminiferous tubules might occur occasionally. It can represent previous areas of injury. The mineralization is composed of basophilic concentric masses that might be amorphous or concentrically laminated.

Hyperplasia of Interstitial (Leydig) Cells. An increase in the relative number of interstitial cells might be associated with testicular atrophy. Focal hyperplasia of interstitial cells can also occur,

and could represent a preneoplastic condition. Diffuse hyperplasia precedes tumor formation in mice with testicular feminization and is associated with estrogenic compounds and 5 gamma reductase inhibitors (Radovsky et al. 1999).

Spermatocoele can occur in any part of the ductular system seminiferous tubules or epididymis and consists of a dilation of the lumen by accumulation of spermatozoa.

Sperm granulomas result from the rupture of the seminiferous tubules or passages conducting spermatozoa. The spermatozoa elicit an inflammatory response because they are coated with proteins that incite an immune response. The granulomatous response might be so florid it is important to differentiate it from a neoplasm.

Neoplastic Lesions

B6C3F1 mice have an overall low incidence of neoplasms in male reproductive organs (Mitsumori and Elwell 1988).

Interstitial Cell Tumors. Both benign and malignant spontaneous tumors of the interstitial cells of Leydig are rare in most strains of mice, but can be seen in both the CD-1 and the B6C3F1 mouse. Neoplasms can be induced with synthetic or natural estrogens in certain strains, particularly BALB/c mice. The cytoplasm of the neoplastic cells is distinctly eosinophilic. Small, well-circumscribed tumors are designated adenomas and large tumors that are invasive or metastasize are referred to as carcinomas. The larger carcinomas occasionally metastasize to the lungs.

Sertoli cell tumors of the testis have been described although they are extremely rare in mice (Rehm et al. 2001). The characteristics of these neoplasms are areas of palisading cells within poorly demarcated tubular structures. The tumor cells are elongated with central nuclei in abundant pale eosinophilic cytoplasm.

Gonadal stromal tumors resemble ovarian granulosa cell tumors. Cells are in nests, cords, or sheets and occasionally in follicular patterns. Cells have scant amphophilic cytoplasm and distinct nuclei.

Other tumors arising within the testis are teratomas, seminomas, yolk sac carcinomas, and adenomas and carcinomas of the rete testis. These are all rare neoplasms.

Accessory Sex Glands

Nonneoplastic Lesions

Prostate. Foci of chronic inflammatory cells are a frequent finding in the interstitial tissue of the prostate.

Preputial Gland. The preputial glands are one of the accessory sex glands of the male mouse composed of modified sebaceous acini and squamous ducts. Acute suppurative and chronic inflammation are common.

Abscesses of the preputial gland are frequently seen in male mice as a consequence of fighting. The abscess is characterized by numerous neutrophils and liquefactive necrosis.

Neoplastic Lesions

Neoplasms of Accessory Sex Glands. Neoplasms of all of the male accessory sex glands (prostate, seminal vesicles, preputial and coagulating glands) are extremely rare. Adenocarcinomas of the prostate can be composed of relatively well-differentiated neoplastic cells. Squamous metaplasia might occasionally be seen. Adenomas and carcinomas of the seminal vesicle and coagulating gland

have been reported very infrequently. Squamous cell papillomas and carcinomas are very occasionally seen in the preputial gland.

Integument

Nonneoplastic Lesions

Alopecia

Alopecia or hair loss on the face and back is common in mice and appears to be associated with friction contact with feeding jars. Alopecia also occurs on the thorax and abdomen of B6C3F1 mice, the cause of which is unknown (Burek et al. 1982).

Amyloidosis (Peckham 1999)

Although amyloidosis of the skin is usually a manifestation of systemic amyloidosis it has been infrequently observed in the skin of aging B6C3F1 mice primarily associated with the chronic inflammation due to mite infestation.

Atrophy

Atrophy of the skin is a sporadic spontaneous finding and is characterized by a very thin epidermis with associated atrophy of sebaceous glands and hair follicles. The causes of atrophy include nutritional deficiencies and hormonal imbalances such as hypothyroidism.

Ulcers

Ulcers are common spontaneous findings in the B6C3F1 mouse in NTP studies. Ulcers are deep excavations in the epidermis that penetrate into the underlying dermis. Ulceration is usually secondary to traumatic injuries or neoplasia. Ulceration secondary to an immune complex vasculitis was observed in groups of C57BL/6N mice (Andrews et al. 1994).

Dermatitis

Inflammation of the skin is a common finding in B6C3F1 mice. Fighting amonggroup-housed animals and parasitism are predisposing causes. Secondary inflammation is commonly associated with large subcutaneous neoplastic lesions as a consequence of impaired blood supply and trauma.

Neoplastic Lesions

General

Subcutaneous mesenchymal tumors are much more common in mice than are epithelial neoplasms (Goodman et al. 1981; Holland and Fry 1982). Malignant mesenchymal tumors are usually named according to the predominant or most malignant pattern observed. However, in mice, these neoplasms appear to represent a spectrum. Because of this, some pathologists do not separate the different types and might group these tumors under a single term such as fibrosarcoma or sarcoma not otherwise specified (NOS). These tumors are common in male B6C3F1 mice, and there is some evidence that they might be associated with fighting and chronic trauma in group-housed male mice (Squire, personal communication,1990).

Fibroma

These benign tumors are poorly to moderately cellular tumors (Ernst, Carlton, et al. 2001). These tumors are composed of fusiform or stellate cells with elongated hyperchromatic or vesicular nuclei. The cells produce interlacing bundles of collagen fibers, which can be densely packed or loosely arranged if separated by edema or a mucinous ground substance. The tumors are relatively well circumscribed and noninvasive although they commonly cause local compression.

Fibrosarcoma

These malignant tumors are more cellular than their benign counterpart (fibromas) and produce less collagen. Pleomorphic spindle cells are arranged typically in either a herringbone pattern or forming interlacing bundles. Mitotic figures are numerous and these tumors are locally invasive although they infrequently metastasize.

Fibrous Histiocytoma

These are the most commonly occurring soft tissue subcutaneous tumor in CD-1 mice. They are rare in B6C3F1 mice. Both benign and malignant histiocytomas occur although in CD-1 mice the latter are more common (Faccini et al. 1990b).

Benign Fibrous Histiocytoma

These tumors have a characteristic storiform pattern of neoplastic cells. The cell type is fibroblast like with a subpopulation of histiocytic cells. There is abundant collagen. Inflammatory cells are scattered throughout the tumor (Bruner et al. 2001).

Malignant Fibrous Histiocytoma

There are two main histological appearances of this malignant tumor of mesenchymal stem cells: fibrous and pleomorphic type. The fibrous type is composed of fibroblastic spindle cells arranged in a storiform pattern. Nuclei are pleomorphic and mitotic figures are numerous. There is abundant collagen. Foci of necrosis and inflammatory cell infiltration are present.

The pleomorphic type is composed of rounded histiocytic cells with numerous bizarre multinucleated tumor giant cells and pleomorphic fibroblast-like cells. There are focal areas of collagen production. Infiltration of surrounding tissue is a characteristic of both varieties of malignant fibrous histiocytoma although the pleomorphic type is more likely to metastasize.

Neurofibroma and Neurofibrosarcoma

These tumors arise from the fibroblasts of the perineural connective tissue. Their histological appearance is similar to fibromas and fibrosarcomas although association with a nerve is necessary for the diagnosis of neurofibromatous tumors.

Benign Schwannoma

These benign tumors arise from nerve sheath cells, the Schwann cell, which originates from the neuroectoderm. There are two varieties of growth patterns in Schwann cell tumors, Antoni type A and Antoni type B. Antoni type A tissue is composed of relatively compacted cells that form whorls, bundles, and foci of nuclear palisading (Verocay bodies). Antoni type B tissue is composed of irregularly arranged cells in an edematous matrix.

Malignant Schwannoma

These malignant tumors are more commonly observed than the benign variety. Cell morphology is more pleomorphic. Local invasion and metastatic lesions are hallmarks of malignancy. Cells can be arranged in either Antoni type A or B patterns.

Sarcomas NOS

Sarcomas NOS are extremely cellular tumors that can contain large bizarre nuclei, mitotic figures, and multinucleated giant cells. A pattern of interwoven bundles of fusiform cells might be apparent but collagen fibers are difficult to demonstrate in any quantity even with polarized light. They could be locally invasive and metastasize.

Mast Cell Tumor

These neoplasms are typically benign and are usually solitary dermal nodules. Mast cell tumors are composed of densely packed polygonal mast cells with granular cytoplasm and a central round nuclei. There are few mitotic figures. Malignant mast cell tumors are very uncommon and generally occur as systemic tumors.

Malignant Melanoma

These are primary tumors of the melanocytes that are of neuroectodermal origin. These tumors occur in the dermis. The neoplastic cells are spindle, epithelioid, or anaplastic. The neoplasm can be locally invasive. They are rare spontaneous tumors in mice that can be induced by topical application of carcinogens.

Epithelial Neoplasms

Epithelial tumors of the skin are rare spontaneous lesions in mice.

Basal Cell Adenoma

Basal cell tumors originate from primary epithelial germ cells of the piliary complex. Basal cell tumors have a variety of gross appearances.

Basal cell adenomas are circumscribed cellular masses with a variety of patterns and cell morphology. The cells are uniform, small, with prominent round or oval nuclei and relatively scant cytoplasm. Mitoses can be numerous. There is a variable fibrous stroma.

Basal Cell Carcinoma

These are distinguished from adenomas by abnormal or atypical cell morphologies, numerous mitoses, and frequently extensive local invasion.

Sebaceous Cell Adenomas and Carcinoma

Sebaceous cell adenomas are composed of sharply demarcated lobules of well-differentiated sebaceous cells. They might have foci of squamous epithelium with keratinization.

Sebaceous gland carcinomas are more irregular, less organized masses than adenomas. The cells also tend to be less differentiated and there could be local invasion.

Trichoepithelioma

These are a subtype of basal cell tumors and are tumors of the hair follicle. They are generally subcutaneous and are characterized by the formation of small keratin cysts or hair shafts.

Basosquamous Tumor

This type of tumor is a mixed piliary complex tumor containing both basal and squamous cells.

Keratoacanthoma

These are benign neoplasms of basal and squamous epithelium that are crateriform or invaginated into the dermis and subcutis. They are thought to originate from hair follicles. Squamous cell carcinomas might arise from keratoacanthomas.

Squamous Cell Papilloma

These are superficial benign tumors arising from the epidermis and are composed of squamous epithelial cells that show acanthosis, hyperkeratosis, and papillary patterns. Chemical carcinogens readily induce squamous cell papillomas in mice during skin painting studies. These induced papillomas can become squamous cell carcinomas (Rehm et al. 1989).

Squamous Cell Carcinomas

These are malignant tumors of squamous epithelial cells that have a variable amount of keratin depending on the degree of maturation of the neoplastic cells. The tumors are composed of irregular masses or cords of squamous epithelial cells invading the adjacent dermis and subcutis.

Liver and Biliary System

Liver

Nonneoplastic Lesions

Extramedullary Hematopoeisis. Extramedullary hematopoeisis is normally present in both the fetal and neonatal mouse liver. The lesion can also occur in the adult mouse liver secondary to an infectious disease, hemorrhage, or neoplasia. When the predominant cell type is the granulocyte, granulocytic hyperplasia might be specified and if nucleated erythrocytes are the prominent cells, erythropoietic hyperplasia is an appropriate term. In the mouse liver, the granulocytic activity seems maximal in the sinusoids adjoining the portal vein (Dunn 1954). Megakaryocytes may be associated with the areas of hematopoiesis.

Fatty Metamorphosis (Fatty Change, Steatosis, Lipidosis Fat Deposition). Fatty metamorphosis can occur spontaneously in mice and also as a response to toxic agents (Harada et al. 1999; Harada et al. 1996). It can be present as a focal, zonal, or diffuse change. The degree of fatty metamorphosis can vary. It occurs spontaneously in 25% to 30% of B6C3F1 mice at 24 months of age. The empty clear vacuoles with the peripherally located compressed nuclei represent lipid that has been removed during tissue processing. The lipid can be confirmed by staining frozen sections with Oil Red O or Sudan Black B. Fatty change is usually reversible but sometimes when severe it can result in both fibrosis and regenerative hyperplasia.

Glycogenic Vacuolation. Glycogenic vacuolation appears as irregularly shaped, clear vacuoles within hepatocytes. Glycogen is dissolved in the aqueous fixative. The glycogen content of the liver is variable depending on the physiological status of the mice (Faccini et al. 1990a). Overnight fasting causes a dramatic reduction in the amount of glycogen present in the liver. It is therefore important to randomize the time of sacrifice between treated and control animals.

Hemosiderosis. Hemosiderin pigment can be found in Kupffer's cells within the liver. The pigment is a distinctive granular, golden color within the cytoplasm of Kupffer's cells. Occasionally special stains such as Prussian blue can be used to confirm the presence of iron and to differentiate it from other pigments such as bile or ceroid.

Ceroid Pigment (Lipofuscin). Ceroid is a lipofuscin pigment that is acid fast and is acid Schiff (PAS) positive. An increase of ceroid pigmentation has been associated in aging mice in certain organs, including the ovaries and adrenals. Ceroid pigment in the liver can also occur as a result of hepatic toxicants. The pigment is slightly darker brown than hemosiderin, and is usually present in the cytoplasm of Kupffer's cells adjacent to portal areas.

Necrosis. Focal hepatic necrosis is a nonspecific entity quite often encountered as an incidental finding in the liver of mice. It can be the result of viruses (mouse hepatitis virus), bacteria (*Bacillus piliformis*), toxicants, and ischemia; however, the etiology is often unknown. It can involve single cells, single lobules, or multiple lobules, and it can vary in distribution. Coagulation necrosis with distinct eosinophilic cytoplasm and pyknotic or absent nuclei is the typical morphological feature. Cell outlines are usually distinct and the presence or type of an associated inflammatory reaction depends on the duration of the lesion. Hepatic necrosis, which is induced by chemicals, could have a zonal distribution. If the extent of necrosis is severe and persistent there might be a resultant fibrosis and compensatory hepatocellular hypertrophy.

Microgranulomas. Microgranulomas are commonly seen in small numbers in the livers of many strains of mice. They consist of small collections of macrophages and lymphocytes within the sinusoids or surrounding a single necrotic hepatocyte. The cause of these lesions is unknown. Periodic showering of bacteria from the intestine through the blood stream has been suggested as a possible cause.

Inflammatory Lesions. Inflammatory lesions might occur as a result of a toxic agent. Inflammation can also result from infection with viruses and bacteria such as mouse hepatitis virus, mousepox, *Bacillus piliformis,* and *Helicobacter hepaticus*. In 1992, a novel chronic active hepatitis, which was associated with a high incidence of hepatocellular neoplasia, was identified in strain A/JCr control mice (Ward 1984). *Helicobacter hepaticus* was found to colonize the GI tract of many mouse strains and to demonstrate strain-dependent pathogenicity. Warthin Starry silver stains are used to demonstrate helical bacterial organisms. In mice of susceptible strains the early liver changes are typically focal, acute, nonsuppurative necrosis of hepatocytes. Later lesions are characterized by chronic active hepatitis with hepatocytomegaly, oval cell hyperplasia, cholangitis, and bile duct proliferation.

Karyomegaly and Cytomegaly. A striking histological feature of the livers of aged mice is the presence of hepatocytes with enlarged nuclei of variable size. The enlarged nuclei can be rounded or elongated and generally are two or more times normal in size. The polyploid cells appear with increasing frequency as aging occurs (Jones 1967). This increase in nuclear size (karyomegaly) might or might not be associated with an increase in cell size (cytomegaly), and cytomegaly can occur either with or without an increase in the size of the nucleus. These changes have also been seen in mice treated with DDT, Phenobarbital (Ward 1984), Aroclor 1254 (Kimbrough and Linder

1974), and other chemicals and in mouse hepatitis virus-infected cells (Ward et al. 1977). Toxins also often induce binucleate and multinucleated hepatocytes.

Inclusions. Both intranuclear and intracytoplasmic inclusions are frequently observed within normal and neoplastic mouse hepatocytes. Intranuclear inclusions are round, often filling most of the nucleus, and are distinctly eosinophilic in appearance. These inclusions have been reported to increase in incidence with age and are usually considered to be invaginations of the cytoplasm into the nucleus (Andrew 1962; Herbst 1976).

Cytoplasmic inclusions are somewhat less common, and are most frequently seen in hepatocytes in or adjacent to hepatocellular neoplasms. Intracytoplasmic inclusions are round, vary markedly in size, and are usually eosinophilic. Some investigators have reported these intracytoplasmic inclusions to be aggregates of smooth endoplasmic reticulum (Hruban et al. 1966), or Mallory bodies. These types of intracytoplasmic inclusions have been reported in mice (Frith and Ward 1980).

Atrophy. Atrophy that is diffuse in distribution might be related to administration of test substance or it might reflect inanition. The hepatocytes are small and appear more eosinophilic due to a decrease in the amount of glycogen.

Angiectasis. Angiectasis consists of widely dilated vascular spaces that are lined by unremarkable epithelial cells. This can range from one or two blood-filled cystic spaces to many.

Bile Duct and Ductular Hyperplasia. Proliferation of hepatic bile ducts can occur in response to toxicants and is sometimes associated with an inflammatory reaction. The lesion is usually diffuse and many bile ducts are usually present in portal triads. Bile ductules (cholangioles, oval cells) can become hyperplastic in response to toxicants as well. Normal and hyperplastic bile ducts and ductules stain for keratin.

Oval Cell Hyperplasia. This is characterized by the proliferation of small, oval cells between hepatocytes frequently in periportal regions sometimes with the formation of incomplete ductlike structures. Oval cell hyperplasia is frequently associated with severe hepatocellular injury such as hepatocarcinogens and infection with *Helicobacter hepaticus*.

Cholangiofibrosis (Adenofibrosis). Cholangiofibrosis is characterized by focal areas of basophilic atypical ducts in a fibrous stroma. It is rarely seen in control mice. The lesion is controversial and much less commonly induced in the mouse than in the rat. Adenofibrosis has been described in the mouse induced by Aroclor 1254 (Kimbrough and Linder 1974).

Cirrhosis. Hepatic cirrhosis, whether postnecrotic, biliary, pericellular, or of some other type, is uncommon spontaneously in mice. It can be seen after a variety of toxicants, including carbon tetrachloride and chronic mouse hepatitis infection in nude mice (Ward et al. 1977). It often takes the form of a focal or diffuse increase of fine reticular fibers rather than distinct fibrous collagenous bands (Ward, Bernal, et al. 1979).

Cholangitis. Cholangitis is inflammation of the bile ducts and is characterized by the presence of inflammatory cells (polymorphonuclear leukocytes or mononuclear cells) within the ducts and periductular tissue.

Hypertrophy. Hypertrophy of hepatocytes can be induced by a large number of compounds. Phenobarbitone causes hepatocellular hypertrophy that is predominately centrilobular and is due to smooth endoplasmic reticulum proliferation. Some hypolipidemic agents cause liver enlargement in mice. Typically the hepatocytes are hypertrophied throughout the liver lobule and exhibit a ground

glass appearance of their cytoplasm. Electron microscopy in these cases reveals a marked increase in peroxisomes.

Hyperplasia, Hepatocellular Regeneration. This occurs as a consequence of liver damage and must be differentiated from a neoplastic lesion (Deschl et al. 2001). There is evidence of hepatocellular insult; for example, inflammation, fibrosis, necrosis, and atrophy.

Hepatocellular Foci of Cellular Alteration. Foci of cellular alteration in mice are somewhat similar to those described in the rat (Squire and Levitt 1975) and can be seen in mice exposed to some carcinogens, including benzidine (Frith and Dooley 1976), Aldrin and Dieldrin (Reuber 1976), N-nitrosodimethylamine, N-nitrosodiethylamine (Ward 1984; Ward et al. 1983), and other chemicals (Frith and Ward 1980). They are occasionally seen in control mice. The incidence of hepatocellular foci is less than in rats. The primary alteration involves tinctorial qualities and textural appearances of the cytoplasm and size of hepatocytes. There is no obvious disruption of the liver architecture, and the affected hepatocytes merge with adjacent hepatocytes with little or no compression of adjacent normal parenchyma. The hepatic plates merge imperceptibly with the surrounding hepatocytes. Foci of cellular alteration can be classified as eosinophilic, basophilic, vacuolated, clear cell, or mixed. They might progress to adenomas and occasionally to carcinomas (Frith and Ward 1980; Ward 1984).

Clear cell foci consist of cells with a clear, ground glass, or sometimes a lacy cytoplasm containing much glycogen. The clear areas stain with PAS stain prior to but not after diastase digestion, suggesting the presence of glycogen. The nuclei of the affected cells are not flattened against the cell membrane as in the vacuolated cells, but often are located in the center of the involved cells and surrounded by clear cytoplasm. Often, clear cell foci also contain many hepatocytes with basophilic cytoplasm.

Basophilic foci consist of hepatocytes that have increased basophilia of the cytoplasm. This basophilia is due to free ribosomes or rough endoplasmic reticulum. The cells are usually smaller than normal hepatocytes. Eosinophilic foci are composed of slightly larger hepatocytes than normal with distinct granular, pale pink cytoplasm. Mixed cell foci contain, in varying proportions, two or more of any of the cell types already described.

Features that distinguish hepatocellular adenomas from foci of cellular alteration are cytomorphological features, growth pattern (i.e., loss of normal architecture), and compression of adjacent hepatic parenchyma.

Focal Fatty Change. Focal fatty change consists of focal areas of hepatocytes that contain lipid-laden, distinct cytoplasmic vacuoles of variable size. The nuclei of these vacuolated cells in microscopic sections are either absent or are flattened against the cytoplasmic membrane. Some pathologists use the term vacuolated cell foci of cellular alteration for this lesion.

Neoplastic Lesions

General. Hepatocellular neoplasms—both induced and spontaneous—are among the most common tumors in the mouse. The incidence of liver tumors in mice is very high in some strains, and the incidence can be affected by various factors. Consequently, the mouse liver tumor has been criticized as an inappropriate endpoint in carcinogenesis bioassays. There is probably no other tumor that has created more difficulty and confusion in the interpretation of its morphology, biology, and significance (Frith 1979; Popp 1984; Tomatis et al. 1973; Turusov and Takayama 1979; Ward, Grieserner, et al. 1979).

The terminology used to describe liver tumors in the mouse has been varied and inconsistent. Up to now, some pathologists have considered a hepatocellular neoplasm to be a "true" tumor only

if it metastasizes, whereas others diagnose all mouse liver neoplasms as hepatocellular carcinoma regardless of their morphology and biological behavior.

A variety of terms, including hyperplastic nodule, nodular hyperplasia, neoplastic nodule, hepatoma, liver cell tumor, liver cell adenoma, hepatocellular adenoma, liver cell carcinoma, and hepatocellular carcinoma have been used to describe neoplastic hepatocellular lesions of mice. *Hepatocellular neoplasm* is a collective term used to describe the progressive stages of tumor development from the benign hepatocellular adenoma to the morphologically and biologically malignant hepatocellular carcinoma. Inasmuch as experimental pathologists, particularly those involved in carcinogenicity testing, need to distinguish between nontumors and tumors as well as between benign and malignant tumors, and because special biological and histochemical methods are either not helpful or simply cannot be used in large-scale studies, an attempt has been made to classify hepatocellular neoplasms on a purely morphological basis. The preferred term is hepatocellular adenoma for benign tumors and hepatocellular carcinomas for malignant tumors (Frith and Ward 1980).

Hepatocellular Adenoma. The term *hepatocellular adenoma* is the preferred term for the morphologically and biologically benign liver cell neoplasm (Butler and Newberne 1975; Gellatly 1975; Reuber 1971; Vesselinovitch and Mihailovich 1983; Ward 1984; Ward and Vlahakis 1978). Adenomas are progressively growing focal lesions and might represent early stages in the formation of carcinomas (Frith and Ward 1980; Ward and Vlahakis 1978). Synonyms for this lesion include benign hepatoma, hyperplastic nodule, nodular hyperplasia (Butler and Newberne 1975), type A nodule (Walker et al. 1972), type 1 or 2 nodule (Gellatly 1975), liver tumor (Tomatis et al. 1972), neoplastic nodule (Squire and Levitt 1975), and hepatocellular carcinoma. Hepatocellular adenomas are usually 1 mm to 10 mm in diameter, consist of cells resembling relatively normal hepatocytes, and usually contain cells similar to those in the foci of cellular alteration. Adenomas exist as distinct nodules that compress adjacent parenchyma and might bulge from the liver surface. Histologically, they are composed of a uniform population of well-differentiated cells that form a solid nodule and that might be composed of larger or smaller than normal hepatocytes that have cytoplasm that is basophilic, eosinophilic, or vacuolated. Adenomas can form regular plates one cell thick. They do not invade adjacent parenchyma or vessels, the lesions do not metastasize, and the small nodules have a lower degree of transplantability than do carcinomas (Gellatly 1975; Reuber 1967; Williams et al. 1979). Transplantability is evidence of their neoplastic nature. More recently, they have been shown to be of clonal origin (Rabes et al. 1982) and some tumor cells contain alpha fetoprotein, which is also evidence of neoplasia (Koen et al. 1983). Some chemicals induce either eosinophilic (Hoover et al. 1980; Ward 1984; Ward, Grieserner, et al. 1979) or basophilic adenomas (Vesselinovitch et al. 1978; Ward, Goodman, et al. 1979). Naturally occurring hepatocellular carcinomas can arise within adenomas (Frith and Dooley 1976; Ward 1984; Ward and Vlahakis 1978). These foci of carcinoma within adenomas appear identical to carcinomas described next.

Hepatocellular Carcinoma. The diagnosis of hepatocellular carcinoma is often made on a distinct trabecular or adenoid pattern as well as on cytological features characteristic of malignancy. Synonyms for this lesion include type B nodule (Walker et al. 1972), type 3 nodule (Gellatly 1975), trabecular carcinoma, and malignant hepatoma. The liver cell plates are three or more cell layers thick, irregular, and composed of well to poorly differentiated hepatocytes. The well-differentiated tumors are composed of uniform cells with a fair amount of cytoplasm. The moderately well-differentiated hepatocellular carcinomas are composed of larger hepatocytes that vary more in size and shape and form thickened trabeculae or a solid pattern. The poorly differentiated tumors are composed of cells with less cytoplasm and more immature nuclei forming prominent plates or a solid pattern; some have extremely large anaplastic cells.

The incidence of metastases has generally been considered very low for mouse hepatocellular tumors, but more recent studies indicate that a thorough examination of step or serial sections of

the lung reveals a much higher incidence than previously expected, especially for tumors induced by dimethylnitrosamine (Kyriazis et al. 1974). Metastases are usually uncommon (0%–5%) in mice with spontaneous hepatocellular carcinomas (Butler and Newberne 1975), but they might be seen in up to 40% of male B6C3F1 or C3H mice with hepatocellular carcinoma that are allowed to live out their life span. Metastases usually occur only when tumors are large (over 10 mm) and increased in weight (Frith and Ward 1980). Some chemicals cause highly metastatic tumors, whereas other cause carcinomas with a low metastatic rate (Ward 1984). Pulmonary metastases can share the trabecular pattern often seen in the primary hepatocellular carcinomas, or they can be more solid in appearance.

Hepatoblastoma. Hepatoblastomas have been described in mice as occurring spontaneously and are also experimentally induced. Turusov and Takayama (1979) described liver tumors in mice that resembled human hepatoblastomas. These tumors are almost invariably found within or adjacent to hepatocellular carcinomas and are readily distinguished by their basophilia with H&E stain. The tumors frequently consist of organoid structures lined by vascular channels. The channels are surrounded by several layers of tumor cells arranged either radially or concentrically. In some areas, the cells are arranged in rows, rosettes, sheets, or ribbons. Foci of osseous metaplasia are occasionally seen. The cell of origin for this particular mouse neoplasm is uncertain, although it has been suggested that this type is of fetal origin. Although similar neoplasms occur in children, hepatoblastomas only occur in aged mice. Other pathologists have referred to similar lesions as poorly differentiated cholangiocarcinomas (Reuber 1967) and cholangiomas (Jones 1967; Vlahakis and Heston 1971). Recently, the presence of keratin in hepatoblastomas has been demonstrated in aged mice but not alpha fetoprotein, suggesting their duct or ductular origin rather than their hepatocellular origin.

Cholangioma and Cholangiocarcinoma. Cholangiomas and cholangiocarcinomas are relatively rare in mice compared to the common occurrence of hepatocellular neoplasms (Reuber 1967; Vlahakis and Heston 1971). Some hepatocellular carcinomas assume a distinctly adenoid or glandular pattern suggestive of an adenocarcinoma, yet in some cases, when the transition is not evident, it is sometimes difficult to differentiate a hepatocellular adenocarcinoma from a cholangiocarcinoma. Mixed carcinomas, or carcinomas containing distinct areas of both trabecular hepatocellular carcinoma and cholangiocarcinoma, have also been described.

Metastatic Tumors. Metastatic tumors of the livers are rare compared to metastatic tumors of the lung. The most common are the hematopoietic neoplasms, including lymphoblastic lymphoma and FCC lymphoma. Histiocytic sarcoma can occur as either a metastatic or a primary neoplasm of the liver. Metastatic tumors in the liver include alveolar or bronchiolar adenocarcinoma, urinary bladder transitional cell carcinoma, osteosarcoma, and pancreatic islet cell carcinoma (Frith 1983b).

Gallbladder

Nonneoplastic Lesions

Gall Stones. Gall stones are rare in mice and have not been chemically characterized. Stones are present in the lumen and consist of concentric laminations or lamellae.

Crystals. Distinct eosinophilic crystals of obscure etiology are occasionally seen in the cytoplasm of the epithelial cells and in the lumen of the gallbladder of mice. They may be similar in etiology to those described in the lungs, and could be products of the epithelium, as the acidophilic cytoplasm of the epithelial cells stains and reacts the same as the extracellular crystals.

Neoplastic Lesions

Adenoma. Gallbladder adenomas are rare tumors in the mouse. They consist of papillary projections covering a connective tissue core.

Adenocarcinoma. A single gallbladder carcinoma has been seen in a control mouse at the National Center for Toxicological Research (NCTR), and a small number have been seen in the B6C3F1 mouse (Yoshitomi et al. 1986). These tumors can be broad-based masses or diffuse thickening of the mucosa.

Nervous System

Nonneoplastic Lesions

Cerebral Mineralization

Mineralized deposits detected in the cerebrum generally occur in the region of the medial, ventral, and posterior thalamic nuclei (Morgan et al. 1983). These deposits are very common. However, the incidence is quite variable and can depend on the level of the brain from which the sections were taken. They vary from single, rounded bodies up to 100 μm in diameter to very extensive, irregular, but usually rounded masses, which in most cases are associated with blood vessels. They appear to develop on vascular basement membranes, and focal malacia of the neuropil can result. Focal osseous metaplasia can occur in the neuropil but is more common in the meninges (Radovsky and Mahler 1999).

Hydrocephalus

Hydrocephalus can be congenital or more commonly is associated with obstructed outflow of cerebrospinal fluid, for example, secondary to compression by an underlying pituitary tumor (Radovsky and Mahler 1999). Hydrocephalus usually involves the lateral ventricles, is bilateral, and is characterized by increased fluid in the ventricular system. The ventricles might be so enlarged as to cause a dome shape to the cranium. The sulci and gyri can be flattened and almost indiscernible in advanced cases. Microscopically, the ventricles are enlarged at the expense of the flattened cerebral cortex.

Vacuolization of the White Matter, Central Nervous System

Spongiosis and vacuolization refer to microscopic cystic degeneration of gray or white matter. Vacuoles are a common entity in the white matter of the brains of mice, particularly in the cerebellum. Vacuoles might be present within neuronal cytoplasm, with or without neuronal dropout and reactive astrogliosis, or caused by distension of myelin sheaths indicative of myelin oedema or demyelination (Radovsky and Mahler 1999). It has been suggested that vacuolization might be an artifact associated with the collection and processing of the brain (Wells and Wells 1989). Pathological vacuolization can be difficult to differentiate from artefactual change, but typically vacuoles have less regular outlines and can contain cellular debris (Radovsky and Mahler 1999).

Infarct

Infarcts are rare in the CNS, but occasionally occur in the cerebrum. They more commonly occur at the surface or less frequently deeper within the parenchyma.

Developmental Abnormalities

Epithelial inclusion cysts are a relatively common developmental abnormality of the brain and spinal cord, and there can be considerable variation in size. Cysts are lined by flattened squamous epithelium and are filled with eosinophilic desquamated keratin. There are seldom any associated clinical signs.

Adipose tissue accumulations (lipomas) have been seen associated both with the meninges and choroid plexus (Morgan et al. 1984). They consist principally of discrete, well-demarcated clusters or masses of mature adipocytes. They can be present in the interstitium of the choroid plexus of the lateral ventricles. Occasionally, a small mass of cartilage-like material is present among the fat cells. The cell or tissue of origin of these lesions is unknown. Others consider these lesions to be a developmental abnormality (Radovsky and Mahler 1999).

Neoplastic Lesions

A number of cell specific markers for the murine nervous system, are useful in differentiation of tumors and include neurofilament markers for neurons, glial fibrillary acidic protein (GFAP) for glial cells, and myelin basic protein (MBP) for oligodendrocytes and Schwann cells. Neuron-specific enolase (NSE) and S-100 protein are less specific markers for neural tissue.

Oligodendroglioma

Tumors of the CNS of the mouse are extremely rare lesions (Morgan et al. 1984). The most common tumor is the oligodendroglioma, which occurs in the cerebrum or diencephalon. It is usually ventrolateral in location and involves much of the thalamus, hypothalamus, and amygdaloid. The oligodendroglioma comprises a poorly demarcated mass of proliferating oligodendrocytes with variable degrees of neuronal satellitosis, nuclear palisading, and mitoses. These cells have scanty cytoplasm, with a distinct perinuclear halo in some cases; the single round to oval nuclei are often hyperchromatic. With immunohistochemical staining they are positive for MBP.

Astrocytoma

Astrocytomas occur much less frequently than oligodendrogliomas in mice. The neoplasms comprise cells with indistinct cytoplasmic boundaries and large, oval, or slightly folded nuclei. The margins of the tumor mass are indistinct and there might be areas of edema, hemorrhage, and microcystic damage. Hemorrhage and necrosis are seen more frequently in astrocytomas than in oligodendrogliomas. Differential diagnoses should include benign mixed glioma and reactive gliosis (Krinke et al. 2001).

Malignant astrocytoma extends over multiple areas in the CNS and can be multicentric. Invasive growth into perivascular spaces and meninges is characteristic of these neoplasms. There is a high degree of cellular atypia, pleomorphism, necrosis, and hemorrhage.

Astrocytomas in mice, unlike those in man, are commonly GFAP negative, despite their fibrillary differentiation.

Meningioma

Meningiomas are typically discrete, expansile masses overlying the brain or spinal cord. They occur rarely in the mouse and can assume a variety of patterns as in other species. These neoplasms comprise a regular pattern of loosely interwoven bundles of delicate spindle cells, with single small hyperchromatic oval nuclei. The nuclei in some areas palisade in an irregular fashion. These cells lie in a faintly basophilic, finely granular ground substance. In some cases, the neoplasms infiltrate

the ventral brain along the adventitia of small blood vessels. Malignant meningioma is diagnosed when there is evidence of local invasion. Major differential diagnoses are metastatic sarcoma and systemic histiocytic sarcoma (Radovsky and Mahler 1999).

Metastatic Tumors

Leukemias and lymphomas commonly involve the brain. Mesenchymal tumors (sarcomas) can also occasionally spread to the brain. Carcinoma of the pituitary gland and nasal cavity can extend locally to involve adjacent brain.

Respiratory System

Upper Respiratory Tract (Nasal Cavity, Larynx, Trachea)

Spontaneous nonneoplastic and neoplastic lesions of the nasal cavity, larynx, and trachea are uncommon in mice.

Lung

Nonneoplastic Lesions

Alveolar Epithelial Hyperplasia. Alveolar epithelial hyperplasia is characterized by a single layer of noncillated cuboidal cells lining all or part of the alveolar wall of the lung. The cells are found on the scaffold of existing stroma. There are no papillary projections into the lumens nor are there solid masses of cells filling alveolar spaces. The proliferating cells are type II pneumocytes (Goodman et al. 1981). These lesions can be precursors of alveolar or bronchiolar neoplasms.

Preneoplastic hyperplasia is not associated with inflammation and necrosis and the parenchymal architecture is usually maintained, which differentiates this lesion from regenerative hyperplasia (Dixon et al. 1999). Regenerative hyperplasia of the alveolar type II pneumocytes or airway epithelium commonly occurs in response to cell loss due to injury or cell death.

Alveolar Hemorrhage. Acute alveolar hemorrhage is a common agonal finding in mice (Percy and Barthold 2001). It is differentiated from older hemorrhage or chronic heart failure by the absence of pigment (hemosiderin)-laden macrophages in the lumina.

Pulmonary Histiocytosis. Focal accumulations of lipid-laden macrophages (some of which might contain cholesterol lefts) are frequently observed in the subpleural areas of aging mice of all types (Percy and Barthold 2001).

Alveolar Lipoproteinosis. Alveolar lipoproteinosis is characterized by intra-alveolar accumulations of vacuolated macrophages (pulmonary phospholipidosis).

Crystal Pneumonitis. Crystal pneumonitis is characterized by variably sized crystals, within macrophages or free in lumina of terminal airways, alveolar ducts, alveolar lumina, and bronchiolar glands in certain strains of mice, particularly aging B6 mice (Percy and Barthold 2001). Immunodeficiency is associated with accelerated lesion formation. Crystals are composed of breakdown products of granulocytes, particularly eosinophils (Murray and Luz 1990). Lesions could be extensive enough to cause dyspnoea.

Inflammatory Lesions of the Lung. Differentiation of lesions associated with underlying infectious disease from those of toxic origin is obviously of critical importance in interpretation of

toxicological bioassays. The presence of infectious agents can also influence the toxic or neoplastic outcome of chemical administration (Dixon et al. 1999). The lesions associated with the principal infectious agents of rodent respiratory tract are outlined next.

Sendai Virus. Sendai virus is an extremely contagious pulmonary disease of mice caused by an RNA virus (Paramyxovirus). Its prevelance is considered to be high worldwide. Grossly, the lungs are plum-colored, atelectic, and exude a serosanginous fluid on cut surface. Microscopically, the lesions consist of necrotizing bronchitis and bronchiolitis with the formation of polypoid proliferations and proliferative alveolitis.

Mycoplasma pulmonis (Murine Respiratory Mycoplasmosis). *Mycoplasma pulmonis* is a gram-negative bacterium that preferentially colonizes the luminal surface of respiratory epithelia. It has also been incriminated in natural infections of the genital tract in rodents. Lung lesions are characterized by a purulent bronchopneumonia with bronchiectasis.

Corynebacterium kutscheri. *C. kutscheri* is a gram-positive bacterium causing septicemia with bacterial emboli in the liver, kidney, lungs, skin, and joints.

Cilia-Associated Respiratory Bacillus (CAR bacillus). *CAR bacillus* is a gram-negative bacterium causing respiratory disease similar to severe murine respiratory mycoplasmosis.

Pneumocystis carnii. This opportunistic organism affects immunosuppressed or immunocompromised animals. Histologically alveoli are filled with eosinophilic foamy material, septae are usually thickened, and there are few inflammatory cells.

Lymphocytic Infiltrates. Lymphoid tissue is normally present in association with the bronchi. Lymphocytic infiltrates are very common around blood vessels and bronchioles or in the pleura as focal lesions. These lesions are usually minimal to mild in severity and are not diagnosed by some pathologists. They probably represent a response to antigenic stimuli, such as previous viral infection. Such infiltrates are also present as part of a generalized perivascular change in lymphoproliferative disorders.

Neoplastic Lesions

General. The most common lung tumors observed in B6C3F1 and CD-1 mice are alveolar and bronchiolar adenomas and carcinomas. Many of these arise from type II pneumocytes. Tumors arising from Clara cell have also been described (Kauffman and Sato 1985a, 1985b; Theiss and Shimkin 1982). On H&E sections, these two tumor types can be difficult to differentiate morphologically. Consequently, in most toxicology studies, the terms alveolar/bronchiolar (A/B) adenoma and carcinoma are used routinely. Squamous cell carcinoma of the lung can be induced by various agents, but are extremely uncommon spontaneous tumors.

Alveolar/Bronchiolar Adenoma. A/B adenomas microscopically might be circumscribed and compress adjacent tissue or the edges can be irregular where the neoplastic cells extend into adjacent airways. The lesion is composed of cells with a normal nuclear/cytoplasmic ratio that are cuboidal or columnar and resemble normal lining cells of the lower airway. Proliferating cells can form papillary projections into alveoli or completely fill them. The central portions of these tumors tend to be more solid than the periphery.

Alveolar/Bronchiolar Carcinoma

A/B carcinomas tend to be larger than adenomas. There is cellular and nuclear pleomorphism, increased nuclear/cytoplasmic ratio, hyperchromatic nuclei, and an increase in mitotic figures. There might be multiple solid areas within the tumor; invasion of airways, blood vessels, lymphatics, and pleural surfaces; extension throughout the mediastinum; and coelomic and distant metastases. Necrosis is common, and when these tumors metastasize, they can have a sarcomatous pattern.

Metastatic Tumors. The lung is the most common site of metastasis of neoplasms in rodents (Dixon et al. 1999). Mammary gland neoplasms and systemic hematopoietic tumors are the two most frequently observed metastatic tumors (Sass and Liebelt 1985; Vaage 1988). Metastatic mammary gland neoplasms must be differentiated from A/B adenocarcinoma of the lung.

Special Senses

Eye

Nonneoplastic Lesions

Nonneoplastic lesions of the eye are occasionally observed in aged CD-1 and B6C3F1 mice. Most commonly observed, although infrequent and sporadic in incidence, are cataracts and retinal atrophy.

Retinal Atrophy. Age-related retinal atrophy in the mouse can be unilateral or bilateral and is characterized by loss of the photoreceptors (predominately rods) and outer nuclear layers. The lesion can progress to loss of the second or third layer of neurons, eventually resulting in a thin fibrous layer containing glial cells and occasional neurons (Geiss and Yoshitomi 1999). Inherited retinal degeneration has been described in mutant and transgenic mice (Gregory and Bird 1995; Sidman and Green 1995; Smith 1992).

The rodent retina, particularly that of the albino strains, is sensitive to increased light intensity (Greenman et al. 1982). Due to its pigmentation, the incidence in the B6C3F1 mouse is less than that in the albino BALB/c mouse (LaVail et al. 1987). Such light-induced changes are morphologically difficult to differentiate from early age-related changes.

Cataract. Cataract is occasionally seen in the lens of the eyes of mice. Morphologically, cataracts appear as multiple globoid circumscribed bodies within the substance of the lens, especially at the periphery or capsular surface. Histologically, all degenerative lesions of the lens capsule, epithelium, or fibers resulting in reduced light permeability or opacity are termed cataracts (Geiss and Yoshitomi 1999).

Early lenticular degeneration consists of irregular swelling of the epithelium or fiber cells with a granular or vacuolated cytoplasm, progressing to liquefaction and Morgagnian globule formation, and, often, dystrophic mineralization.

Senile cataract formation is associated with decreased superoxide dismutase activity and increased free-radical-induced damage.

Accumulations of polyol and glycolation of protein are important in the pathogenesis of cataract formation in diabetic mice.

Neoplastic Lesions

Primary tumors of the eye are extremely rare in all mouse strains (Squire et al. 1978). Secondary tumors such as malignant lymphoma and Harderian gland carcinoma can spread to the eye (Geiss and Yoshitomi 1999).

Ear

Vestibular Syndrome

Signs of vestibular disease such as head tilt and circling are common in mice. Causes include bacterial otitis, CNS disease, and necrotizing arteritis. The latter is characterized by segmental necrosis and inflammation of medium-sized arteries in tissue surrounding the structurally normal internal and middle ear.

The aetiology of the arteritis is unknown but in some strains it might be immune-mediated (Andrews et al. 1994; Plendl et al. 1996).

Harderian Gland

Nonneoplastic Lesions

Small focal lymphocytic infiltrates and ectasia of the ducts are occasionally observed.

Neoplastic Lesions

Harderian Gland Adenoma. These tumors are frequently well-demarcated lesions that cause compression of the adjacent parenchyma, but usually do not have a well-defined capsule. The architecture consists of pseudoglandular structures from which arborizing and folded fingerlike fronds project into the lumen. The fronds are composed of a delicate fibrovascular core covered by cells that are usually tall columnar with foamy amphophilic cytoplasm. The cells usually form a single layer, but areas are frequently present that give the appearance of having a basal layer of normal-appearing cells "capped" by smaller cells adjacent to the lumen.

Harderian Gland Adenocarcinoma. These tumors can be well differentiated, differing from adenomas by greater cellular atypia, invasion, and metastases. Undifferentiated adenocarcinomas contain areas of varying size that are solid and composed of pleomorphic cells, many of which contain a single large vacuole. Mitotic figures are uncommon.

Urinary System

Kidney

General

Mouse Urinary Protein. Mouse urinary protein (MUP) is a protein similar to α 2u-globulin produced by male rats but unlike α 2u-globulin, it is not reabsorbed by the kidney. Hyaline droplet nephropathy therefore does not occur in the mouse (Lehman-McKeeman and Caudill 1992).

Sexual Dimorphism of the Kidney. The glomerular tuft of the adult male mouse kidney of certain strains has a unique morphological characteristic that is not a pathological lesion. In females, the parietal layer of Bowman's capsule is composed of a single layer of flattened epithelium. In the male mouse, the parietal layer of most of the glomeruli is composed of simple cuboidal epithelium. It is not present in each glomerulus and the proportion of glomeruli showing this characteristic varies in different strains. The presence of cuboidal epithelium is thought to be due to metaplasia of flattened cells under the influence of testosterone (Leibelt 1986).

This characteristic is seen only in adult male mice possibly associated with their greater requirement for reabsorption of albumin and prealbumin. Proteinuria, due to the high albumin and

prealbumin content, is normally seen in mice but is increased in mature males because of the influence of testosterone (Percy and Barthold 1993).

Congenital Lesions. Congenital lesions of the kidney vary among strains. Agenesis, fusion, hypoplasia, and displacements have been reported. Polycystic renal disease has been reported in several mouse strains.

Double Renal Pelvis. The kidney of the mouse contains a single large renal papilla surrounded by the renal pelvis. Very rarely, a congenital anomaly consisting of a double renal pelvis can occur.

Hydronephrosis. Hydronephrosis is fluid-induced dilation of the renal pelvis, which can be minimal, resulting in only a slight dilation of the renal pelvis, to severe, causing marked compression atrophy of the kidney. It can be unilateral or bilateral, congenital or acquired, and frequently results in tubular dilatation. A high incidence of congenital hydronephrosis occurs in the C3H, C57L, and DDD strains. Progressive renal failure has been reported in association with congenital hydronephrosis in C57BL/6 mice (Horton et al. 1988).

Aquired hydronephrosis might follow obstruction of one or both ureters or the urethra due to calculi, tumors, or inflammation (Bendele and Carlton 1986; Carlton and Gries 1983). Papillary necrosis secondary to amyloidosis can also result in hydronephrosis.

Degenerative Lesions

Nephropathy. Chronic nephropathy similar to that seen in rats also occurs in mice (Montgomery 1986; Wolf and Hard 1996) but lesions tend to be less severe. The condition can occur spontaneously or be drug induced (Wolf 1996).

The incidence and severity of lesions is greater in males than in females. Microscopic changes include focal to multifocal tubular basophilia and nuclear crowding. Tubular basement membranes might be thickened, and in more advanced lesions eosinophilic proteinaceous casts are present within the lumina (Wolf 1996). The interstitium can contain variable mononuclear cell infiltrates. Glomerular changes progress from hypercellularity of the glomerular tuft and dilatation of Bowmans space to glomerulosclerosis.

Hyaline Glomerulonephropathy. Eosinophilic hyalinization of glomeruli associated with PAS positive subendothelial deposits resembling immune mediated glomerulonephritis has been reported in B6C3F1 mice (Wojcinski et al. 1991).

Renal Papillary Necrosis. Necrosis of the renal papilla can occur in the mouse kidney and is usually associated with renal amyloidosis (Frith and Ward 1988) or toxins. Papillary necrosis can be seen secondary to ascending pyelonephritis such as occurs following bite wounds in group-housed males.

Renal Mineralization. The degree of renal mineralization varies among strains and does not appear to have a sex predisposition in mice as it does in rats. Mineralization is seen microscopically as densely basophilic material associated with tubular basement membranes or blood vessels, particularly in the corticomedullary junction. Intraluminal mineral can also be seen. Renal calculi can occur within the renal pelvis (Frith and Ward 1988).

Renal Infarct. Renal infarcts in the mouse are morphologically comparable to renal infarcts in other species. They commonly appear as triangular-shaped areas of coagulation necrosis that extend from the capsular surface through the cortex to or into the medulla. The tubules can be intensely eosinophilic, and fibrosis might be present in older lesions. The surface of the infarct is depressed

below the normal renal surface. In recent lesions, polymorphonuclear leukocytes might infiltrate the border and some tubules can contain hemoglobin casts. Infarction might occur secondary to arteriothrombosis, disseminated intravascular coagulation, pyelonephritis, amyloidosis, or neoplasia (Seely 1999).

Neoplastic Lesions

The most common renal proliferative changes are tubular epithelial hyperplasia, adenoma, and carcinoma. Renal cell adenoma and carcinoma can occur spontaneously (Shinohara and Frith 1980) or can be induced.

A progression of change from hyperplasia through adenoma to carcinoma can be seen with administration of renal carcinogens but is not inevitable ((Shinohara and Frith 1980). Renal sarcomas have been induced in mice by polyoma virus infection (Frith et al. 1994).

Renal Tubular Hyperplasia. Renal tubular hyperplasia has been interpreted as a precursor of neoplasia on several occasions (Shinohara and Frith 1980; Terracini et al. 1966; Terracini and Testa 1970). Single or multiple foci of change involving a single tubule can be seen. There is a lack of compression of surrounding parenchyma and lesions are less than three times the diameter of a normal tubule. Hyperplastic epithelial cells, with a normal to minimally pleomorphic appearance, partially to completely fill the lumen. Occasionally lumina might be dilated (Seely 1999).
Tubular hyperplasia should be differentiated from tubular epithelial hypertrophy consisting of normal numbers of enlarged, typically eosinophilic tubular epithelial cells, and from adenoma.

Tubular Cell Adenoma. Most renal tubular cell adenomas are single lesions that are sharply defined, often with some compression of the surrounding parenchyma and greater than five tubules in diameter. They can be morphologically classified as cystic, papillary, or solid (Shinohara and Frith 1980). The papillary type is the most common. Several growth patterns can coexist within the same tumor.

The cells forming the adenomas are cuboidal with eosinophilic, basophilic, or clear cytoplasm and relatively small nuclei. There is minimal pleomorphism with some variation in cell size. Mitotic figures are rare.

Renal Tubular Cell Carcinoma. Renal tubular cell carcinomas tend to be larger than adenomas, with marked compression or infiltration of surrounding parenchyma. There might be marked hemorrhage and necrosis. Histologically, they can be solid, cystic, papillary, mixed, or anaplastic in appearance. Component epithelial cells can have eosinophilic, basophilic, or clear cytoplasm and vary from small and uniform to large and pleomorphic. The nuclei are small and round or oval to highly pleomorphic with prominent nucleoli. The mitotic index in the tubular cell carcinomas varies from tumor to tumor (Seely 1999). Some of these tumors contain areas of sarcomatous change with multinucleated giant cells (Frith et al. 1994). Carcinomas occasionally metastasize to the lungs.

Nephroblastoma. Nephroblastomas are rare tumors of undifferentiated (blastema) cells that might contain primitive tubular and glomerular structures (Leibelt et al. 1989; Turusov 1992).

Neoplastic Associated Changes. Eosinophilic, hyaline droplets consisting of lysozyme secreted by monocytes and macrophages (Hard and Snowden 1991) are found within renal tubules in association with histiocytic sarcoma of the kidney (Luz and Murray 1991).

Renal Toxicity. The kidneys are highly susceptible to toxic injury by virtue of their large blood supply; their functions of filtration, concentration, and excretion of xenobiotics; and metabolic capability resulting in tubular enzymatic bioactivation of some xenobiotics (Stine and Brown 1996).

Renal tubular toxic changes are most readily recognized morphologically and comprise necrosis and degeneration and regeneration, particularly of the proximal convoluted tubule (Seely 1999). Degeneration is characterized by increased cytoplasmic eosinophilia or vacuolation. In tubular necrosis there is intense cytoplasmic eosinophilia, nuclear pyknosis or karyorrhexis, and epithelial desquamation into tubular lumina resulting in eosinophilic tubular casts. Regenerative changes comprise increased tubular basophilia, with nuclear crowding. Cytomegaly, karyomegaly, and dysplasia have also been reported (Abdo et al. 1984; Nakanishi et al. 1982). Increased mineralization in female mice has been reported with administration of 11-aminoundecanoic acid (Dunnick et al. 1983). Papillary necrosis has been reported in association with chemical administration in mice (Wolf et al. 1992).

Ureter

Nonneoplastic Lesions

Ureteral Diverticulum. Diverticula are not common spontaneous lesions in the mouse but have been associated with inflammation and the administration of 4-ethylsulfonyl naphthelene-l-sulfonamide (ENS), acetazolasmide, and oxamide (Frith et al. 1984; Jackson et al. 1979). The lesion exists as a downgrowth into the ureteral wall that might be confused with a carcinoma. Downgrowths of the surface epithelium can extend through the muscularis to the adventitia. The lesion appears to be associated with crystalluria.

Urinary Bladder

Nonneoplastic Lesions

Bladder Calculi. Urinary calculi can occur spontaneously or can be associated with the administration of compounds such as ENS (Frith et al. 1984; Jackson et al. 1979). Calculi are usually accompanied by chronic cystitis, and could result in papillary and nodular urothelial hyperplasia.

Lymphoid Aggregates. Focal lymphoid aggregates can occur in the lamina propria of the urinary bladder. Their incidence and severity increase with age.

Urothelial Hyperplasia. Hyperplasia of the transitional epithelium of the mouse urinary bladder is a rare spontaneous lesion, but a common finding in association with chronic cystitis, calculi formation, and toxic compounds such as cyclophosphamide and methyl or ethyl methanesulfonate.

Hyperplasia is also seen in animals treated with bladder carcinogens and can be considered a preneoplastic lesion with the potential to progress to a tumor (Koss 1977). Hyperplasia can be focal, multifocal, diffuse, simple, papillary, or nodular (Frith 1979).

Papillary hyperplasia is associated with thickening and folding of subjacent lamina propria and tends to be multifocal to diffuse, differentiating this lesion from the usually solitary transitional cell papilloma (Gaillard 1999).

Nodular hyperplasia (transitional epithelial downgrowths extending into the lamina propria) can occur in conjunction with either simple or papillary hyperplasia and can be focal or multifocal. Nodular hyperplasia is comparable morphologically to von Brunn's nests or cystitis cystica in humans (Frith 1979). Although the areas of nodular hyperplasia often appear to have no connection with the surface epithelium, serial sections usually reveal such a connection. The lesion might regress if the etiological stimulus is removed (Frith and Rule 1978).

Urothelial Metaplasia. Urothelial transitional epithelium can undergo metaplasia to squamous or rarely glandular epithelium, in association with inflammation, hyperplasia, or neoplasia (Greaves 1998; Roe 1964).

Neoplastic Lesions

Urinary Bladder Papilloma. Naturally occurring papillomas of the urinary bladder are rare in mice. The epithelium shows no plemorphism, atypia, or anaplasia and is well differentiated. Papillomas induced with 2-acetylaminofluorene (2-AAF) might have a slender narrow stalk or a broad base (Frith 1979).

Urinary Bladder Carcinoma. Naturally occurring bladder carcinomas are extremely rare in mice (Frith, Farmer, et al. 1980). Experimentally induced malignant neoplasms of the urothelium can be classified according to histological pattern, cell type, and depth of invasion. Malignant epithelial lesions classified by histological pattern can be divided into papillary and nonpapillary (solid, polypoid) carcinomas. Papillary carcinomas project into the lumen and might or might not show invasion. Nonpapillary or solid carcinomas invade the bladder wall.

Bladder carcinomas classified according to histological cell type are divided into transitional cell, transitional cell with squamous metaplasia, squamous, undifferentiated, and adenocarcinoma types. The most common type induced is the transitional cell carcinoma.

Musculoskeletal System

Bone

Nonneoplastic Lesions

Fibro-osseous Lesion, Bone. This lesion has been called by a variety of terms: fibroosseous lesion, myelofibrosis, senile osteodystrophy, or osteoporosis (Burek et al. 1982; Frith and Ward 1988; Goodman et al. 1981; Sass and Montali 1980). This lesion is seen in the sternum and other bones in aging mice, particularly in females. There is replacement of all or part of the marrow cavity with an eosinophilic fibrillar matrix containing abundant collagen fibers. Fibroblast-like cells and osteoclasts are present throughout the matrix. Small foci of normal marrow might be present. Although the lesion resembles fibrous osteodystrophy associated with renal disease, the pathogenesis appears to be different as both kidneys and parathyroids are normal. Similar lesions have been induced with estrogen administration.

Anklylosis. Ankylosis of the hock joints has been reported in group-caged male B6C3F1mice (Rao and Lindsey 1988). The incidence of ankylosis increases rapidly after 6 months of age. The cause is unknown but the incidence decreased when mice were individually caged. The joints are enlarged. Microscopically, there are bony proliferations (exostoses) on the tarsal bones. There is bridging of the joint and, in severe cases, obliteration of the joint with new bone. Chronic exposure of CD-1 mice to sodium fluoride is associated with ankylosis of the stifle joint (Maurer et al. 1993).

Bone Atrophy. Bone atrophy is the proportional loss of mineralized and unmineralized matrix resulting from decreased formation or increased resorption of bone. It can occur as a spontaneous age-related lesion, predominately in females. Localized bone loss can occur secondary to immobilization (Long and Leininger 1999). Senescence-accelerated mouse (SAM) strains have accelerated bone loss and associated spontaneous fractures (Takahashi et al. 1994).

Other Nonneoplastic Lesions. Osteoarthrosis and bone necrosis with associated fractures have also been reported sporadically (Burek et al. 1982).

Neoplastic Lesions

Osteomas and osteosarcomas are occasionally observed in mice. Oncogenic tumor virus-induced osteomas can occur spontaneously in a number of different strains of mice (Hoger et al. 1994;

Leib-Mosch et al. 1986). Activation of these viruses is thought to be associated with osteoma of the skull in mice treated with estradiol or gold thioglucose (Nilsson and Stanton 1994). Osteosarcomas are characterized by formation of osteoid by malignant stromal cells and might exhibit a high level of anaplasia and mitotic activity. Lung metastases are common (Long and Leininger 1999). Some osteosarcomas (as evidenced by the presence of metastases) can resemble osteoma histologically (Nilsson and Stanton 1994).

Cartilaginous tumors, chondromas, and chondrosarcomas are extremely rare in mice (Lombard 1982).

Chondromas are expansile, well-circumscribed masses composed of irregular lobules of cartilage, containing well-differentiated but disorganized chondrocytes. Chondromas with areas of osseous metaplasia and/or chondroid formation should be differentiated from osteoma (Long and Leininger 1999).

Chondrosarcomas are lobulated, highly cellular tumors with occasional pleomorphic, binucleate, or multinucleate giant cartilage cells, and rare mitotic figures. More anaplastic tumors contain spindle-shaped cells ressembling fetal chondroblasts. There is minimal invasion (Ernst, Long, et al. 2001).

Skeletal Muscle

Both neoplastic and nonneoplastic spontaneous lesions of skeletal muscle are uncommon in laboratory mice (Burek et al. 1982; Lombard 1982; Squire et al. 1978).

Multiple Systems

Amyloidosis

The CD-1 mouse is widely used for chronic toxicology studies. The major cause of death of aged CD-1 mice is amyloidosis. Amyloidosis occurs much less frequently in other strains of mice. It is uncommon in B6C3F1 mice. The incidence can be increased in association with fighting among group-housed males and with ectoparasitism (Percy and Barthold 2001).

The disease can be generalized (systemic) or localized. The systemic forms of amyloid can be primary or secondary. Primary myeloma associated amyloid comprises fragments of immunoglobulin light chains (AL). Secondary amyloid is derived from serum amyloid A (SAA); polymorphic, acute phase response proteins produced by the liver in response to release of monokines (including IL-1 and TNF) by macrophages during inflammation. Secondary amyloidosis can occur spontaneously in laboratory mice, related to chronic inflammation and acariasis.

Generalized amyloid deposits can be seen in many tissues. Amyloid deposition in the small intestine and glandular stomach generally occurs in the lamina propria and submucosa. In the adrenal glands, amyloid deposits are common in the inner cortex surrounding the medulla. Lymph node involvement is primarily in the mesenteric lymph node and the amyloid deposits occur at the periphery of the node in the subcapsular sinuses. Amyloid deposits in the thyroid and parathyroid involve the interstitium in a diffuse fashion. Amyloid deposits in the kidney primarily involve the glomeruli, but also the interstitium. Splenic perifollicular areas, hepatic periportal areas, pulmonary alveolar septae, male and female reproductive organs, myocardium, and aorta can also be involved.

Involvement of the glomeruli of the kidneys is usually the cause of death in animals that die with amyloidosis. Amyloidosis is often associated with atrial thrombosis and left or right congestive heart failure. Mice with amyloid deposits in the renal medullary interstitium can develop papillary necrosis.

Localized forms of amyloid, all with differing composition, are formed in the brain in association with endocrine tumors. Localized amyloidosis of ovarian corpora lutea is frequently seen in CBA and DBA mice in the absence of the systemic disease.

Morphology

Amyloid is a chemically diverse family of proteins characterized by a beta-pleated sheet molecular conformation, which imparts its characteristic staining properties. With the light microscope and H&E stain, amyloid appears as an amorphous, eosinophilic, hyaline, extracellular substance, with progressive accumulations encroaching on and producing pressure atrophy of adjacent cells. The most common stain used to confirm amyloid is Congo red, which imparts a pink or red color to amyloid and results in a green birefringence with polarized light. Special stains sometimes used to confirm the presence of amyloid include thioflavin T (secondary fluorescence with ultraviolet light) and metachromasia with crystal or methyl violet.

Genetically Engineered Mice in Toxicology

For many years, the 2-year, two-species (rat and mouse) bioassay has been the accepted standard for carcinogenicity studies intended for submission to regulatory agencies. For some time, however, there has been debate in the profession as to the utility of the traditional mouse bioassay. It has been shown that about half of all known human carcinogens are positive in both rat and mouse, and that very few human carcinogens have been identified only in the mouse. A number of potential alternatives to identify carcinogens have been suggested and explored. These include fish models, structure–activity relationships, artificial intelligence programs, receptor-mediated models, the infant mouse model, the rat liver initiation and promotion model, and genetically engineered mice.

In the past few years, there has been an incredible proliferation of genetically engineered mouse models. These include transgenic mice, in which foreign genetic material is inserted into the mouse genome and remains functional; as well as knockout mice, in which one (heterozygous) or both (homozygous) alleles of a gene of interest are "knocked out." Although most genetically engineered mice are used in basic research, several different models show promise in the area of regulatory toxicology. The advantages of these genetically engineered mouse models when compared to the 2-year bioassay is that they are faster (due to decreased tumor latency), cheaper (fewer animals required with shorter protocols), they require lower exposures, they might minimize strain differences, and they are often targeted toward a specific mechanism of tumor formation. The principal hypothesis regarding the use of genetically engineered mice is that the models represent mice with specific genetic alterations critical to tumorigenesis, but that by themselves are insufficient to produce tumors before the end of a short (generally 6-month) study. Thus the exposure of these mice to transpecies carcinogens would be expected to result in the rapid induction of compound-specific tumors.

Several large validation studies have been completed in the past several years by the National Institute of Environmental Health Sciences (NIEHS) and the International Life Sciences Institute (ILSI) consortium for Alternatives to Carcinogenicity Testing (ACT), including more than 50 industrial, government, and academic laboratories in the United States, Europe, and Japan. The ILSI results were published in a supplement to the journal *Toxicologic Pathology* (ILSI/HESI 2001). At this time, several models appear to be the best candidates for use in short-term bioassays, either as replacements for the 2-year mouse bioassay, or as additional studies to be used in the weight of evidence for a submission (Mahler et al. 1998; Maronpot et al. 2000).

The three models discussed next have all been through one or more validation studies and have been used as part of submission packages for pharmaceuticals or chemicals both in the United States and in other countries.

p53+/- Knockout Mouse

This knockout mouse has a single wild-type p53 allele and one inactivated allele of p53. p53 is a tumor suppressor gene that is important in repairing DNA. It has been called the gatekeeper

of the genome that, in the presence of DNA damage, causes arrest of the cell cycle in G1 to allow time for DNA repair. When DNA repair is successful the cell cycle is allowed to progress. If repair does not occur cells are directed by p53 to undergo apoptosis. With one copy of p53 inactivated, the p53 heterozygous mice are therefore more susceptible to carcinogens, because it only takes a loss or mutation of the remaining copy of wild type p53 to result in loss of repair function with subsequent tumor development. p53 mice respond to genotoxic carcinogens by the development of tumors. The p53 heterozygous knockout is most often produced on a C57BL/6 background; however at least five different strains have been used, resulting in different (strain-related) spontaneous tumors.

Spontaneous tumors include a number of sarcomas: subcutaneous sarcomas, osteosarcomas, meningeal sarcomas, and urinary bladder sarcomas, as well as A/B adenomas in the lung, granulocytic leukemia, and malignant lymphoma (Mahler et al. 1998).

The subcutaneous sarcoma complex generally presents as masses of mesenchymal cells in sheets, fascicles, or whorling patterns. These tumors might differentiate to fibrosarcomas, myxosarcomas, rhabdomyosarcomas, and hemangiosarcomas, or they might be so poorly differentiated as to require a diagnosis of subcutaneous sarcoma. They occur spontaneously, and have been induced by melphalan administered intraperitoneally, by subcutaneous injection of test compounds, and by the use of subcutaneous microchips used for identification purposes.

Malignant lymphoma has been reported in about 2% of control male and female p53 heterozygous mice. Increased rates of malignant lymphoma in p53 heterozygous mice were associated with exposure to melphalan. The lymphoma is usually thymic in origin, and a thymic mass or enlargement is the most common gross manifestation.

The neoplastic cells are large lymphoblasts with sparse cytoplasm, round to vesicular nuclei, and one or more nucleoli. Mitotic figures are common. The tumor often becomes systemic, with neoplastic cells present in the spleen, lymph nodes, lung, heart, liver, and kidney.

An unusual lesion reported in the p53 knockout mouse is atypical hyperplasia of the thymus. There is an absence of a distinction between thymic cortex and medulla. One or both lobes of the thymus can be affected. The lobes are smaller, and contain sheets of large atypical lymphocytes, which appear neoplastic mixed with smaller, more normal lymphocytes. There is a proliferation of this mixed cell population, but it is characteristic that it does not extend beyond the capsule. It has been suggested that affected thymuses first undergo atrophy, followed by a proliferation of remaining cells. This lesion has been induced by several unrelated compounds and is considered to be a preneoplastic, precursor lesion of thymic lymphoma.

Tg.AC Mouse

The Tg.AC transgenic mouse uses the FVB/N background, and has a mutated Harvey-ras oncogene, with multiple copies on chromosome 11. The expression of this transgene is inducible by carcinogens. The transgene expresses in the skin, which is genetically initiated by the presence of the transgene. The induction of multiple squamous papillomas following application by skin painting represents the reporter phenotype.

There are many spontaneous tumors present in Tg.AC mice at 6 months of age. Significantly, the incidence of skin papillomas is around 4%, so that the presence of papillomas is a reliable indicator of a positive response. Other spontaneous tumors include papillomas of the forestomach, and odontogenic tumors, which can present as ameloblastomas with anastamosing cords of squamous cells surrounded by loose undifferentiated stroma, or odontomas with abortive tooth structures formed by enamel, dentin, and well differentiated ameloblasts and odontoblasts. There are also benign and malignant A/B tumors in the lung, and a carcinoma of the duct of the submandibular salivary glands, which grossly resembles the tooth tumors, and often replaces the salivary gland with a cystic structure lined by well-differentiated nonkeratinizing stratified squamous epithelium. Systemic neoplasms include erythroleukemia, and malignant lymphoma, which generally arises in

the thymus, and presents as a well-differentiated lymphocytic lymphoma. With the exception of the odontogenic tumors and the forestomach papillomas, all of these tumors are present at an extremely low incidence (< 5%).

Induced tumors are present at the site of application, and consist primarily of multiple squamous papillomas. The original intention with the Tg.AC mouse was to count the papillomas, without any additional pathologic evaluation. Generally speaking, the number of papillomas and the time of onset are recorded up to 30 tumors. Occasionally, these tumors present as keratoacanthomas, or transform from papillomas to squamous cell carcinomas. A few cases of metastasis have been reported.

In addition to the induction of skin papillomas, it has been shown that carcinogens can induce an increase in the number and size of squamous papillomas of the forestomach, as well as the incidence of malignant lymphoma. An unusual lesion described as myelodysplasia has also shown to be induced by some chemicals. This lesion presents as a wide spectrum of changes in the liver and spleen, and might resemble exuberant extramedullary hematopoiesis, granulocytic leukemia, or lesions that have characteristics of both. As the liver involvement becomes more severe, there is periductular hyaline degeneration, bile duct proliferation, and perivascular fibrosis.

rasH2 Transgenic Mouse

This genetically engineered mouse was developed in Japan. This is the only transgenic mouse used in short-term bioassays that has inserted human genetic material. Based on a CB6F1 background, it has normal coding of the human c-Ha-ras gene, with a mutation in the last intron. There is overexpression of the gene in both normal and neoplastic tissue. There are few lesions present at 6 months of age. The suggested advantages of this transgenic mouse are that it carries a human oncogene, has more rapid onset of tumors, develops more tumors than in a traditional bioassay, and the tumor types correspond to those present in traditional mouse bioassays.

Spontaneous tumors in the rasH2 mouse include forestomach papillomas and squamous cell carcinomas, alveolar epithelial neoplasms, thymic lymphomas, and splenic hemangiosarcomas (Mitsumori et al. 1998). The most common spontaneous tumors are hemangiosarcomas, which have been reported in the spleen, uterus, and subcutaneous tissue. These tumors are of the endothelial type with neoplastic endothelial cells surrounding blood-filled vascular spaces, as well as solid cellular masses. Mitoses are common, and there is a delicate to broad-banded collagen stroma in the tumor. Typical transitional cell carcinomas were present in the bladder of rasH2 mice following administration of p-cresidine, a potent bladder carcinogen.

METABOLISM

The mouse is the most extensively used experimental animal in biomedical research. In contrast to its popularity as an animal model, information on xenobiotic metabolism in the mouse is much less available compared with information in the rat and dog. This makes proper assessment of the suitability of this species for predicting xenobiotic metabolism in humans difficult. Although the metabolic patterns of some xenobiotics in the mouse are similar to those in the rat, there are many examples where xenobiotic metabolism is quantitatively and qualitatively different between these species, resulting in a difference in toxicity. The mouse is, in general, a more active oxidizer compared to the rat. Sex differences in xenobiotic metabolism are much less frequently observed in the mouse than in the rat.

Kato (1966) studied species differences in cytochrome P-450 content in relation to the activity of hepatic microsomal mixed-function oxidase. Among the species studied (male and female mice, male and female rats, male rabbits, and male cats), the mouse has the highest content of cytochrome P-450 with the highest activity of NADPH oxidase, aminopyrine N-demethylase, and aniline

hydroxylase (Kato 1979). The mouse has the highest liver–body weight ratio (66.2 ±2.8 g/kg body weight) among the commonly used laboratory animals (e.g., rat, 40.4 g/kg; guinea pig, 40.2 g/kg; rabbit, 34.5 g/kg; dog, 23.2 g/kg) but the microsomal protein concentration in the mouse liver (22.3 mg/g liver) was similar to that of the other species (Gregus et al. 1983). Consequently, the amount of cytochrome P-450 (0.5–1.1 nmol/mg) in the mouse (table 2.24) was similar to that in the rat and guinea pig when calculated on the basis of milligrams of protein (Chhabra et al. 1974; Gregus et al. 1983; Litterst et al. 1975). However, the value in mouse liver was slightly higher than that

Table 2.24 Summary of Hepatic Xenobiotic Drug Metabolizing Enzymes in Mice

Enzyme	Concentration or Activity	Comments and References
Cytochrome P-450 (nmol/mg protein)	1.1[a], 0.48[b] 1.08 ± 0.05 for M & 1.04 ± 0.07 for F[c], 0.6 ± 0.02[d]	[a]Chhabra et al. (1974) [b]Souhaili-el Amri et al. (1986) [c]Kato (1979) [d]Flyn et al. (1972), determined at 27°C for 1 hr
Cytochrome b (nmol/mg protein)	0.3 ± 0.01[d], 0.543 ± 0.069[e]	[d]Flyn et al. (1972), determined at 27°C for 1 hr [e]Gregus et al. (1983)
NADPH: Cytochrome c reductase (nmol/min/mg protein)	113.0[a], 127.5[b], 109[f], 28.0 ± 1.4[d]	[a]Chhabra et al. (1974) [b]Souhaili-el Amri et al. (1986) [f]Litterst et al. (1975) [d]Flyn et al. (1972), determined at 27°C for 1 hr
NADPH: Cytochrome P-450 reductase (nmol/min/mg protein)	1.43 ± 0.18[d]	[d]Flyn et al. (1972), determined at 27°C for 1 hr
Hydrocylase (nmol/min/mg)		
Aniline hydroxylase	16.1 ± 0.1[a], 1.5[f], 1.21 ± 0.06 for M & 1.18 ± 0.06 for F[c], 0.53 + 0.02d	[a]Chhabra et al. (1974) [f]Litterst et al. (1975) [c]Kato, (1979) [d]Flyn et al. (1972) determined at 27°C for 1 hr
Benzo[a]pyrene hydroxyl-lase	114 ± 11 unit/mg/min[a]	[a]Chhabra et al. (1974)
biphenyl-4-hydroxlyase	6.35 ± 0.67[a] 2.8[f]	[a]Chhabra et al. (1974) [f]Litterst et al. (1975)
Hexobarbital hydroxylation	1.51 ± 0.10 for M & 1.53 ± 0.07 for F[c]	[c]Kato (1979)
Sotyrene 7,8-oxide hydrolase		
Ethoxycoumarin O-demethyl	1.4[g]	[g]Oesch and Wolf (1989)
N-Demethylase (nmol/min/mg)		
Aminopyrene N- demethylation	11.0 ± 1.9[f], 2.33 ± 0.14[d]	[f]Litterst et al. (1975) [d]Flyn et al. (1972), determined at 27°C for 1 hr
Ethymorphine N- demethylation	6.75 ± 0.64[a]	[a]Chhabra et al. (1974)
N-hydroxylase dibenzylamine	0.627 ± 0.0.13[h]	[h]Beckett and Gibson (1975)
UDP-Glucuronosyl transferase (nmol/min/mg)		
1-naphthol	2.6[e]	[e]Gregus et al. (1983)
4-nitrophenol	6.1[e]	[e]Gregus et al. (1983)
Glutathion S-transferase (nmol/min/mg)		
1-chloro-2, 4- Dinitrobenzene	617g (cytosol), 147-15[g,i] (microsomes)	[g]Oesch and Wolf (1989) [i]Morgenstern et al. (1984)
Herachloro-1,3-butadiene	0.109g (cytosol)	[g]Oesch and Wolf (1989)
Protein estimates		
Microsomal (mg/g)	34.3 ± 9.4[f]	[f]Litterst et al. (1975)
	22.3 ± 1.3[e]	[e]Gregus et al. (1983)
Cytosolic (mg/g)	79.0 ± 3.2[e]	[e]Gregus et al. (1983)

in the dog (0.347 nmol/mg). The level of NADPHcytochrome c reductase activity (109 ±33 nmol/mg/min) was much lower in the mouse compared with the rat (187 ±51 nmol/mg/min). The activities in mixed-function oxidase measured by hydroxylase activity for aniline and biphenyl were about twofold higher in the mouse than in the rat. Aminopyrine N-demethylase activity was similar in these species. In contrast, the activities of benzo(a)pyrene hydroxylase and ethylmorphine N-demethylase in the mouse were about half the activities in the rat (Chhabra et al., 1974). Table 2.25 summarizes CYP 450 isoenzymes characterized in the mouse.

The distribution pattern of mixed-function oxidases for xenobiotics between the smooth and rough microsomal membranes of the liver is known to be species dependent. In the mouse liver microsomes, concentrations of all components of a mixed-function oxidase system were approximately the same between smooth and rough membranes (Gram et al. 1971), whereas in some animals (e.g., monkey, guinea pig), the concentrations were approximately twofold higher in the smooth membrane.

It has been shown that the levels of mixed-function oxidase are generally highest in the mammalian liver compared with the levels in other organs. In the mouse, the content of cytochrome P-450 was approximately fourfold and twofold higher in the liver than in the lung and kidney, respectively. Activity of NADPH-cytochrome c reductase was similar in these organs. Hydroxylase activity for aniline and biphenyl and aminopyrine N-demethylase activity were much higher in the liver than in the lung and kidney. However, beta-glucuronidase activity for phenolphthalein in the mouse spleen was threefold to tenfold higher than that in the liver depending on the strains.

Table 2.25 CYP Activity in the Mouse

7-Ethoxyresorufin *O*-dealkylation	1A1/2 , Ryan and Levin (1990)
7-Methoxyresorufin *O*-dealkylation	1A1/2
Caffeine 3-demethylation	1A2, Fuhr et al. (1992); Casley et al.(1997)
Benzphetamine *N*-demethylation	2D10, 2B10, 2C29, Taniyama et al. (1989); Honkakoski et al. (1992)
7-Benzoxyresorufin *O*-dealkylation	2B10
7-Pentoxyresorufin *O*-dealkylation	2B10, Honkakoski et al. (1992); Honkakoski and Negishi (1997)
Coumarin-hydroxylation	2A5, Jung et al. (1985)
7-Ethoxy-4-trifluoromethylcoumarin deethylation	
Ethoxycoumarin *O*-dealkylation	1A, 2B, Riley et al. (1993)
Tolbutamide methyl-hydroxylation	2C29, Koop (1992)
Chlorzoxazone 6-hydroxylation	2E1, Koop et al. (1985)
4-Nitrophenol hydroxylation	2E1, Koop et al. (1985); Adas et al. (1999)
N-Nitrosodimethylamine *N*-demethlation	2A5
Androstenedione 15α-hydroxylation	
Androstenedione 16α/β-hydroxylation	
Dextromethorphan *O*-demethylation	
Dextromethorphan *N*-demethylation	
Testosterone$\rightarrow$Androstenedione*	
Testosterone 2α-hydroxylation	2D, Sharma and Shapiro (1995)
Testosterone 2β-hydroxylation	3A11/13, Liu et al. (1995); Yanagimoto et al. (1992)
Testosterone 6β-hydroxylation	3A11/13, Liu et al. (1995); Yanagimoto et al. (1992)
Testosterone 7α-hydroxylation	
Testosterone 15α-hydroxylation	2A4, Iwasaki et al. (1993); Nelson et al. (1993)
Testosterone15β-hydroxylation	
Testosterone 16α-hydroxylation	2D9, Harada and Negishi (1984); Nelson et al. (1993); Noshiro et al. (1988)
Testosterone 16β-hydroxylation	2B10, 2C29, Honkakoski et al. (1992)
Lauric acid 11-hydroxylation	2E1, Adas et al. (1999)
Lauric acid 12-hydroxylation	4A10/12, Bell et al. (1993); Adas et al. (1999)

Sex difference in the activity of microsomal mixed-function oxidase is a peculiar phenomenon that is observed clearly in the rat with higher activity in the male than in the female. However, sex differences in mice are not consistently observed (Davis et al. 1969; Kato 1974; Westfall et al. 1964) and appear to be strain dependent. There was no sex difference in the contents of cytochrome P-450, NADPH-cytochrome c reductase, or NADPH oxidase in the mouse. Sex differences were not evident in aminopyrine N-demethylase and aniline hydroxylase activities (Souhaili-el Amri et al. 1986), whereas clear sex differences were demonstrated in the hexobarbital hydroxylase (Castro and Gillette 1967; MacLeod et al. 1987) and N-demethylation activity of ethylmorphine (Catz and Yaffe 1967; Kato and Onoda 1966; MacLeod et al. 1987) with significantly higher maximal velocities (V,a) in female than in male mice of some strains. UDP Glucuronyltransferase activity in the male mouse was similar to or significantly greater than that in the female depending on the strain of the mouse (Boutin et al. 1984).

Strain differences in the metabolism of xenobiotics are frequently observed in the mouse as also observed in the rat and rabbit. For example, a marked strain difference in the oxidative metabolism of hexobarbital was observed in mice. The duration of sleeping time after a single dose of the drug, which is determined by the rate of its oxidation, ranged from 20 min in the CS7BL mouse strain to 60 min in the 129J strain. Interestingly, there appears to be an inverse relationship between toxicity and sleeping time in the strains of mice studied. This relationship in time correlates with the strain-dependent rates of hepatic metabolism, perhaps suggesting an intermediate toxic product.

The mouse is known to have a high activity of oxidative enzymes compared with other laboratory animals and humans. The biological half-life of oxidative metabolism of phenobarbital in the mouse is 20 to 60 min, whereas the half-lives in the rabbit, rat, dog, and human are 60, 140, 260, and 360 min, respectively.

As discussed in another chapter (see the “Xenobiotic” section in chapter 8), N-dealklyation is most frequently used by the dog and human and least frequently used by the rat. The mouse uses this pathway for many compounds and appears to have high N-dealkylase activity in general. For example, the mouse has been shown to excrete major amounts of the N-dealkylated metabolite of tiaramide (Schwarz et al. 1973), whereas the other species (e.g., rat, dog, and monkey) favor N-oxide formation or side chain oxidation. Oxaminase has been shown to be metabolized by N-dealkylation in the mouse but not in the rat. When N-demethylation activity for aminopyrine was examined in the mouse, rat, rabbit, and cat, the enzyme activity in the mouse was highest and at least threefold higher than in the male rat (Kato 1966). In addition, there was no sex difference in the N-demethylase activity in the mouse, whereas the enzyme activity in the male rat was approximately fourfold higher than that in the female rat. Castro and Gillette (1967) studied species difference in N-demethylation of ethylmorphine using hepatic microsomes of the mouse, rat, guinea pig, and monkey. Ethylmorphine N-demethylase activity in the oak and female mouse was higher than that in the other species except for the male rat. N-demethylase activity in the male mouse (Vmax of 139 mmol/mg/10 min) was approxmately half the activity in females. Later Van Den Berg et al. (1977) found that ethylmorphine N-demethylase activity differed between the sexes in one strain of mouse (PB-SE) but not in another strain (CPB-V). In the rat, the ethylmorphine N-demethylase activity in males (Vmax of 203 mmol/mg/10 min) was approximately fivefold higher than in females.

Diazepam is metabolized oxidatively by two metabolic pathways; one is ring hydroxylation, which is the main pathway in the rat, and the other is oxidative N-demethylation, which is the main pathway in the mouse and man. Although concentrations of diazepam in blood and brain are similar in the mouse and rat, antimetrazol activity is longer lasting in the mouse than in the rat owing to the accumulation of active N-demethylated metabolite in the mouse brain (Marcucci et al. 1968). In humans, the N-demethylated metabolite was the major metabolite detected in plasma.

Amphetamine is metabolized oxidatively by two metabolic pathways: one is ring hydroxylation and the other oxidative deamination (Dring et al. 1966). N-deamination of amphetamine occurs in

the mouse as in humans (Caldwell 1976). In the rat, amphetamine was shown to be extensively metabolized by aromatic hydroxylation and poorly metabolized by oxidative deamination. Based on the available metabolism data, the mouse appears to be a relatively good animal model, in general, for N-dealkylation and N-deamination. However, the extents of N-dealkylation and N-deamination are highly compound dependent and caution must be exercised for this generalization.

There are several microsomal aromatic hydrocarbon hydroxylases (AHHs) whose occurrence depends not only on species, but also on the nature of the aromatic compound. Generally, the activities of these enzymes are much greater in the mouse than in humans, as observed with amphetamine and benzo(a)pyrene (Souhaili-el Amri et al. 1986). Aromatic hydroxylation of amphetamine occurred to the extent of 10% to 19% in the mouse and about 3% in humans. In the rat, it was about 60% (Parke 1968). In contrast to amphetamine hydroxylase activity, the activity of benzo(a)pyrene hydroxylase in the mouse was approximately threefold higher than that of the male inbred Sprauge-Dawley rat (Oesch et al. 1973). The AHH activity in human liver was approximately 30% to 60% of the hepatic activity of the male rat (Pelkonen et al. 1975). Although the bezo(a)pyrene hydroxylase activity was similar in male and female mice, a sex difference has been shown in the rat, with the female displaying about 20% to 40% of the hepatic activity of the male.

Biphenyl was metabolized in the mouse to 2-hydroxybiphenyl as well as 4-hydroxybiphenyl, which is the metabolite of all species examined (Creaven et al. 1965). However, in rats the 2-hydroxy metabolite was formed only in young animals but not in the adults, although rats are known to be a good animal model for aromatic hydroxylation. Therefore, the distribution and activity of the microsomal hydroxylases among species cannot be predicted for all compounds. If a difference is found among species with a given compound, then it is likely, but not always certain, that a similar species difference can consistently occur with its derivatives (Williams 1974).

Many unreactive compounds can be metabolized to chemically highly reactive intermediates and act as mutagenic or carcinogenic agents. Unreactive aromatic hydrocarbons and olefinic compounds are converted to highly active arene oxides and alkene oxides that are formed from the epoxidation of double bonds in aromatic rings and olefinic double bonds, respectively. The mouse is reported to have high epoxygenase activity (Gregus et al. 1983). In a comparative *in vitro* metabolism study of naphthalene using microsomal preparations from lung, liver, and kidney of mice, rats, and hamsters, it has been shown that metabolism of naphthalene to an epoxide was most extensive with microsomal preparations from the mouse lung (Buckpitt et al. 1987). Furthermore, metabolism of naphthalene was stereoselective to form (IR,2S)-naphthalene 1,2-oxide with the mouse lung, which is the target tissue for acute toxicity in this species. In contrast, with microsomal preparations from the mouse liver and kidney, and with all microsomal preparations from the rat and hamster, metabolism was not stereoselective.

The epoxides are metabolized to much less reactive vicinal diols by epoxide hydrolases and to glutathione conjugates by glutathione S-transferase. Therefore, the level and activity of epoxide hydrolases among species might be of great importance for the mutagenic or carcinogenic risks. With styrene oxide as the substrate the epoxide hydrolase activity in the mouse (1.4 nmol/mg protein/min) was less than one-fortieth of that in humans (59.3 nmol/mg protein/min) (Oesch and Wolf 1989). However, in the diabetic mouse (male and female db/db strain) the styrene oxide hydrolase activity was increased approximately threefold (Watkins and Klueber 1988). In addition, genetic polymorphism of microsomal epoxide hydrolase activity in the mouse has been reported (Lyman et al. 1980).

A species, such as mice, having high epoxygenase activity (Gregus et al. 1983) but low epoxide hydrolase activity (Oesch and Wolf 1989) might be much more susceptible than the human to toxicity related to epoxide formation. However, epoxides are also detoxified via glutathione conjugation. The mouse liver has a high level of glutathione transferase activity (149 ± 13 nmol/mg/min) compared to that of rats (87 ± 10 nmol/mg/min) and humans (25 ± 4 nmol/mg/min; Pacifici et al. 1981). For example, following acrylonitrile administration, concentrations of an oxide metabolite (2-cyanoethylene oxide) in rat blood were 6 to 11 times higher than those in the mouse, although

mouse liver oxidized acrylonitrile to the oxide at a much greater rate (approximately 3 times) than rat liver (Kedderis 1989). Acrylonitrile was metabolized to thiodiglycolic acid, a metabolite of the glutathione conjugate, 7 times faster in the mouse than in the rat. Therefore, to what extent these interspecies differences in the epoxide toxicity occur for other substrates remains to be further evaluated.

It has been reported that N-hydroxylase activity is predominately mediated via the polycyclic hydrocarbon-inducible PI-450 (Felton et al. 1976) and is of great importance for toxicity evaluation. The mouse is the most susceptible to acetaminophen-induced hepatic injury and the rat had the lowest susceptibility. This difference in toxicity was found to be related to the rates of N-hydroxylation of the drug by the hepatic microsomes (Davis et al. 1974). An increase in N-hydroxylation enhances the need for reduced glutathione, and glutathione depletion in the liver precedes marked increases in covalently bound acetaminophen. Formation of metabolites covalently bound to microsomal protein and depletion of hepatic glutathione were highest in the mouse but only minor extents of covalent binding and depletion of glutathione were observed in the rat. As a result, in the mouse, acetaminophen induced hepatotoxicity. Increases in covalently bound metabolites of the drug were found to be highly correlated with the Ali′ allele (Thorgeirsson et al. 1977).

Lotlikar et al. (1967) studied species differences in the relative N- and ring hydroxylation of 2-acetylaminofluorene by liver microsomes of the mouse and other species. The N-hydroxylase activity in the mouse was lower than that in the hamster and rabbit but higher than that in the rat and guinea pig. The N-hydroxy metabolite was practically undetectable in the guinea pig, which explains resistance of the guinea pig to hepatoma induction by 2-acetylaminofluorene. Later Razzouk and Roberfroid (1982) reported that Vmax values for N-hydroxylase of aminofluorene and acetylaminofluorene in the mouse (510 and 225 pmol/mg/min, respectively) were higher than those in the hamster (260 and 140 pmol/mg/min, respectively) and at least tenfold higher than those in the rat (41 and 14 pmol/min/min, respectively). N-hydroxylase activities were also studied in several species with dibenzylamine (Beckett and Gibson 1975) and 4-aminobiphenyl (McMahon et al. 1980). In contrast to acetylaminofluorene N-hydroxylase activity, N-hydroxylase activity of dibenzylamine (18.8 nmol/mg/30 min) and 4-aminobiphenyl (13.2 gmol/80 min) in the mouse were approximately twofold lower compared with guinea pig liver. The activity of dibenzylamine N-hydroxylase in the mouse was slightly higher than that in the rat (12.5 nmol/mg/30 min), whereas 4-aminobiphenyl N-hydroxylase activity in the mouse was fivefold higher than that in the rat (2.4 gmol/80 min). Thus, based on the limited information available, N-hydroxylase activity in the mouse is moderate and similar to or higher than that of the rat.

The mouse is not reported to display any defects in conjugation reactions such as those observed in the cat (glucuronidation), pig (sulfation), and dog (acetylation). In acetylation of certain aromatic amines, both fast and slow acetylators have been identified with some mouse strains such as C57BL/6J and A/J as with the human population (Elves et al. 1985). Lower and Bryan (1973) studied acetylation of the carcinogens 2-aminofluorene, 4-aminobiphenyl, and 2-aminonaphthalene by the mouse liver cytosol enzymes. The most readily acetylated carcinogen was 2-aminofluorene (332 --t 25 nmol/50 mg liver/5 min), followed by 4-aminobiphenyl (215 -t 15 nmol/50 mg liver/5 min) and 2-aminonaphthalene (209 ± 9 nmol/50 mg liver/5 min). These enzyme activities in the mouse liver were about half the activities in the hamster liver but 6-10 times higher than those in the rat liver. Recently, Calabrese (1988) reported that the mouse was about 4.6-fold more efficient acetylator than the human fast acetylator for 2-aminofluorene. The rat displayed about half the activity of the human fast acetylator and sevenfold greater activity to acetylate 2-aminofluorene than the human slow acetylator, which is consistent with previous findings (Lower and Bryan 1973).

Lower and Bryan (1976) also studied deacetylase activites of the acetylated derivatives of each of the same three compounds in the mouse, hamster, rat, guinea pig, and dog. Deacetylase activity of 4-acetylaminobiphenyl was highest in the mouse and more than 20 times that observed in the rat. However, deacetylase activities for 2-acetylaminofluorene (54.7 ± 6.3 nmol/mg/hr) and 2-acetylaminonaphthalene (21.1 ± 6.3 nmol/mg/hr) in the mouse were approximately threefold and sixfold

lower compared with those of hamster liver. The guinea pig and rat were generally the least efficient in deacetylation of these compounds. The relative capacity of the animal model to acetylate and deacetylate is an important variable with respect to arylamine-induced cancer. In the mouse, as in the rat, the ratio of N-acetyltransferase to deacetylase activity is very high relative to the dog. This may provide rodents with some protection against bladder cancer, but not hepatocarcinogenesis.

Surprisingly there is little information available for glucuronidation activity of drugs in the mouse. Based on limited information, UDP-glucuronosyltransferase activities in rodents and primates are generally high compared with that in cats (Caldwell 1978). Gregus et al. (1983) studied hepatic UDP-glucuronosyltransferase activity toward I acceptors in the mouse, rat, guinea pig, rabbit, cat, quail, and trout. The transferase activity in the mouse, when compared to the activity in other species, was high toward phenolphthalein, diethylstilbestrol, morphine, valproic acid, and bilirubin, whereas the activity was medium or low toward I-naphthol, p-nitrophenol, testosterone, estrone, chloramphenicol, and digitoxigenin. Therefore, the UDP-glucuronyltransferase activity appears to be highly substrate specific and no general pattern in its activity could be established among the species studied. Considerable evidence has accumulated that suggests that multiple forms of UDP-glucuronyltransferase exist, and this might explain why glucuronidation activity is high for some substrates but not for others.

A strain difference in UDP-glucuronosyltransferase activity in the mouse has been reported (Tsyrlov and Lyakhovich 1978). After pretreatment with 3-methylcholanthrene, the microsomal UDP-glucuronosyltransferase activity increased threefold in C57BL mice but not in AKR mice, although the transferase activity in the control animals was similar between these two strains.

Hydrolysis of glucuronide conjugates is carried out by the lysosomal enzyme betaglucuronidase, which is present in most tissues, particularly liver, kidney, spleen, intestinal tract, endocrine, and reproductive organs. The level of beta-glucuronidase in multiple tissues has been studied in a variety of animal species as well as in humans. Genetic polymorphism of beta-glucuronidase activity was observed in the various tissues of the mouse in which the activity in the high-activity strain is 4 to 10 times greater than that of the low activity strain. The enzyme levels in the liver, kidney, and spleen of the high-activity strain of the mouse were similar to the activity in the respective human tissues, whereas the activities in the rat were approximately tenfold higher than those in humans. The beta-glucuronidase of the cecal contents and feces were also similar in mice and humans (Rowland et al. 1986). However, beta-glucuronidase activity in the mouse intestine was much higher than that in humans and rats. The activities of betaglucuronidase in the spleen were similar to or higher than those in the liver regardless of species and strain of mouse.

As discussed earlier, GSH transferase activity in the mouse liver was reported to be higher than that in the rat with 1-chloro-2,44nitrobezene as a substrate (Igarashi et al. 1983; Igarashi et al. 1986) and also higher than human liver with styrene oxide as a substrate (Pacifici et al. 1981). Recetitly, Oesch and Wolf (1989) determined glutathione transferase activities in liver microsomes and cytosol from various species, including mice and humans with hexachloro-1,3-butadiene and 1-chloro-2,4-dinitrobenzene. Contrary to the earlier findings, the glutathione transferase activities in the mouse were lower than those in the rat with the same substrate and also lower than those in humans. This difference might be, in part, due to strain difference of the mouse and substrate difference in humans among the studies. The transferase activity in the microsomes (0.16 nmol/min/mg) and cytosol (0.109 nmol/min/mg) were similar in the mouse, whereas in humans the enzyme activity was much greater in microsomes (1.17 nmol/min/mg) than in cytosol (0.031 nmol/min/mg) with hexachloro-1,3-butadiene as a substrate (Oesch and Wolf 1989). However, with 1-chloro-2,4-dinitrobenzene, GSH transferase activity in the cytosol fraction in mice (617 nmol/min/mg) and humans (1,666 nmol/min/mg) was about 11- and 30-fold higher than the activities in the microsomal fraction, respectively. Therefore, the GSH transferase activity appears to be highly compound specific and it is difficult to make any broad generalization.

Thiol methylation is an important pathway in the metabolism of many sulfhydryl drugs. At least two enzymes, thiol methyltransferase (TMT) and thiopurine methyltransferase (TPMT) can

catalyze the thiol methylation. Thiol methyltransferase is membrane associated and catalyzes the S-methylation of aliphatic sulfhydryl compounds such as 2-mercaptoethanol, captopril, and D-penicillamine. It is inhibited by SKF 525A but not by the benzoic acid derivatives that are potent inhibitors of TPMT. Ottemess et al. (1986) measured TMT activity in hepatic microsomes from 10 different strains of mice using 2-mercaptoethanol as the methyl acceptor substrate and compared the activity with that in human red blood cell membranes and kidney microsomes. Because the properties of TMT in the mouse liver were similar to those of the enzyme in the human tissues, the authors concluded that the inbred mouse is a useful experimental animal model to study the action and function of TMT.

Very little work has been reported on the conjugation reactions of xenobiotics with stiffate, taurine, and amino acids in the mouse. Based on the limited information, the mouse appears to have moderate sulfate conjugation capability as illustrated with acetamisophen and 4-hydroxy-3-methoxyphenyl ethanol. The concentration of adenosine 3phosphate-5-phosphosulfate (PAPS), which is required for sulfation reactions as the sulfate donor, in the male mouse liver (29.4 ± 1.6 mmol/g of tissue) was lower than that of rats (67.9–76.8 mmol/g), similar to hamsters and rabbits, but much higher than that in the dog (16.1–17.3 mmol/g). The concentrations of PAPS in the mouse kidney, lung, and intestine were approximately half that of liver.

Emudianughe et al. (1978; Emudianughe et al. 1987) studied amino acid conjugation of the isomeric naphthylacetic acids in seven subprimate animal species, including the mouse. With 2-naphthylacetic acid, the taurine conjugate was the major urinary conjugated metabolite (57%), whereas the glucuronide conjugate was 40% of the urinary conjugated metabolites. However, with 1-naphthylacetic acid, the majority of the urinary conjugate metabolites in the mouse were glucuronide (68%) and the glycine conjugate was minor (16%). In general the dog appears to be the most extensive taurine conjugator, man and rhesus monkey are weak species for this conjugation pathway, and the mouse is intermediate. An exception to this is the conjugation metabolism of an arylacetic acid, oxepinac. Oxepinac was metabolized to the taurine conjugate in the mouse as well as in the rat (Hakusui et al. 1978). However, in the dog and man it was conjugated with glucuronic acid, although the dog is known to be an extensive taurine conjugator with some other compounds. Glutamine conjugates of naphthylacetic acids were not detected in the mouse urine, although other rodents such as the rat and hamster formed substantial amounts of this conjugate.

The drug-metabolizing enzymes in the mouse are generally induced or inhibited with compounds known to be enzyme inducers or inhibitors, respectively, in the other species. An exception to this is that DDT, an enzyme inducer for the rat, pig, and sheep, did not produce any enhancement of the oxidative metabolism of drugs in the mouse (Gabliks and Maltby-Askari 1970; Hart and Fouts 1965). Furthermore, with some inducers marked strain differences were observed in the induction of xenobiotic metabolism in the mouse (Glass and van Lier 1988). A typical example includes AHH induction by polycyclic hydrocarbons. The AHH assay is a reliable, simple, and very sensitive assessment of aromatic hydrocarbon responsiveness following treatment of animals with polycyclic hydrocarbon inducers. Using AHH induction as an indicator of phenotype at the Ah locus, several laboratories have found that about half or slightly more than half of all inbred mouse strains examined were responsive and the remaining mouse strains were nonresponsive (Nebert and Jensen 1979).

SKF-525A inhibited aminopyrine N-demethylase and hexobarbital hydroxylase in mouse hepatic preparations as in rat hepatic preparations. However, the inhibition was quasicompetitive in the mouse, whereas in the rat it was competitive, thus indicating differences in the nature of the monooxygenases between these two species.

Not many in-depth works have been published about stereoselectivity in metabolism and toxicity of xenobiotics in the mouse. One interesting example, however, is stereoselective toxicity of thalidomide. Both S-(-)thalidomide and the R-(+)-enantiomer are transformed into I-N-phthaloylglutamine and I-N-phthaloylglutaminic acid in various species. Although conflicting reports have been published on potential racernization of thalidomide enantiomers and the sensitivity of different mice and rat

strains as to thalidomide toxicity, in SWS mice only the glutaminic acid metabolite derived from the S-(-)-enantiomer and not the one derived from the R-(+)-isomer was shown to be embryotoxic and teratogenic (Ockenfelz et al. 1976).

Species differences in toxicity of a compound might be due to factors other than metabolism differences. Some of these factors include protein binding and biliary excretion in addition to absorption and elimination. When protein binding of some drugs (clofibric acid, etodolac, tolrestat, perrinone, benoxaprofen) was examined in the rat, mouse, dog, rabbit, rhesus monkey, and human, the binding was, in general, highest in human serum, weakest in the mouse, and somewhat variable with other species.

Differences in biliary excretion can have pronounced toxicological implications, especially when followed by reabsorption; that is, enterohepatic circulation takes places. For example, the intestinal toxicity of indomethacin in five species of laboratory animals (rat, dog, monkey, guinea pig, and rabbit) is inversely proportional to the exposure of the intestinal mucosa to the drug as a consequence of enterohepatic circulation. In general, the mouse, rat, and dog are very efficient in biliary excretion, and guinea pigs, monkeys, and human are relatively inefficient. For example, the biliary excretion rate of methyl mercury was substantially higher in the mouse, rat, and hamster (approximately 0.8 nmol/min/kg) compared with that in the guinea pig and rabbit (0.15 and 0.03 nmol/min/kg, respectively; Stein et al. 1988). The biliary excretion rate of GSH-related thiols and disulfides was also highest in the mouse.

As reported for other species, the drug-metabolizing enzymes in newborn mice are not fully developed. When phenobarbital was injected in mice of various ages, the newborn animals failed to metabolize any of the drug over a 3-hr period. One-week-old rnice metabolized about 18% and 3-week-old mice about 22%. A hexobarbital sleeping time study also revealed striking differences with age (Jondorf et al. 1958). The sleeping time of 1-week-old mice was about 107 min, whereas the sleeping time of adults was less than 5 min.

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CHAPTER 3

The Rat

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CONTENTS

TOXICOLOGY

History

The Norway rat (*Rattus Norvegicus*) is believed to have originated in Asia and spread throughout the world with modern civilization as an economic pest. The rat, considered to be the first animal to be domesticated for strictly scientific purposes (Richter 1959), was first used experimentally in France in the study of adrenal gland function (Philipeaux 1856, as cited in Lindsey 1979). Early research with rat in the areas of nutrition, endocrinology, physiology, and behavior led to discoveries such as the nutritional quality of various amino acids in mammals, the existence of vitamins, the characterization of the hormones of the anterior pituitary, and the existence of circadian rhythms. The rat has become a species of choice for almost every area of biological and medical research because of its size, relatively docile nature, life span, and gestation period.

For the purpose of reducing variability and emphasizing various desirable characteristics, several early researchers established breeding programs. Henry Donaldson and his colleagues at the Wistar Institute (Philadelphia, Pennsylvania) were prominent in this effort. Many of the rat strains commonly used in toxicology today, including the Wistar, Sprague-Dawley, and Long Evans, can be traced to the Wistar lineage. The Fischer 344, another commonly used strain, was developed for use in cancer research at the Crocker Research Institute of Columbia University in New York City.

The use of the rat in toxicology studies has paralleled its use in other fields. The rat continues to be the rodent species of choice for most toxicology studies.

Choice of the Rat in Toxicology Research

Ideally, safety testing of products intended for use in humans, or to which humans could be exposed, should be done in humans. The data from humans would apply without reservation to complex human physiology and cellular and biochemical mechanisms and human risk assessment. Unfortunately humans cannot be used for this purpose. Therefore, the choice of an appropriate species for toxicology studies should be based on a comparison of the pharmacokinetics and metabolism of the test compound in different laboratory species and man. In the absence of these data this choice is often based on practicality and economics. The rat has become a species of choice because of metabolic similarities, as well as their small size, relatively docile nature, short

life span, and short gestation period. The extensive use of the rat in research has led to the development of a large historical database of their nutrition, diseases, and general biology.

Species Differences

Although the rat is a species of choice in toxicology research because of the many physiological similarities and anatomical characteristics, differences exist that must be considered when designing and conducting studies with this animal. Rats are obligate nose breathers, and as such an inhaled test material is subject to nasal filtration and absorption. The placenta is considerably more porous in the rat. This difference could increase the chance of fetal exposure to an administered test material or increase the overall level of fetal exposure to an administered test material. The overall distribution of intestinal microflora is different in the rat, which could lead to differences in the metabolism of an orally administered test material. These and other differences in the rat might lead to positive signs of toxicity to a test material that might not be present in a different species.

Strain Differences

Breeding rats for specific characteristics has produced some physiological differences among strains of rats. Some of these differences are known to affect how the various strains react to toxicants. Among others, strain-specific differences have been found in sensitivity to thiourea (Dieke and Richter 1945), sensitivity to acetaminophen nephrotoxicity (Newton et al. 1985), the incidence of spontaneous glomerular sclerosis (Bolton et al. 1976), sensitivity to the carcinogenic actions of 7,12-dimethylbenz(a)anthracane (Boyland and Sydnor 1962), the effects of trimethyltin on operant behavior and hippocampal glial fibrillary acidic protein (GFAP) (MacPhail et al. 2003), differences in renal carcinogenesis (Hino et al. 2003), differences in cytochrome P-4501A1 gene expression caused by 2,3,7,8-tetrachlorodibenzo-p-dioxin in the liver (Jana et al. 1998), susceptibility to 4-nitroquinoline 1-oxide induced carcinoma (Kitano et al. 1992), and differences in the levels of drug-metabolizing enzymes (Page and Vesell 1969). In recent years, research and breeding programs have focused on producing inbred and outbred strains focused on specific disease models and susceptibility to the development of certain carcinomas. When choosing a strain for use, it is important to consider these differences.

Of importance for carcinogenicity studies, strain differences have been found in the incidence of spontaneous tumors. Table 3.1 gives the incidence of spontaneous tumors found in commonly used strains in carcinogenicity studies. The historical incidence is important to the analysis of a study in that a high spontaneous rate might mask a small test-material-related increase in tumor incidence.

Table 3.1 Incidence of Common Spontaneous Tumors in Fischer 344 and CD (SD)IGS Rats

		% Tumors in Untreated Rats							
		CD(SD)IGS		CD (SD)		Fischer		Wistar	
Organ	Tumor Type	Male	Female	Male	Female	Male	Female	Male	Female
Adrenal gland	Pheochromocytoma	10.0	2.3	11.3	2.3	11.9	3.2	3.2	1.3
Mammary gland	Fibroadenoma	1.4	44.5	1.3	16.7	0.8	7.1	1.2	30.2
Pancreas	Islet cell adenoma	3.6	1.4	4.0	0.3	1.5	0.2	5.3	1.9
Pituitary gland	Adenoma pars distalis	33.6	56.8	35.7	50.3	12.4	28.2	41.1	65.8
Testis	Interstitial cell tumor	1.8		7.0		74.6		4.3	
Thyroid gland	C-cell adenoma	10.5	5.0	5.0	5.7	12.5	8.2	10.1	10.7

Source: Adapted from Charles River (2001), Mitsumori et al. (2001)

Normal Physiological Values

General values for selected physiological parameters are given in table 3.2 and table 3.3. Normal values will vary based on the strain of animal, supplier, feed, and housing conditions. These tables should be used as a point of reference only.

Table 3.2 Selected Normative Data

Husbandry	
Room temperature (°C)	18–26
Relative humidity (%)	30–70
Ventilation (air change/hr)	10
Light/dark cycle (hours)	12–14/12–10
Minimum cage floor size	
Housed individually (cm^2)	350
Breeding with pup (cm^2)	800
Group housed (cm^2 adult)	250
General	
Life span (years)	2.5–3.0
Surface area (cm^2)	0.03–0.06
Chromosome number (diploid)	42
Water consumption (mL/100g/day)	10–12
Food consumption (g/day)	20–40
Average body temperature (°C)	37.5
Reproduction	
Puberty (males and females)	50 ± 10 days
Breeding season	All year
Type of estrous cycle	Polyestrous
Length of estrous cycle	4–5 days
Duration of estrous	10–20 hr
Mechanism of ovulation	Spontaneous
Time of ovulation	7–10 hr after onset of estrous
Time of implantation	Late day 4 or 5[a]
Length of gestation	21–23 days
Litter size	8–16 pups
Birth weight	5–6 g
Eyes open	10–12 days
Weaning age/weight	21 days/40–50 g
Cardiovascular	
Arterial blood pressure	
Systolic (mmHg)	116–145
Diastolic (mmHg)	76–97
Heart rate (beats/min)	296–388
Cardiac output (mL/min)	10–80
Blood volume (mL/kg)	64
Pulmonary	
Respiration (breaths/min)	100–140
Tidal volume (mL)	1.1–2.5
Compliance (mL/cm H_2O)	0.3–0.9
Resistance (cm H_2O/mL*s)	0.1–0.55
Pattern	Obligate nasal
Renal	
Urine volume	15–30 ml/24 hr
Na^+ excretion	200 mmol/L/24 hr
K^+ excretion	150 mmol/L/24 hr
Urine osmolarity	2,000 mOsm/kg H_2O
Urine pH	7.3–8.5
Urine specific gravity	1.01-1.07
Urine creatinine	6 mol/L/24 hr
Glomerular filtration rate	1.0 mL/min/100 g body weight

[a] The estrous cycle length can vary from 4 to 5 days between strains. Time of implantation can vary based on the length of the estrous cycle and is dependent on Day 0 or the first day sperm is found in the vagina.
Source: Data from Baker et al. (1979), Bivin et al. (1979), Peplow et al. (1974), Waynforth (1980), Sharp and La Regina (1998), and Van Zutphen et al. (1993).

Table 3.3 Growth Rates in Selected Rat Strains

	Age (Days)							
	Crl:CD (SD)IGSBR		Crl:(WI)BR		Crl:(LE)BR		CDF(F-344)/CrlBR	
Weight (g)	M	F	M	F	M	F	M	F
Up to 50	Up to 23	Up to 23	Up to 23	Up to 25	Up to 21	Up to 21	Up to 23	Up to 23
51–75	24–28	24–29	24–28	26–30	22–25	22–26	24–29	24–29
76–100	29–34	30–35	29–32	31–34	26–29	27–31	30–34	30–35
101–125	35–37	36–39	33–35	35–40	30–34	32–36	35–39	36–42
126–150	38–42	40–44	36–40	41–47	35–37	37–43	40–45	43–55
151–175	43–45	45–50	41–44	48–56	38–42	44–50	46–50	56–72
176–200	46–49	51–56	45–48	57–64	43–46	51–55	51–57	73–105
201–225	50–52	57–70	49–52	65–81	47–49	56–69	58–63	105+
226–250	53–56	71–84	53–56	82–105	50–55	70–86	64+	
251–275	57–59	84–105	57–61	106+	56–58	87–102		
276–300	60–65	106+	62–67		59–64	103+		
301–325	66–71		68–73		65–70			
326–350	72–77		74–79		71–80			
351–375	78–87		80–87		81–90			
376 +	88+		88+		91+			

Source: Adapted from Charles River (2004).

Husbandry

The environment to which a rat is exposed can have profound affects on the result of a research study. Subtle or short-term changes in the environment can alter the response to a test material. Good animal husbandry is essential to maintain the health of the animals on study, increase the reproducibility of the results, and eliminate variables that could confound the results of a study. A good animal husbandry program requires proper oversight of the facility to ensure the health of the animals and takes into account the nutritional and environment needs of the animals.

Facilities

Temperature and Relative Humidity

Current specifications for temperature and humidity are 18°C to 26°C and 30% to 70% relative humidity (Clough 1991; ILAR 1997). These ranges are designed to allow homeotherms to maintain a minimum metabolic rate or to be within their thermoneutral zones (Bligh and Johnson 1973). Rats have the ability to adapt to changes in temperature and humidity through physiologic, behavioral, or metabolic mechanisms, but this requires time, causes stress to the animal, and could alter the outcome of an experiment. The degree to which an individual is able to adapt is dependent on the conditions under which the animal is housed; that is, group housing, type of cage, and bedding.

The stress caused to the animal during a time of adaptation to changes in the environment has been shown to affect basic physiologic function and behaviors. Animals housed at temperatures below the recommended range have been shown to have increased food intake, increased weight gain, increased energy expenditure, but a decrease in efficiency (Rothwell and Stock 1986). Conversely, animals housed at temperatures above the recommended range have been shown to exhibit decreased food consumption and body weights (Hamilton 1967). In addition, male rats develop testicular atrophy consistent with degeneration of the seminiferous epithelium and failure of spermatocyte maturation when housed at temperatures exceeding 26.7°C for more than 48 hr (Pucak et al. 1977). Extremes in temperature have been shown to produce significant differences in body weights, food intake, hematologic and serum biochemical parameters, and reproductive parameters across generations of animals housed under these conditions (Romanovsky et al. 2002; Yamauchi et al. 1981).

The influence of temperature on the toxic response has been well reviewed (Clough 1982; Fuhrman and Fuhrman 1961; Weihe 1973). Fuhrman and Fuhrman (1961) postulated three patterns by which toxicity can vary with temperature: Toxicity might increase at temperature extremes, toxicity might increase linearly with temperature, or toxicity might remain constant with increasing temperature to a threshold, then begin to increase.

The relative humidity within the colony room environment or the micro-environment within micro-isolator cages should be maintained between 50% and 70% for optimal health. Although the guide to animal welfare allows for a great range, research has shown that animals housed in relative humidity below 40% can develop lesions such as ring tail and food consumption can be elevated (Clough 1982; Fox et al. 1984).

Lighting

Appropriate lighting and light cycle play a key role in maintaining the physiology and behavior of the rat. Lighting within the animal colony should be of a sufficient level to provide for animals' well-being and allow for animal care activities and safe working conditions for the animal care staff. Light in the animal rooms should provide for adequate vision and for neuroendocrine regulation of diurnal and circadian cycles (Brainard 1989, as cited in ILAR 1997). The current guideline for the laboratory rat is that levels be 325 lux approximately 1 m above the floor (ILAR 1997). This level of light is considered to be adequate for the performance of animal care activities with a limited risk for the development of retinopathies (Bellhorn 1980).

Variations in light intensity should be taken into consideration when arranging animals on cage racks for toxicology studies. There can be as much as an 80-fold difference in light intensity between a polycarbonate box positioned at the top of a rack and one positioned at the bottom (Weihe et al. 1969). This difference can be even greater when using wire mesh caging and should be taken into consideration when planning long-term subchronic, chronic, and carcinogenicity studies. It is good practice to arrange animals on the caging rack such that dose groups are placed sequentially in vertical order and cage position is rotated periodically (monthly). This will allow for randomization and control of possible retinopathies that might occur over the life of the study.

Most research facilities operate on a 12-hr light/12-hr dark cycle, but a 14-hr light/10-hr dark cycle is also acceptable. Maintenance of an appropriate light–dark cycle for the rat is important for the health and well-being of the animals. The rat is a nocturnal animal and is more active at night. Often, feeding studies are conduct on a reverse light–dark cycle that requires acclimation of the animal prior to the start of a study. The reversal of the light–dark cycle allows for the performance of study function during normal working hours.

It is important that monitoring procedures are in place to assure that the light cycle is operating correctly. In addition to retinal damage, exposure to continuous lighting could have effects on the pharmacokinetics of a test compound. Continuous lighting tends to equalize the day and night food consumption and GI transit rate (Siegel 1961; Wong and Oace 1981; Zucker 1971). Because gastric emptying can be affected by the amount of food in the stomach, the speed of absorption and hence blood levels can be changed, especially in dietary studies. In addition, continuous lighting can affect activity levels of the animals and thereby reduce or increase metabolism.

Ventilation

The purpose of an adequate ventilation system is to provide fresh air, control temperature and humidity, remove or dilute chemical pollutants such as ammonia from animal waste, and help control transmission of infectious agents. Current guidelines recommend a ventilation rate of 10 to 15 air changes per hour (ILAR 1997). In addition, if recycled air is used within the facility it must be mixed with at least 50% fresh air for every air exchange, the recycled air must be returned

to the room or area from which it was generated, and the preparation of recycled air must be sufficient to minimize toxic gases and odors during husbandry practices (ILAR 1997).

When placing racks in the room, the special arrangement should be considered to reduce the possibility of cross-contamination. This is especially important for dietary studies where the food and test article might be aerosolized. It is recommended that racks be placed at least 2 m apart to minimize the possibility for cross-contamination and the spread of microorganisms. As with light exposure, ventilation can be different based on position on the cage rack. Randomization of dose groups throughout the cage rack and periodic rotation of cages will help to control for varied exposure.

The ideal arrangement for proper ventilation of an animal room is a colony facility that is positive to an anteroom that is negative to both the animal room and the hallway. This will prevent the spread of disease between colonies, reduce the possibility of exposure to test compounds administered in the diet, and control odors. If the facility does not allow for this type of design, the animal room can be either positive or negative to the hallway depending on the purpose of the study. In clean–dirty corridor systems, air should flow from the access corridor (clean), through the animal room, to the dirty corridor (Sontag et al. 1976).

Noise

The effects of noise on the laboratory rat have been extensively researched. Exposure to various noise levels has been associated with the development of a variety of abnormalities and stress indicators. Changes in uterus and adrenal weights as well as reproductive disturbances have been observed (Geber 1973; Nayfield and Besch 1981; Sackler et al. 1959). In addition, the noise associated with normal husbandry activities has been associated with a marked increase in plasma corticosterone levels (Barrett and Stockham 1963). Noise within the animal rooms might be unavoidable, but current guidelines suggest that noise levels should be maintained below 85 dB whenever possible. Taking this into consideration, animal care staff should be trained to reduce noise as much as possible and rodents should be housed in facilities separate from large animal species such as dogs or nonhuman primates.

Caging

Different types of caging have been developed for the housing of rodents, taking into account the requirements for temperature and humidity, ventilation, lighting, noise, and the health and well-being of the animal. The type of caging used might create a different microenvironment within the cage than that found in the colony room (macroenvironment). When designing a study, the type of caging to be used should be taken into consideration. For rodents, two main types of caging are available. These are the shoebox or solid bottom cage and the hanging or wire mesh cage.

Shoebox Caging

The use of shoebox caging has several benefits when conducting toxicology studies. The use of solid bottom caging might reduce the incidence of foot lesion in chronic and carcinogenicity studies (Peace et al. 2001). The Association for the Assessment and Accreditation of Laboratory Animal Care has strongly recommended that solid bottom caging be used for all rodent studies unless justification for the use of other types of caging can be provided. Shoebox cages are either rectangular or square boxes with solid bottoms and walls. They can be made of stainless steel, but molded plastics such as polycarbonate (clear) or polypropylene (translucent) are more common. Lids for this type of cage are made of wire mesh capable of holding both feed and a water bottle, metal with ventilation holes, or filter tops. Cages can also be suspended from shelves in a rack, in which case the shelf acts as the cover for the cage.

Traditional filter top lids provide some protection from airborne contaminants (Brick et al. 1969; Kraft 1958; Simmons and Brick 1970). A disadvantage of this type of caging is that ventilation within the cage is reduced, leading to increased temperature, humidity, and levels of carbon dioxide and ammonia (Gordon and Fogelson 1994; Serrano 1971; Simmons et al. 1968). This has led to the development of caging that is entirely enclosed. The animal cage in conjunction with the cage rack creates a microenvironment suitable for the rat. The rack system has a fresh air supply that can be HEPA filtered and an air return system that removes the air and filters for contaminants (see figure 3.1). This type of caging also has the added benefit of reduced husbandry. Due to the constant airflow in the cage, urine and feces are quickly dried and ammonia is removed; as a result, bedding does not require changing as often (Kanzaki et al. 2001).

Typical shoebox caging has sufficient floor space for up to three adult rats group housed or a singly housed dam with litter. The cage can be designed to accept a water bottle or might have openings in one end that allow the cage to be placed on a rack with an auto-watering system. Animals might have access to feed via feed jars placed in the cage or via a specially designed lid that has a bin for holding block diet. Shoebox cages can also be designed with a rotating feed

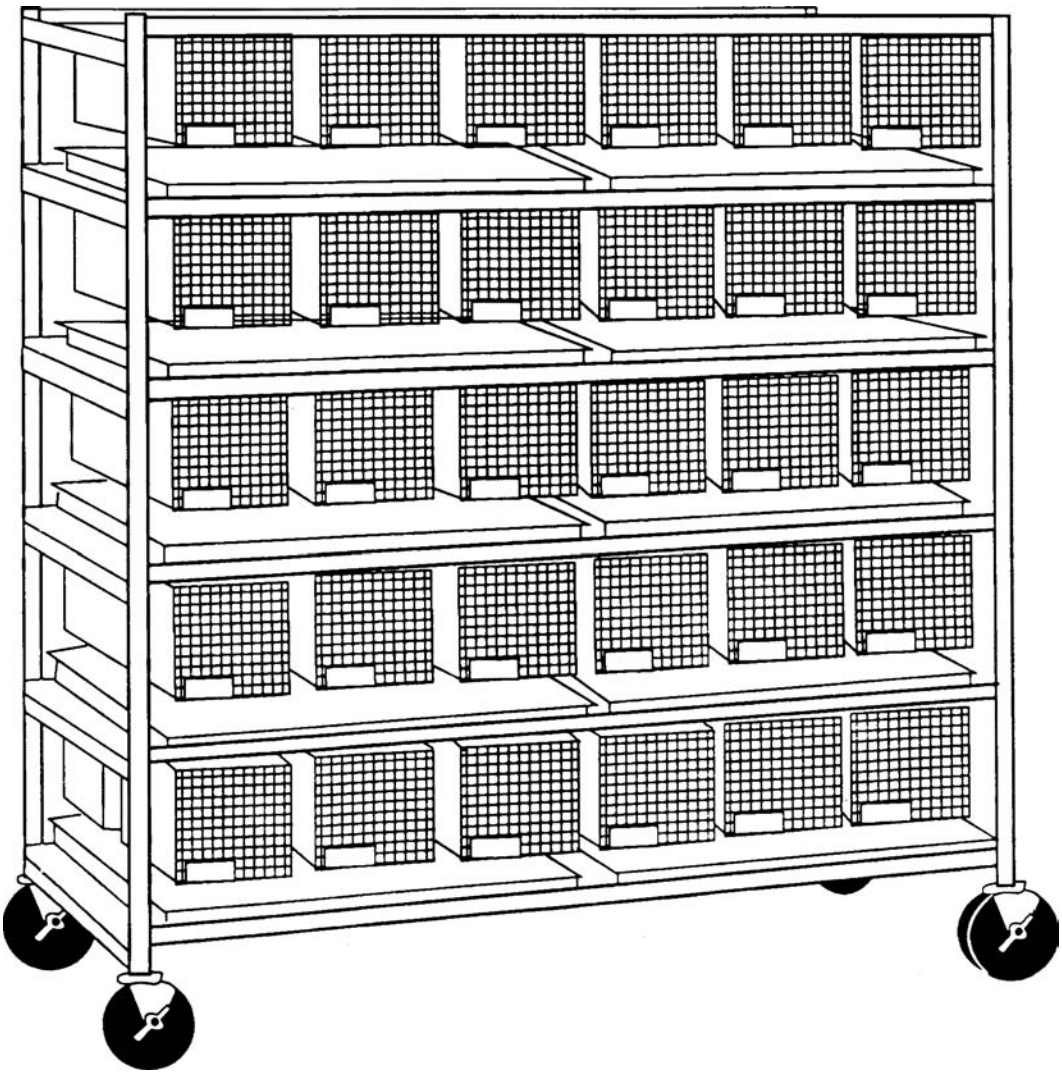

Figure 3.1 Rack of suspended wire mesh cages.

system that allows for controlled administration of fixed amounts of diet. Recent studies have shown that controlled or restricted feeding in long-term studies might decrease the incidence of some tumors and might increase survival rates in carcinogenicity studies (Clinton et al. 1997; Toriyama-Baba et al. 2001). The floor of the shoebox cage is usually filled with absorbent bedding. The choice of bedding should be made with care. The use of cedar or soft wood bedding should be avoided, as these materials have been associated with increases in adverse events such as hepatic microsomal enzyme levels (Weichbrod et al. 1988) and increased pup mortality (Burkhart and Robinson 1978).

Shoebox cages also have several disadvantages when used in rat toxicology studies. Rats are corpophagy and might receive additional exposure to the test compound or its metabolites that have been excreted in the feces. This is of particular importance when evaluating pharmacokinetic data or metabolite profiles. Rats also have a tendency to eat the bedding, which can reside in the stomach or impede gavage dosing. In dietary studies, bedding can serve as an alternate food source if the test article alters feed palatability. Due to these factors, alternate caging types should be considered.

Suspended Caging

Suspended wire bottom cages are typically made entirely of metal. The wire mesh floor is designed to allow excrement to fall through to absorbent paper or bedding placed under the cage. This design reduces the need to disturb the animal during routine husbandry. In addition, this type of caging reduces the possibility of increased drug exposure through the consumption of excrement and eliminates the risk of an impeded or obstructed stomach or esophagus during gavage dosing. This cage design also allows for increased ventilation in the cage, thereby reducing ammonia levels.

However, this cage design offers little protection against airborne transmission of disease or contaminants such as test compound in diet admixture studies (Sansone et al. 1977). Wire mesh floor cages have also been associated with a higher incidence of decubital ulcers in studies greater than 1 year in duration (Peace et al. 2001).

When designing a study, the type of caging to be used should be chosen carefully. Both solid bottom and wire mesh cages have advantages and disadvantages. For short-term subchronic toxicology studies, wire mesh cages might be appropriate as they reduce the risk of increased drug exposure. For chronic and carcinogenicity studies, solid bottom or a combination of solid bottom and wire mesh cages can be used.

Minimum Space Recommendations

Current minimum space recommendations for the rat are given in table 3.4. When deciding how many rats to house per cage, remember that the population density can affect heat, moisture, oxygen, carbon dioxide, and ammonia levels in the cage (Clough 1976). Several studies have been

Table 3.4 Minimum Cage Sizes for Rats

	Floor Area/Animal [a]	
Weight (g)	**in.^{2}**	**cm^2**
Up to 100	17	110
101–200	23	150
201–300	29	190
301–400	40	260
401–500	60	390
Over 500	70	460

[a] Cage height should be at least 7 in. (17.78 cm).

Source: ILAR (1997).

done comparing the effects of group versus individual housing. Overcrowding has been shown to increase plasma corticosterone levels (Barrett and Stockham 1963). Rats individually housed for 3 months develop larger adrenal and thyroid glands, have smaller spleens and thymus glands, and display an increased sensitivity to the cardiotoxicity of isoprenaline (Balazs et al. 1962; Dairman and Balazs 1970). The rat is a social animal and whenever possible should be housed in pairs or groups of three. For the purpose of most toxicity studies, this might not be practical, but should be considered.

Food

Physical Form and Presentation

Feed is typically supplied as either pellets or meal to rats. Pellets are usually presented to rats in a metal hopper attached to the front of the cage for suspended wire mesh cages and in wire bins in the lid of shoebox cages. Pelleted diets are considered a more efficient form in that they are less likely to be scattered by the animals and are more easily handled by caretakers. Pelleted diets are also made to be relatively hard and require gnawing, which helps to wear down the incisors of the animal. Pelleted diets are available from a variety of commercial manufacturers and can be formulated to specific needs. The meal form of diet is useful when test compounds must be mixed with the diet or for conducting food consumption. The feed is presented to the animals in wide-mouthed jars with lids. In addition to the lid, a perforated metal disk can be placed in the jar, which helps to reduce spillage and contamination by excreta. Devices have also been developed for pair-feeding studies or for studies in which feed aliquots must be presented throughout the day (Loveless et al. 1972; Quarterman et al. 1970). For specific applications, semimoist diets made with powdered ingredients mixed with water (50% v/v) and agar (0.1%) or liquid diets can be used.

Nutrition

Nutrient Requirements

Nutrient requirements for the laboratory rat have been specified by the National Research Council (1995). Recommendations for dietary nutrient contents are presented in table 3.5. Several complete rat diets adequate for normal growth, reproduction, and maintenance are available from commercial sources. These diets are available in natural ingredient and purified diet forms. Natural ingredient diets use unrefined or minimally processed ingredients such as cereals, cereal bioproducts, fish meals, soya-bean meals, skimmed milk, meat and bone meals, and molasses (Clarke et al. 1977). Purified diets use refined ingredients such as casein, vegetable oil, starch, and sucrose. Cellulose is used as a nonnutritive filler in purified diets.

Natural ingredient diets are more economical and more widely used; however, the variability of the nutrient content could make interpretation of toxicology results difficult. For the purpose of toxicology studies conducted under GLP, diets should be purchased from a vendor that certifies the diet. A certificate of analysis should be obtained for each lot of feed purchased (Environmental Protection Agency 1979; Food and Drug Administration 1978; ILAR 1997; National Academy of Sciences 1978).

Food Restriction in Chronic Studies

Food restriction in chronic studies has been found to increase life expectancy and decrease the incidence of some types of tumors. Keenan et al. (1998) found that a 25% and 50% restriction of food consumption resulted in decreased benign and malignant tumor incidence and increased

Table 3.5 Recommended Dietary Content of Nutrients for Rats

Nutrient	Amount in Diet (90% Dry Matter)[a]	Amount in Diet (PMI Certified Diet)[b]
Protein (%)	15.0	20.1
Fat (%)	5.0	5.1
Digestible energy (kcal/kg)	3800	4040
L-amino acids		
Arginine (%)	0.6	1.13
Asparagines (%)	0.4	
Glutmic acid (%)	4.0	4.2
Histidine (%)	0.3	0.49
Isoleucine (%)	0.5	1.03
Leucine (%)	0.75	1.58
Lysine (%)	0.7	1.18
Methioine (%)	0.6	0.43
Phenylalanine (and tyrosine) (%)	0.8	0.88
Proline (%)	0.4	1.47
Threonine (%)	0.5	0.78
Tryptophan (%)	0.15	0.24
Valine (%)	0.6	1.05
Nonessential (%)	0.59	
Minerals		
Calcium (%)	0.50	0.8
Chloride (%)	0.005	0.47
Chromium (mg/kg)	0.3	2.0 (ppm)
Copper (mg/kg)	5.0	11 (ppm)
Fluoride (mg/kg)	1.0	13 (ppm)
odine (mg/kg)	0.15	0.77 (ppm)
ron (mg/kg)	35.0	210 (ppm)
Magnesium (%)	0.05	0.21
Manganese (mg/kg)	10.0	75 (ppm)
Phosphorous (%)	0.30	0.60
Potassium (%)	0.36	0.86
Selenium (mg/kg)	0.15	0.25 (ppm)
Sodium (%)	0.005	0.30
Sulfur (%)	0.03	0.25
Zinc (mg/kg)	12.0	76 (ppm)
Vitamins		
A (IU/kg)	2,300	18,000
D_3 (IU/kg)	1,000	2,200
E (IU/kg)	27.0	66
K_1 (mg/kg)	1.0	0.4 (ppm)
Choline (mg/kg)	750	1,800 (ppm)
Folic acid (mg/kg)	1.0	4.0 (ppm)
Niacin (mg/kg)	15.0	95 (ppm)
Pantothenate (calcium) (mg/kg)	8.8	17 (ppm)
Riboflavin (mg/kg)	3.0	8.0 (ppm)
Thiamin (mg/kg)	4.0	16 (ppm)
Vitamin B_6 (mg/kg)	6.0	6.0 (ppm)
Vitamin B_{12} (mg/kg)	0.05	0.02

[a] Source: Values taken or calculated from National Research Council (1995).

[b] Source: PMI® Nutrition International.

survival rates (see table 3.6). In addition, animals placed on feed restriction were found to have lower overall body weights, which can be correlated to the incidence of plantar lesions. When planning a chronic toxicity or carcinogenicity study, consideration should be given to placing animals on feed restriction.

Table 3.6 Effects of Feed Restriction in Chronic Toxicity Studies

	Ad Libitum		25% Restriction		50% Restriction	
Parameter	**M**	**F**	**M**	**F**	**M**	**F**
Body weight (g)	765 ± 145	611 ± 87	572 ± 49	296 ± 22	357 ± 24	216 ± 15
Tumor incidence (%)	78	94	78	87	58	54
Benign tumor (%)	70	94	66	82	46	50
Malignant tumor (%)	26	30	22	40	16	10
Survival (%)	18	18	68	56	78	82

Source: Keenan et al. (1998)

Sterilization

Diet sterilization is mandatory for gnotobiotic animals or immunocompromised animals and is recommended for diets used for specific pathogen-free animals (Coates 1984; Wostman 1975). Common means of sterilization are heat, ionizing radiation, and fumigation. Heat can affect the nutrient value of diets and the physical nature of the diet by causing the pellets to disintegrate or become hard or increasing the tendency for mold growth. Fumigation with ethylene oxide has proven effective, but might change the nutrient value; care must be taken to ensure all traces of ethylene oxide have been removed. The use of ionizing radiation has proven to be the most effective means of sterilization without affecting the nutrient value.

Water

Bottle or automatic watering systems are used to supply water to the animals. Where bottles are used, they are capped with rubber stoppers fitted with stainless steel tubes that have constricted aperatures or ball valves. When the bottles are inverted and attached to the cages, rats can lick the aperature or valve and a drop of water will be delivered. Water bottle systems are useful when water consumption is to be measured or the test compound is to be delivered in the water. This is inexpensive to set up, but incurs increased labor costs due to bottle washing, sterilization, and replacement; bottles are commonly changed every 2 to 3 days.

Autonomic watering systems connect the cage and rack to the laboratory water supply by a series of pipes and tubes. Animals activate the system by licking a valve attached to the cage. The system requires less maintenance, but can malfunction if air becomes trapped in the rack, laboratory water pressure fails, or the rack is incorrectly attached to the laboratory plumbing. Because cages and racks are connected by plumbing, there is an increased risk of pathogen spread. Flushing the system minimizes microorganism build-up in the plumbing. In both systems, a malfunction could result in flooding of shoebox cages and animals should be monitored periodically each day.

Because the water supply can be a source of environmental contamination, current toxicology guidelines and regulations require periodic analysis of water for contaminants that could affect the outcome of the study.

Prevention of Infectious Diseases

The result of a toxicology study will be affected by a disease outbreak that results in the death or severe clinical illness of the test animals. Less pathogenic agents causing subclinical or less severe signs could also affect study results by suppressing or modifying the immune response, affecting fetal viability or organogenesis, or causing specific histologic changes (Hsu et al. 1980; Jacoby and Barthold 1981). By suppressing immune response, a mycoplasma infection can result in an increase in the incidence of lung tumors (Schreiber et al. 1972). If only a portion of the animals in the study are affected, this could lead to misleading or uninterpretable results. It is recommended that sentinal animals be placed in the room for subchronic toxicity studies greater

than 90 days in length and chronic toxicity and carcinogenicity studies. On a weekly basis a small amount of soiled bedding from other colony animals is transferred to the sentinal animal cage. This exposes the animals to the microbial status of other study animals. Periodically throughout the course of the study these animals should be submitted for clinical pathology and necropsy evaluation, which well help to monitor the health status of the study animals over the course of the study.

Microbial Status

As study results can be affected by infectious disease, researchers have moved toward using animals of known health status. The range of different levels of microbic association goes from *axenic* (or germ-free) animals, which are produced by hysterectomy and reared behind germ-free barriers, to conventional rats from which microbes have not been excluded. *Specific pathogen free* (SPF) or *barrier reared* (BR) rats are animals derived from axenic animals, reared in barrier-protected colonies, and shown to be free of certain pathogens. The SPF animal is preferred by researchers, especially for longer term studies.

Disease Prevention

One challenge facing the toxicologist and laboratory management is maintaining the microbial and health status of the animals once they arrive at the facility.

Animals should be purchased from a vendor breeding animals for the purpose of research. These vendors typically ship animals in groups in boxes with polyester fiber filters that help to avoid contamination of the animals in transit. This is especially important if using immunocompromised animals. To minimize the stress animals face during transportation to the laboratory, the vendor should provide sufficient feed and water, and, if possible, ship the animals in environmentally controlled vehicles. To avoid contamination from the outside of the shipping containers, these containers should not be brought into the room where animals will be quarantined.

Once received at the facility, animals should be quarantined to allow them to recover from the stresses of transit, resume normal weight gain, and allow existing disease to express itself. The period recommended for quarantine varies from 48 hr to 4 weeks, but a period of 2 weeks is typical for most facilities. Animals can be quarantined in a specially designed room or in the room to be used for the study. For longer term studies, baseline values for a disease-surveillance program can be established during this period: Groups of excess animals obtained for this purpose can be euthanized and examined on receipt and at preselected intervals during the course of the study for various serological, clinical laboratory, and histological examination.

Elements in barriers against disease introduction include (a) how materials, including equipment, feed, and bedding will be handled as they are brought into the room; (b) the personnel entry procedures; and (c) how the environmental system is handled. ILAR (1997) discusses procedures to be used for various classifications of barrier areas. Specific procedures will be determined by the research objectives, the health of the animals, and the cost of repeating a compromised study.

Jacoby and Barthold (1981) discuss various options available to the investigator if an outbreak occurs. These include ignoring the infection, quarantine of specific animal or colony, purposeful exposure of animals to generalize the infection, culling, and termination of the study or population. The selection of an appropriate option is dependent on the type of infection and chances for control, continued health of the animals, research objectives, husbandry practices, and facility design. It is strongly recommended that if an outbreak occurs the researchers should consult with the attending veterinarian to determine the best course of action.

Study Design

The length and design of toxicology studies used to predict human risk are governed by guidelines issued by regulatory bodies such as the U.S. Food and Drug Administration (FDA), the

International Council on Harmonization (ICH), the Environmental Protection Agency (EPA), and their counterparts worldwide. Toxicology studies are divided into a series of three sets of studies that are required for each phase of clinical trials. For initial approval to begin clinical trials, the following studies are required. The length of dosing in the toxicology studies varies depending on the intended length in clinical trials. A test compound intended to be a repeat dose study for up to 28 days in duration initially requires a two-phase study in which a maximum tolerated dose (MTD) following a single administration is determined, followed by a second phase during which the test compound is administered daily at dose levels based on the MTD for 5 to 7 days (table 3.7). Following the completion of the MTD study, a 14- or 28-day repeat dose study should be conducted (table 3.8 and table 3.8a). These studies assess the effects of a test compound at dosages that do not cause immediate toxic effects.

Table 3.7 Maximum Tolerated Dose Study in Rats

	Oral MTD Study	
Phase A	**Males**	**Females**
Dose Level 1	3	3
Dose Level 2	3	3
Dose Level 3	3	3
Dose Level 4	3	3

	7-Day Oral Range Finding Study			
	Main Study		Toxicokinetics	
Phase B	**Males**	**Females**	**Males**	**Females**
Control	5	5	—	—
Low dose	5	5	9	9
Mid dose	5	5	9	9
High dose	5	5	9	9

Experimental Design: In Phase A, the dose level will be increased until the MTD is determined. The MTD is a dose that produces neither mortality nor more than a 10% decrement in body weight nor clinical signs of toxicity. In Phase B, animals will be dosed daily for 7 days at fractions of the single-dose MTD to estimate a repeat dose MTD.
Dose Route/Frequency: As requested Phase A: Once / Phase B: Once per day for 7 consecutive days
Observations: Twice daily in both phases (mortality/moribundity)
Detailed Clinical Observations: Daily in both phases
Body Weights: Daily in both phases
Food Consumption: Daily
Clinical Pathology (Phase B only): Hematology, clinical chemistry, and urinalysis evaluations on all surviving main study animals at termination
Necropsy (Phase B only): Tissues saved for possible future histopathological evaluation
Organ Weights (Phase B only): Adrenals, brain, heart, kidneys, liver, lungs, ovaries with oviducts, pituitary, prostate, salivary glands, seminal vesicles, spleen, thyroid with parathyroid, thymus, testes, uterus
Toxicokinetics: Blood collected on Days 1 and 7 (3 cohorts consisting of 3 animals/sex/treatment group bled twice to equal six time points), calculation of C_{max}, T_{max}, $AUC_{0\text{-}24}$, and $T_{1/2}$.

In support of Phase 2 clinical trials, longer term subchronic and chronic toxicity studies (table 3.9) should be conducted. Subchronic and chronic toxicity studies are designed to assess the test compound effects following prolonged periods of exposure. The highest dosage level in each of these studies should produce a toxic effect such that a target organ can be identified. The lowest dosage level should provide a margin of safety that exceeds the human clinical dose and ideally allows for the definition of a no observable effect level (NOEL). Alternatively, when effects related to the pharmacological mechanism of the test compound or observed effects can be related to treatment with the test compound but might not be of toxicologic significance, a no observable adverse effect level (NOAEL) can be determined.

Table 3.8 14- or 28-Day Repeat Dose Toxicity Study in Rats

	Main Study		Toxicokinetics	
	Males	**Females**	**Males**	**Females**
Vehicle control	10	10	—	—
Low dose	10	10	9+3*	9+3*
Mid dose	10	10	9+3*	9+3*
High dose	10	10	9+3*	9+3*

* Three additional animals/sex/treatment group included as replacement animals.

Dose Route/Frequency: As requested
Observations: Twice daily (mortality/moribundity)
Detailed Clinical Observation: Weekly
Functional Observational Battery: Pretest and Day 14 or 25
Body Weights: Weekly
Food Consumption: Weekly
Ophthalmology: All animals prior to test article administration; all surviving main study animals at study termination
Clinical Pathology: Hematology, clinical chemistry, and urinalysis evaluations on all surviving main study animals at termination
Toxicokinetics: Blood collected on Days 1 and 14 or 27 (3 cohorts consisting of 3 animals/ sex/treatment group bled twice to equal six time points); toxicokinetic modeling
Necropsy: All main study animals; toxicokinetic animals euthanized and discarded
Organ Weights: Adrenals, brain, heart, kidneys, liver, lungs, ovaries with oviducts, pituitary, prostate, salivary glands, seminal vesicles, spleen, thyroid with parathyroid, thymus, testes, uterus
Slide Preparation/Microscopic Pathology: All animals in the vehicle control and high-dose groups and all found dead animals: full set of standard tissues; low- and mid-dose group target organs (to be determined); gross lesions from all animals

Table 3.8a 28-Day Repeat Dose Toxicity Study with Immunophenotyping in Rats

	Main Study		Toxicokinetics	
	Males	**Females**	**Males**	**Females**
Vehicle control	10	10	—	—
Low dose	10	10	9+3*	9+3*
Mid dose	10	10	9+3*	9+3*
High dose	10	10	9+3*	9+3*

* Three additional animals/sex/treatment groups included as replacement animals; the control animals will not be evaluated for toxicokinetics.

Dose Route/Frequency: As requested/Once daily all animals for 28 days.
Observations: Twice daily (mortality/moribundity)
Detailed Clinical Observation: Weekly
Functional Observational Battery: Pretest and Day 25
Body Weights: Weekly
Food Consumption: Weekly
Ophthalmology: All animals prior to test article administration; all surviving main study animals at study termination
Clinical Pathology: Hematology, clinical chemistry, and urinalysis evaluations on all surviving main study animals at termination
Immunotoxicology: Immunophenotyping of blood leukocytes by flow cytometry on all surviving main study animals at termination; NK cell assay on blood leukocytes of all surviving main study animals at termination
Toxicokinetics: Blood collected on Days 1 and 27 (3 cohorts consisting of 3 animals/sex/ treatment group bled twice to equal six time points)
Necropsy: All main study animals; toxicokinetic animals euthanized and discarded
Organ Weights: Adrenals, brain, heart, kidneys, liver, lungs, ovaries with oviducts, pituitary, prostate, salivary glands, seminal vesicles, spleen, thyroid with parathyroid, thymus, testes, uterus, two lymph nodes (e.g., mesenteric, axillary, popliteal, etc.) including the lymph node draining the route of administration
Slide Preparation/Microscopic Pathology: All animals in the vehicle control and high-dose groups and all found dead animals: full set of standard tissues (add Peyer's patch, extra lymph node); low- and mid-dose group target organs; gross lesions from all animals

Table 3.9 Subchronic and Chronic Toxicity Study in Rats

	Main Study		Toxicokinetics	
	Males	**Females**	**Males**	**Females**
Vehicle control	15	15	—	—
Low dose	15	15	9+3*	9+3*
Mid dose	15	15	9+3*	9+3*
High dose	15	15	9+3*	9+3*

* Three additional animals/sex/treatment group included as replacement animals.

Dose Route/Frequency: As requested
Observations: Twice daily (mortality/moribundity)
Detailed Clinical Observation: Weekly
Body Weights: Weekly
Food Consumption: Weekly
Ophthalmology: All animals prior to test article administration; all surviving main study animals at study termination
Clinical Pathology: Hematology, clinical chemistry, and urinalysis evaluations on all surviving main study animals at termination
Toxicokinetics: Blood collected on Days 1 and 90 (3 cohorts consisting of 3 animals/sex/ treatment group bled twice to equal six time points); toxicoknetic modeling
Necropsy: All main study animals; toxicokinetic animals euthanized and discarded
Organ Weights: Adrenals, brain, heart, kidneys, liver, lungs, ovaries with oviducts, pituitary, prostate, salivary glands, seminal vesicles, spleen, thyroid with parathyroid, thymus, testes, uterus
Slide Preparation/Microscopic Pathology: All animals in the vehicle control and high-dose groups and all found dead animals: full set of standard tissues; low- and mid-dose group target organs; gross lesions from all animals

In addition to the subchronic and chronic toxicity studies in support of Phase 2 clinical trials, reproductive safety studies might also be required. Reproductive toxicity studies are typically required for test compounds intended to be administered to women of childbearing age or that might affect male reproduction. These studies include an assessment of the potential effects of the test compound on general fertility and reproductive performance (Segment I), developmental toxicity (Segment II), or perinatal and postnatal development (Segment III). The highest dose in reproductive studies should be chosen so that administration causes some minimal toxicity. Typically, a pilot study to find dose range in a small number of animals should be conducted prior to initiating the definitive reproductive toxicology studies. Examples of protocols designed to meet the ICH guidelines are presented in table 3.10, table 3.11, and table 3.12.

In support of Phase 3 clinical trials, a carcinogenicity study might be required (table 3.13). Typically 18 months to 2 years in duration, this type of study is designed to assess the potential of the test compound to induce neoplastic lesions. The highest dosage in a carcinogenicity study should cause minimal toxicity when administered via the intended route for clinical use. The preclinical studies required in support of the clinical trials are dependent on the intended route and frequency of administration of the test compound and the intended age group to be treated.

Table 3.10 Study of Fertility and Early Embryonic Development to Implantation in Rats

	Males	Females
Vehicle control	25	25
Low dose	25	25
Mid dose	25	25
High dose	25	25

Dose Route/Frequency: As requested; males dosed beginning 28 days before mating and continuing until euthanasia; females dosed beginning 14 days before mating and continuing through Day 7 of gestation (implantation)

Observations: Twice daily (mortality/moribundity)

Clinical Examinations: Observations for clinical signs, body weights, and food consumption measurements recorded during the study period; beginning at initiation of test article administration, females examined daily to establish estrous cycle

Uterine Examinations: Performed on dams on Day 13 of gestation; gravid uterine weight and the weight of the ovaries recorded; total number of corpora lutea and implantations, location of resorptions, and embryos recorded; females subjected to necropsy, and reproductive organs and gross lesions fixed for possible microscopic evaluation.

Evaluation of Males: Following disposition of females, the males euthanized and subjected to a necropsy; the testes and epididymides weighed, and analysis of sperm parameters (concentration, motility, and morphology) performed; reproductive organs and gross lesions fixed for possible microscopic evaluation

Statistical Analysis: Standard

Table 3.11 Embryo-Fetal Development in Rats

	Time Mated Females
Vehicle control	25
Low dose	25
Mid dose	25
High dose	25

Dose Route/Frequency: As requested; dosing will initiate on Day 6 of gestation and continue to and include Day 17 of gestation

Observations: Twice daily (mortality/moribundity)

Clinical Examinations: Daily gestation Days 6 through 20

Body Weights/Food Consumption: Gestation Days 0, 6, 9, 12, 15, 18, and 20

Cesarean Section/Necropsy: Litters will be delivered by cesarean section on Day 20 of gestation; gravid uterine weight will be recorded; total number of corpora lutea, implantations, early and late resorptions, live and dead fetuses, and sex and individual body weights of fetuses will be recorded; external abnormalities of fetuses will be recorded;approximately one-half of the fetuses will be processed for visceral abnormalities, and the remaining fetuses will be processed for skeletal abnormalities; all fetuses will be examined for visceral and skeletal abnormalities; dams will be subjected to a necropsy and gross lesions and target organs (if known) will be saved.

Table 3.12 Pre- and Postnatal Development, Including Maternal Function in Rats

	P Generation		F_1 Generation	
	Males	**Females**	**Males**	**Females**
Vehicle control	N/A	25	25	25
Low dose	N/A	25	25	25
Mid dose	N/A	25	25	25
High dose	N/A	25	25	25

Number in Study: P Generation: 100 females; F_1 Generation: 100 males, 100 females
Dose Route/Frequency: As requested; once daily to P animals from gestation Day (GD) 6 to postnatal Day (PND) 21; F_1 animals not dosed
Observations: Twice daily (mortality/moribundity)
Clinical Observations: P females: daily during treatment; F_1 adults: weekly
Body Weights:
P females: GD 0, 6, 10, 14, 17, and 20; PND 0, 7, 10, 14, and 21
F_1 males: weekly through termination
F_1 females: weekly until evidence of copulation detected, then GD 0, 7, 10, and 13
Food Consumption: P females: on corresponding body weight days during gestation/lactation
Vaginal Smears: All F_1 females during a 21-day cohabitation period until evidence of copulation is detected
Litter Evaluations: All F_1 offspring, count, body weight, sex, clinical observations on PND 0, 4, 7, 14, 21; behavioral and developmental evaluation of four males and four females from each litter for static righting, pinna detachment, cliff aversion, eye opening, air drop righting reflex, neuropharmacological evaluation, and auditory response; one male and one female (selected for the next generation) tested for sexual maturation (vaginal opening, preputial separation), motor activity/emotionality, and passive avoidance
Cesarean Section: On GD 13, F_1 females for location of viable and nonviable embryos, early and late resorptions, number of total implantations, and corpora lutea
Sperm Evaluation: Can be conducted on F_1 males if evidence of reduced fertility is noted (additional cost)
Necropsy: Gross lesions and target organs fixed for possible microscopic evaluation (additional cost)
All P females at PND 22 as well as all F_1 weanlings not selected for F_1 generation
All F_1 females at GD 13

Table 3.13 Carcinogenicity Study in Rats

	Main Study		6-Month Satellite	
	Males	**Females**	**Males**	**Females**
Vehicle control	60	60	20	20
Low dose	60	60	20	20
Mid dose	60	60	20	20
High dose	60	60	20	20

Dose Route/Frequency: As requested
Observations: Twice daily (mortality/moribundity)
Detailed Clinical Observations: Once weekly
Body Weights: Weekly for first 13 weeks, monthly thereafter
Food Consumption: Weekly for first 13 weeks, monthly thereafter
Ophthalmology: All animals pretest and all survivors prior to terminal sacrifice
Clinical Pathology:
Main study: Hematology at termination
6-month satellite: Hematology, clinical chemistry, and urinalysis evaluations on all surviving satellite animals at termination
Necropsy: All animals
Slide Preparation/Microscopic Pathology: All animals, full set of standard tissues, all masses and all lesions
Statistical Analysis: Standard

Dosing Techniques

A wide range of dosing techniques has been developed for evaluating the effects of test compounds in the rat. Due to the requirement in toxicology studies to extrapolate results to human risk, convention and guidelines dictate that rats should be dosed by a route closely approximating that of human exposure. For mechanistic studies, where specific organs might be targeted, the route of administration will be dictated by the research objectives.

Oral Route

Rodents have several unique characteristics to be considered regarding the oral administration of test compounds. One of the most important characteristics is the lack of an emetic response. The lack of this response allows for a higher dose of a potential emetic compound to be administered and evaluated. Many compounds and excipients can cause emesis in dogs or other large animal species and could lead to a low level of exposure and erratic blood levels. A second factor to consider is that rodents are nocturnal and eat most of their food at night. When maintained on a 12-hr light–dark cycle, rats have been found to consume 75% of their daily food intake during the dark cycle (Wong and Oace 1981). This should be taken into consideration when designing an oral gavage study and determining when the animal can be dosed. Early in the light cycle, animals are more likely to have a full stomach and complications associated with dosing can occur if large volumes of test article are administered. In addition, a full stomach could affect gastric emptying and the rate of absorption of an orally administered test compound.

Techniques for oral administration of test compounds include mixing in the diet, via gavage or stomach tube, via capsule, or in drinking water. The most widely used methods of oral administration are the dietary and gavage techniques.

Dietary vs. Gavage Methods

The choice between dietary and gavage dosing techniques is typically based on several factors. A scientific decision can only be made with knowledge of the pharmacokinetics of the test compound administered by both methods. Other considerations that can be used in making this decision are described next.

The dietary method can be used if a compound can be mixed with the diet, is stable under storage conditions in the diet, and is palatable to the animal. A major advantage of the dietary method is that it requires less manpower to perform the study. The diet mixing process can be performed weekly or, if stability allows, less often. The mixing and feeding process is less labor intensive than gavaging rats on a daily basis. Several disadvantages also exist in using the dietary method. Methods must be developed and validated to prove homogeneity and stability. This is not as easy a process as with a suspension or solution. The dietary method is also less exact than the gavage method, in that the concentration of compound mixed in the feed is based on predicted feed consumption and body weights. In addition, if the feed is not palatable to the animal, or the test compound makes the animal ill, feed consumption might be reduced, thereby reducing exposure to the test compound. In addition, the facility and control animals might be exposed to the test compound through dust or vapors.

The gavage method can be used when the test compound is not stable in the diet or might not be palatable to the animals. In addition, the gavage method is preferable when evaluating toxicokinetics or pharmacokinetics. As with dietary mixtures, test compound administered via gavage as a solution or suspension should be analyzed for homogeneity, stability, and concentration. Methods for solution or suspension might be easier to develop than those required for dietary mixtures. For

GLP studies, evaluation of homogeneity, stability, and concentration should be conducted for every study. If the same methodology and batch size are used for multiple studies, homogeneity can be established one time. Stability of the test compound in solution or suspension should be determined under the testing conditions in the proposed vehicle. Typically, stability for toxicology studies is established for between 7 and 14 days. If the test compound is not found to be stable, stability of shorter duration can be established. Finally, concentration analysis should be established for each dose level and should be periodically evaluated during longer term studies.

With the gavage method of dosing, a more precise amount of the test compound can be delivered, and this might reduce the amount of test compound required to complete the study. This becomes important when evaluating the effects of a pharmaceutical, as the required dose levels and exposure levels to show safety can be lower than that required for a pesticide or chemical. A disadvantage of the gavage method is that it involves handling of the rat for each dosing. Handling of the rat has been shown to increase corticosterone levels (Barrett and Stockham 1963) and could affect study results. Additionally, daily intubation might lead to death due to esophageal puncture or inhalation pneumonia.

Dietary Method

When utilizing the dietary method, the test compound is mixed with the diet and administered to the animals either ad libitum or the diet is presented to the animals for a fixed amount of time each day. The dosage received by an animal is regulated by varying the concentration of test compound in the diet based on the predicted food consumption and body weight. Food consumption and body weight predictions are based on historical laboratory data for early time points in a study. As the study progresses, growth and food consumption curves can be established for each group and group mean data can be used to predict future food consumption. Different concentrations of the test compound and diet should be made for each sex.

Test compounds and diets are mixed in two steps: First, the compound and about 10% of the total amount of diet are blended in a premix, then the premix and the remainder of the diet are mixed. The total amount of diet to be mixed is first weighed out, and the 10% is separated into the premix. To make the premix, all of the test compound and an aliquot of the diet (from the 10%) are put into a mortar. These ingredients are ground with a pestle until the mixture appears homogeneous. The mixture and the remainder of the premix are then layered in a small-capacity mixer and mixed for 5 to 10 min. The time for this mixing process can be varied if analysis shows the total mixture is not homogeneous. For the final mix, the premix and the remainder of the diet are layered in a large-capacity mixer. The mixing time will vary with the type of blender and can be varied if the analysis shows the total mixture is not homogeneous.

Several types of blenders are available for the mixing process, including open-bowl “kitchen” mixers, V or PK blenders, and Turbula mixers. Metal parts should be ground to eliminate electrostatic forces. Food jars and other equipment for presentation of compound diet mixtures have been previously discussed. In addition, alternative methods of dietary administration such as microencapsulation can be used for volatile, reactive, or unpalatable chemicals.

Gavage Method

In the gavage procedure, the test compound is administered by passing a feeding tube or gavage needle attached to a syringe down the esophagus into the stomach.

Test Article Preparation. If not already a liquid, the test compound is prepared for administration by adding it to the appropriate vehicle. The choice of vehicle depends on the characteristics of the compound and whether it is to be administered as a suspension or a solution. In addition, consideration must be given to the effects of the vehicle on the rat (Gad and Chengelis 1988). Common

vehicles used include water and food-grade oils such as corn oil. Suspensions are made when aqueous vehicles are desired and the test compound is not soluble. Suspending agents such as methylcellulose are added to increase the viscosity and hold the compound in suspension. Other agents such as Tween 80, ethanol, polyethyleneglycol 400 (PEG 400), and others can be used as wetting or stabilizing agents.

Equipment. Soft catheters made of silastic or polyethylene (e.g., infant feeding tubes), stainless steel gavage needles with smooth ball-shaped tips, or polyethylene gavage needles with ball-shaped tips are commonly used. All are commercially available and are relatively inexpensive. Although the soft catheter minimizes the chance of esophageal trauma, liquid can leak past the catheter and back up the esophagus and be aspirated. The ball-shaped tips of the stainless steel gavage needles reduce the chances of tracheal injections; however, if an animal struggles while the needle is in the esophagus, the rigid needle increases the chances of perforating the esophagus. The polyethylene gavage needle incorporates the best of both the soft catheter and the stainless steel needle, but due to the flexible nature of the needle, the risk for tracheal injection is increased.

Conybeare and Leslie (1988) found deaths in gavage studies were a result of aspiration of small amounts of irritant solutions or acidic, hypertonic solutions. They also found that the use of a ball tip 4 mm in diameter helped to eliminate deaths related to dosing. With gentle handling, the animals will be acclimated to the techniques used and dosing will become easier.

Aspiration and tracheal administration of test compound as well as esophageal trauma have been associated with gavage dosing and could lead to difficulty in interpretation of the study. The catheter and the needles all have risks inherent in their use, therefore care should be used when using these tools and animal technicians should be properly trained. The choice of the appropriate catheter or needle should be left up to the dosing technician based on training and comfort level.

Technique. The following description is appropriate for either a gavage needle or catheter; for simplicity, only the needle is mentioned in the description. The method for holding a needle and syringe combination while dosing is illustrated in figure 3.2; the method for the catheter and syringe combination is shown in figure 3.3.

Prior to picking up the animal, the syringe should be attached to the needle and filled with the appropriate amount of test compound to be delivered. Any air bubbles should be eliminated and the needle wiped clean of residual test compound. This is done so that the animal does not taste the test compound and residual test compound is not aspirated as the needle is passed down the esophagus. If the dosing liquid is distasteful, the animal might struggle after repeated dosing and increase the chances of being injured.

To position the animal for gavage, it should be grasped by the skin of the back and neck (see figure 3.2), ensuring that the head, neck, and back are in a straight line. Alternatively, the animals can be grasped about the shoulders, with the index finger and thumb on either side of the head (see figure 3.3). The objective is to firmly hold the animals to control any struggling if it occurs and to also prevent the animal from being able to bite the technician. For even more control, the animal can be placed on a table or brought up against the operator's chest.

Once the animal is in position, the needle can be inserted into the mouth of the animal, moved over the tongue, and down into the esophagus. The length of the needle should be inserted into the animal. A slight rotation of the needle might help with insertion into the esophagus. If the needle is inserted into the trachea, the animal might struggle. The syringe should be grasped lightly such that if the animal does struggle, the chances of an esophageal tear are minimized. If the animal continues to struggle, the needle should be withdrawn to allow the animal to calm down, then dosing should be attempted again. Alternatively, if a catheter is used, as the tube is placed into the mouth, it should be placed to the side between the molars. This is done because the tube might be bitten or transected if passed too close to the front teeth.

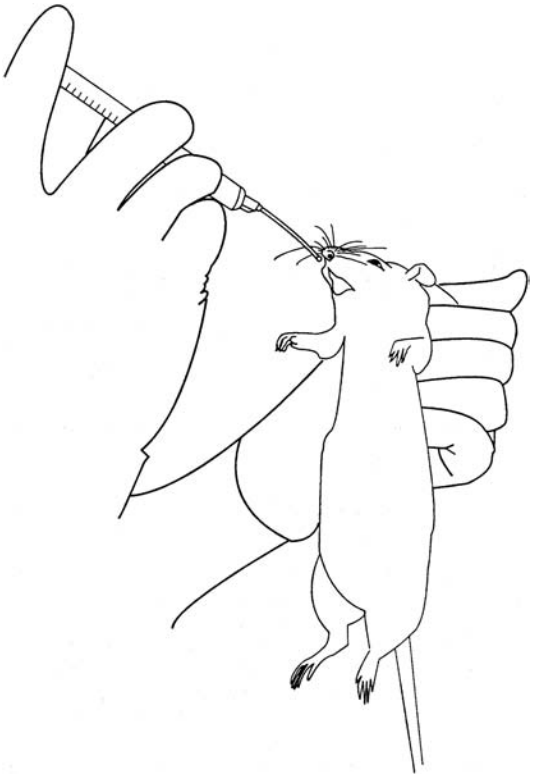

Figure 3.2 Gavage dosing with ball-tipped needle. Note method of restraint.

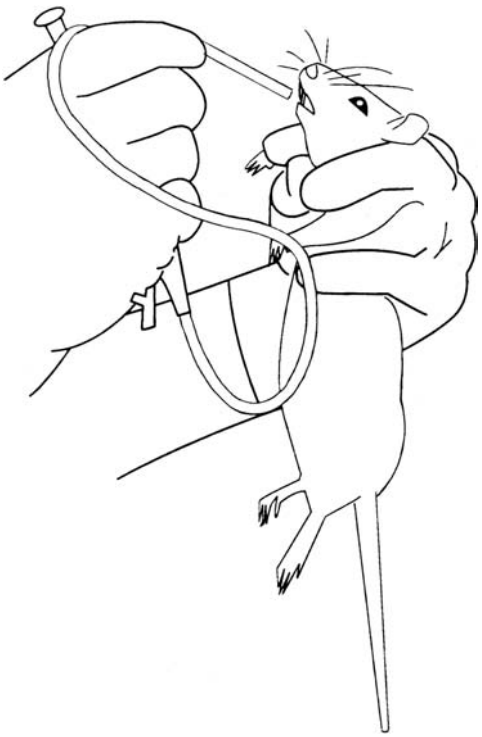

Figure 3.3 Gavage dosing with infant feeding tube. Alternate method of restraint.

With the needle in place, the test compound should be slowly expelled into the animal. If administered too rapidly, reflux could occur and the test compound might back up into the esophagus, resulting in an inaccurate dose being given and possible aspiration of the test compound. Once the dose has been delivered, the needle should be withdrawn and the animal observed for any signs of distress or respiratory difficulty. An experienced technician should be able to dose between five and seven animals per minute without causing discomfort to the animals and with minimal dosing-related deaths.

Gavage liquids are commonly administered at a volume of 5 to 10 ml/kg body weight. The volume should be enough to be delivered accurately, but not so much that it will adversely affect the animal. The maximum volume should be no more than 20 ml/kg. If using volumes greater than 10 ml/kg, it might be advisable to fast the animals for several hours prior to dosing. This will ensure that the stomach is empty prior to dosing and able to handle the larger volume. This option should be considered carefully, as fasting can affect the rate of absorption and clearance from the stomach. In addition, the choice of housing and bedding should be considered when dosing with large volume, as rats have the tendency to eat bedding, which could hinder gavage dosing. In addition, the volume chosen can have an effect on the results of the study and volumes greater than 10 ml/kg should only be used when issues of solubility and exposure exist. Ferguson (1962) found that a change in dose volume of from 5% to 1% of body weight could reduce mortality rate from approximately 95% to 5%, respectively, at equivalent doses.

Neonatal Administration. Neonatal intragastric injections can be made orally with thin silicone tubing (Gibson and Becker 1967; Smith and Kelleher 1973) or by intragastric injection with a 27-gauge needle through the abdominal wall (Bader and Klinger 1974; Worth et al. 1963). The oral method using silicone tubing is performed in a similar manner to the previously described method in adult rats. The intragastric injection through the abdominal wall is performed by first locating the stomach in the upper left quadrant of the abdomen and then carefully inserting the needle through the abdominal wall into the stomach, taking care that the animal does not move. The syringe should be gently aspirated to ensure proper placement and then the injection completed and the needle withdrawn.

Capsule

To eliminate the possibility of dosing errors and to deal with compounds that cannot be delivered through conventional means, methods have been developed for the administration of capsules into the esophagus of the rat. The test compound can be prefabricated into a small capsule or the test article can be weighed and placed into commercially available capsules. An individual capsule is then placed into a specially designed cup in the end of a gavage needle and the needle is then inserted into the esophagus of the rat. The capsule is then pushed out of the cup into the esophagus using either air or a rod inside the needle. The needle is then withdrawn and the capsule moves down into the stomach by peristaltic action. Only small amount of test compound can be administered as a single dose using this method, but multiple capsules can be administered sequentially in the same dosing session.

Water

As an alternative to dietary administration, compounds that are water soluble, palatable to the rat, and stable in water can be administered via the drinking water. This method offers similar advantages to adding a test compound to the diet. Additionally, compounds will be more easily mixed and analyses will be more easily developed than when a compound is in the diet. However, spillage of water makes measurement of the actual dose received difficult.

Intravenous Route

One of the most common methods of administration of test compound is via intravenous (IV) injection or infusion. The IV route is often the route of choice for compounds that have poor bioavailability via the oral route or have a short half-life. Several issues must be considered when administering a test compound intravenously. The compound must be soluble in an acceptable IV vehicle or excipient, must be able to be administered as a solution, and should be sterile or sterile filtered prior to administration. In addition, when designing a study, the pharmacokinetic profile of the test compound administered intravenously should be considered. Study activities such as clinical observations and functional observational battery should be planned around the expected time of greatest plasma concentration.

A variety of veins can be used for IV injections. These include the lateral tail (caudal), jugular, femoral, saphenous, lateral marginal, dorsal metatarsal, sublingual, and dorsal penile vein. Although most of these are superficial, and easily available for injection, several require the use of anesthesia or more than one technician and might be of limited use in repeat dose studies. Although anesthesia might be acceptable for acute studies or surgical model, its repeated use could have an effect on the toxicity of a test compound.

Lateral Tail Vein

The lateral tail veins are currently the most widely used for IV injections in the rat. The veins are easily visible, especially in young animals, and one person can perform injections without the use of anesthesia. The technician performing the function should be well trained and care should be taken to ensure that the lateral veins are being accessed and not the dorsal or ventral artery of the vein.

Bolus Injection Technique. The animal should be placed in an appropriate restrainer. This typically consists of a solid tube into which the animal is placed headfirst. The tube has a stop that is placed behind the animal with a hole that allows the tail to hang out the back (see figure 3.4). The restraint tube is designed to be secure enough that the animal cannot move, back out, or turn, but can still breath comfortably. Once the animal is secure, the tail should be cleaned and the vein can be dilated with heat. This can be accomplished by placing the tail in warm water (40–45°C), placed under a heat lamp, or wrapped with warm gauze. Care must be taken to avoid using excessive heat as tissue damage could result. Minasian (1980) describes a tourniquet made from a plastic syringe and thread. If used, this should not be left on for an extended period of time.

When performing an injection, the end of the tail should be held firmly and taut with the thumb and index finger of one hand. A 23-gauge needle attached to an appropriately sized syringe should be held with the bevel up at a shallow angle parallel to the vein. The skin of the tail is then pierced and the needle advanced until resistance is no longer felt. The plunger of the syringe should then be aspirated to ensure proper placement of the needle. The use of a needle with a clear or transparent hub will facilitate confirmation of correct placement. Blood backflow into the needle confirms entry into the vein. Alternatively, a butterfly needle with an extension line can be used. The butterfly needle with an extension set precludes the need to hold the tail, needle, and syringe. When using this type of setup, the butterfly needle is attached to an extension set and syringe that is filled with the test compound. The tail can be taped to the table and the butterfly needle is then inserted into the vein; placement is verified by aspiration on the syringe. Once confirmed, the butterfly needle can also be taped in place. This prevents the needle from pulling out of the vein during dosing. This type of setup can be very useful when administering large volumes of test article as a slow bolus over several minutes or when the test compound could be irritating or mildly caustic. Taping the animal's tail in place prevents the animal from pulling the tail out of the fingers of the dosing technician.

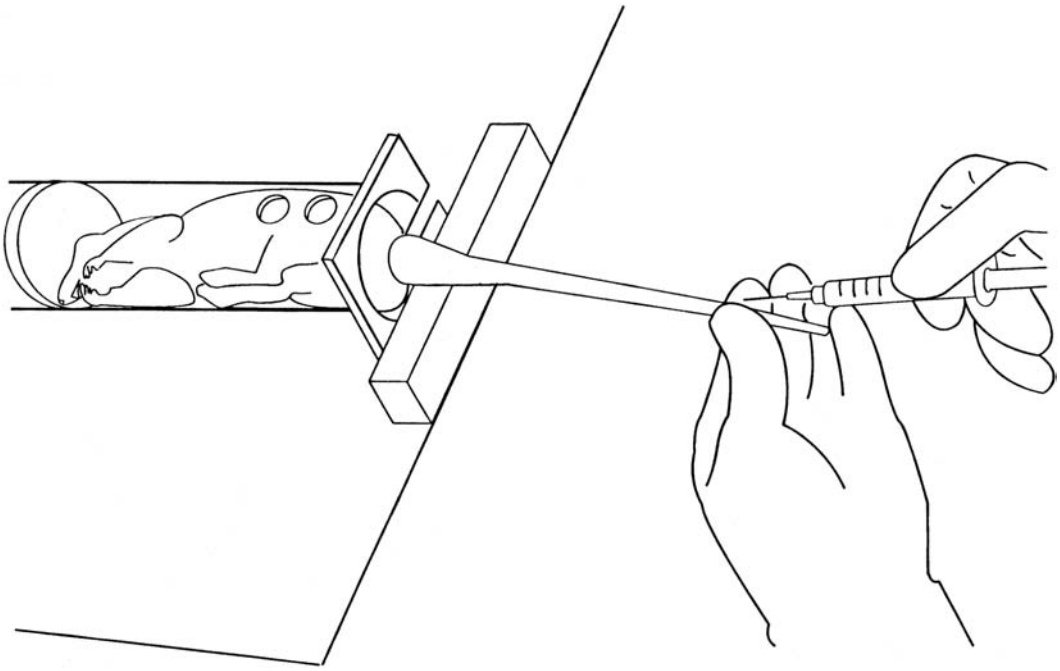

Figure 3.4 Tail vein injection.

If repeated dosing is to be performed, the initial venipunctures should be performed as close to the tip of the tail as possible. During the injection, if the needle comes out of the vein, a bleb will form under the skin. The needle should be repositioned immediately to prevent infiltration of the solution around the vein. Infiltration of an irritating solution can cause necrosis and make future injections difficult or impossible. Injection of 2 ml/100 g body weight can be accomplished without stress to the rat. Barrow (1968) found that injections of volumes over this amount produced respiratory difficulty and pulmonary edema.

Tail Vein Infusions

Tail vein infusions are convenient because catheter placement can be accomplished without anesthesia. A 23-gauge or smaller needle connected to an extension set is inserted into the tail. The needle and extension set is then secured to the tail with tape. The extension set is attached to a syringe that that is placed on a pump and the test compound can be infused. The tail can be taped to a wooden stick or tongue depressor to further protect the needle from being dislodged. Over the needle catheters are also commercially available and offer the advantage that the needle is removed once the catheter is placed in the vein and might help to prevent further penetration of the vein wall and subsequent perivascular dosing (Rhodes and Patterson 1979). Advantages that this technique has over permanent indwelling catheters are that the catheter is removed following dosing and will not become occluded. In addition, the animal doses not have to undergo anesthesia and a surgical procedure to place the catheter. Permanent catheters have a tendency over time to develop a fibrin flap or become clotted, thus losing patency. A major disadvantage is that the animals have to be restrained during the infusion, which could cause stress and alter the results of the study. When using this technique, the duration of the infusion should be limited so that the length of time the animal is restrained is limited.

An alternative technique using the lateral tail vein involves placing a catheter in the vein and wrapping the tail in a similar manner as previously described, then a lightweight protective cover attached to a tether system is placed around the tail to hold the catheter or needle in place.

Jugular Vein

Although this route has been used for bolus injections, it is most widely used as a site for cannulation for indwelling catheters. The indwelling catheter required surgical implantation under anesthesia.

Bolus Injection Technique. Although injections can be made by exposing the jugular vein by incision, this method is not acceptable for repeated dosing. The jugular vein can be accessed for test compound administration without exposing the vein. The animal can either be anesthetized or restrained on the back. The head is positioned to either the left or the right for access to the respective jugular vein. A 23-gauge needle fitted to a syringe with the bevel up is inserted in a cephalocaudal direction into the angle made by the neck and shoulder. The needle should enter the vein anterior to the point at which it passes between the pectoralis muscle and the clavicle. When about one-half the length of the needle has penetrated the skin, the bevel should be in the lumen of the vessel. Insertion of the needle through the muscle stabilizes the needle and minimizes bleeding. Caution should be used when using this technique as it is considered to be a "blind stick" into the vessel and damage to the vessel could occur. Repeated access of the vessel is not recommended.

Infusion. For the purpose of continuous infusion of the test compound over extended periods of time or for repeated short-term infusions, implanted catheters in the jugular vein can be used. For implantation of a jugular catheter, the animal is first anesthetized and placed in a dorsal recumbancy and the surgical site is prepared. A midline incision is then made in the neck and a section of the jugular vein is dissected free. Manipulation of the vein should be limited to prevent vasospasm. A cephalic ligature is then tied and the vein elevated. A small incision is then made in the vein and the catheter is passed into the vein and tied in place. The other end of the catheter is then tunneled subcutaneously to between the scapula where the catheter is exteriorized. The catheter should be filled with an anticoagulant solution such as heparin when not in use. When correctly positioned, the tip of the cannula will be at the junction of both vena cava. If placing catheters into young animals, enough of the catheter should be inserted to allow for growth of the animal. Care should be taken that the catheter is not inserted too far, as the tip could then be pushed into the right ventricle of the heart. Improper placement of the catheter could lead to administration of the test compound directly into the heart, which can cause complications.

Similar to the jugular vein, administration of test article via the femoral vein requires an implanted catheter. For implantation of a femoral catheter, the animal is first anesthetized and placed in a dorsal recumbancy and the surgical site is prepared. A midline incision is then made in the inguinal area and a section of the femoral vein is dissected free. Manipulation of the vein should be limited to prevent vasospasm. A ligature is then tied and the vein elevated. A small incision is then made in the vein and the catheter is passed into the vein and tied in place. The other end of the catheter is then tunneled subcutaneously to between the scapula where the catheter is exteriorized. The catheter should be filled with an anticoagulant solution such as heparin when not in use. When correctly positioned, the tip of the cannula will be position in the vena cava. For longer term infusions, the femoral vein catheter might be preferable as patency is easier to maintain and the risk of damage to heart from the catheter is avoided.

Several commercial vendors offer surgical support services and for an additional fee will implant either jugular or femoral catheters. These vendors will typically have a specific methodology for implant, but will accept requests for modifications such as catheter type, exteriorization site, and so on. The typical catheter implanted might be manufactured from polyethylene, polypropylene, or silastic. In recent years, manufacturers have developed catheter impreganated or ionically bound with heparin. These catheters might help to prolong the life of the catheter (Joint Working Group on Refinement 1993).

The useful lifetime for jugular and femoral catheters is quite variable; the lumen of the cannula might eventually become obstructed by a blood clot or fibrous mass. The position of the tip of the catheter is important. Clot formation is less likely to occur if the tip of the catheter is placed in the venous stream rather than in the jugular vein (Popovic and Popovic 1960). It is recommended for repeated short-term infusions, that when test article is not being infused, a slow infusion of saline will help to prolong the life of the catheter.

Prior to use, the patency of the catheter should be checked by removing the anticoagulant lock, checking for blood drawback, and then flushing with saline or Lactated Ringers Solution. Alternatively, patency can be checked by injecting 3mg to 6 mg of pentobarbital solution (0.05–0.10 ml of a 60 mg/ml solution) into the catheter (Weeks 1972). If the catheter is patent, the rat will lose its righting reflex and become ataxic with 10 to 15 sec of injection. The rat will recover in 10 to 15 min.

Rats will destroy the catheter if left unprotected or in easy reach of the forepaws. By exteriorizing the catheter between the scapula, the rat will not be able to chew on the catheter. For the purpose of continuous infusion, several manufacturers have developed tether systems and catheter sheaths made of metal that prevent the animal from chewing on the catheter. These systems typically consist of a jacket with an attached tether through which the catheter passes. The catheter then attaches to a swivel that prevents the catheter from becoming kinked. The swivel then attaches to a second catheter that can be attached to a syringe or pump for administration of the test compound (Davis 1966; Guo and Zhou 2003). When performing long-term infusion studies, the effects of the catheter and harness should be considered. Infections, septicemia, a variety of visceral lesions, endothelial lesions, and increased platelet consumption have been observed in canulated animals (Hysell and Abrams 1967; Meuleman et al. 1980; Vilageliu et al. 1981). Decreased or erratic weight gains and decreased liver and thymus weights have been observed in tethered animals. These changes can be attributed to the stress involved in chronic tethering of the animals.

An alternative to an exteriorized catheter is to attach a subcutaneous port to the catheter that can be accessed via a transcutaneous needle stick. This type of setup helps prevent infections that can occur with transcutaneous catheters. One of the pitfalls of this subcutaneous port is that it can only be accessed a finite number of times. In addition, care has to be taken to ensure the port and catheter are properly flushed of all test compound and blood as clots can easily form. Administration of small volumes of test compound can be accomplished using a subcutaneously implanted osmotic pump. This type of pump is connected to the catheter after being filled with the test compound and implanted in a subcutaneous pocket. This allows for a continuous administration of small amounts of compound without the need for a jacket and tether system.

Saphenous, Lateral Marginal, and Metatarsal Veins

These veins in the leg and foot are easily visualized and can be injected without anesthesia; however, assistance is required. Shaving the area over the saphenous or lateral marginal vein makes visualization easier. During injection it is necessary for one technician to restrain the animal and occlude the vessel to cause it to dilate. Wiping the skin over the vein with 70% alcohol or with gauze soaked in hot water will help to dilate the vessel and increase the possibility of success. The second technician then performs the injection; a 26- to 27-gauge needle should be used.

Dorsal Penis Vein

When administering test article via the dorsal penis vein, it is preferable to use anesthesia. Lightly anesthetizing the animals with an inhaled anesthetic such as isoflurane or CO_2/O_2 will prevent the animal from struggling and increase the possibility of a successful injection. This procedure requires two technicians to perform the injection. One technician holds the animal by the skin on the back and the feet and tail. The vertebral column is then hyperextended. The second

technician then grasps the tip of the penis between the thumb and forefinger, and injects the test solution into the dorsal vein using a 26- to 30-gauge needle.

Sublingual Vein

Although the method of sublingual vein injection has the disadvantage of requiring anesthesia, it only requires one technician. Ideally, the animal should be anesthetized with an inhaled anesthetic such as isoflurane or CO_2/O_2, but injectable anesthesia can also be used. The animal should be placed in a dorsal recumbancy with the head toward the operator. The test compound can be administered by holding the tongue between the thumb and forefinger. Using a 26- to 30-gauge needle, the vein is entered at a very shallow angle and the injection is performed. After completion of the injection, the bleeding can be stopped using direct pressure. Once the bleeding has stopped, a small cotton-wool pledget should be placed over the vein and the tongue placed back in the mouth. The animal will spit the cotton out on regaining consciousness.

Intraperitoneal Route

Test compounds injected into the peritoneal cavity will be absorbed into the portal circulation and transported to the liver. As a result, the compound will be subjected to the metabolic activity of the liver prior to being circulated to the remainder of the animal. Based on the level of blood flow and circulatory surface area in the peritoneal cavity, compounds injected intraperitoneally will be absorbed quickly.

One person can perform intraperitoneal administration of test compounds in the rat. The animal should be picked up by the scruff of the neck and back and held firmly in a dorsal recumbancy. This position allows for proper access to the peritoneal cavity. The belly of the animal should be visually divided into quadrants and a needle (< 21 gauge) should be inserted anteriorly into one of the lower quadrants just lateral to the midline (figure 3.5). Aspiration of the syringe prior to injection will help the investigator to determine if the needle is positioned appropriately.

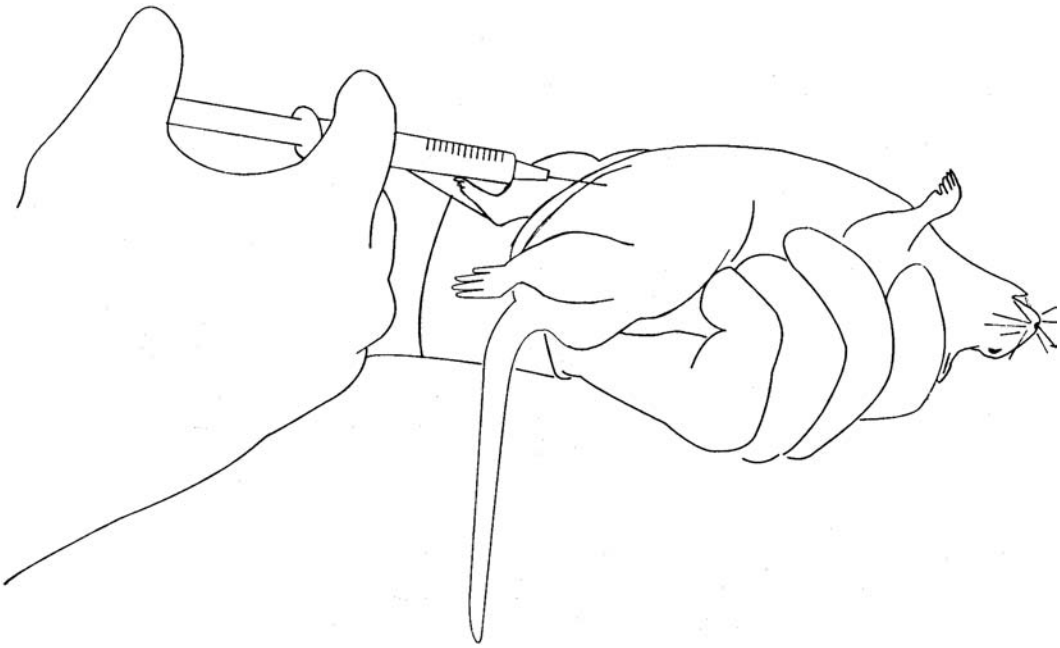

Figure 3.5 Intraperitoneal injection technique.

Intramuscular Route

Intramuscular injection of compounds will result in rapid absorption into general circulation due to the abundant supply of blood vessels. However, the speed of absorption will not be as fast as with an intraperitoneal injection. Acceptable sites in the rat are the quadriceps, the thigh, and the triceps. This procedure can be done with one or two technicians. The selected muscle mass should be stabilized with the thumb and forefinger of one hand while restraining the animal and the needle is guided into the muscle with the other hand. A 21-gauge or smaller needle should be used. The needle should be lightly aspirated to ensure the tip of the needle is not in a blood vessel and then the compound is slowly injected. A slow injection with a minimal volume will help to minimize pain. Approximately 1 ml/kg of solution can be injected per site. If larger volumes are required, multiple injection sites should be used.

Subcutaneous Route

Absorption following subcutaneous injection is typically slower than following intramuscular injection. This could be advantageous if a relatively sustained period of absorption is desired. Another advantage of the subcutaneous route versus the intramuscular route is a much larger volume of test compound can be administered. Five to 10 ml can be easily injected with little to no discomfort to the animals. This can be beneficial for test compounds that have limited solubility. Suitable sites for injection are the ventral body, the flank, and shoulders. To perform the injection, the skin is grasped between the thumb and forefinger and raised to make a tent. The needle (< 20 gauge) is inserted through the skin to make the injection. Injection sites can be varied for multiple-dose studies where the solution is a potential irritant.

To minimize the stress of manipulation and to provide a means for continuous infusion, a perforated cannula or catheter can be implanted transcutaneously. The cannula can than be secured using a tether system similar to that used for IV infusions. Using this system, the test compound can be infused continuously. Mucha (1980) injected sodium pentobarbital directly into the cannula and showed it was absorbed much more rapidly than following injection. An alternate method of infusion of small volumes over an extended period of time is through the use of an osmotic minipump. The pump is filled with the test compound and implanted subcutaneously under anesthesia. These pumps are commercially available and offer the advantage of a continuous infusion at a constant rate without the animals being encumbered with the infusion apparatus. This method would work only when the solutions are stable at body temperature for the duration of the infusion period. In recent years several types of absorbable microspheres have been developed that can act as carriers for the test compound.

Topical Route

The rat has not traditionally been used as a model in skin irritation or sensitization studies. However, the rat has been used in systemic toxicity studies where the skin is used as a portal of entry for whole body exposure or in skin painting studies where the carcinogenic potential is being assessed. In a comparison of absolute absorption rates of several compounds, Bartek et al. (1972) found dermal absorption rates in the rat tended to be slightly lower than in the rabbit, and higher than in the monkey, swine, and human.

Exposure in dermal studies is usually to the anterior dorsal portion of the back. The skin should be shaved weekly or 24 hr prior to skin painting. Care should be taken to ensure that the skin is not damaged during shaving, as this can increase the rate of absorption of the test compound. The test area for application should be clearly marked; for repeat dose studies the area of this site is often 10% of the body area. Usually 0.25 to 1.0 ml of the test solution is applied in skin painting

studies. The amount of a cream or ointment applied will vary with the test compound and desired total dose administered. Dosing is typically performed every day.

The actual dose in a dermal toxicity study is determined by the amount of compound absorbed, so factors that influence absorption should be considered. Several design features in a topical study can affect absorption: Abraded skin will tend to absorb faster than intact skin; test compound might adhere or build up at the site of exposure and could impede absorption (and test compound can be chemically changed owing to exposure to air or light); the test compound might be licked or scratched from the site; and the test compound could be ingested by the animals.

Several techniques have been developed to avoid removal or ingestion of the test compound. For acute studies, Rice and Ketterer (1977) described a cable-type restrainer attached to a stainless steel plate. Loops just behind the front and just in front of the hind legs hold the animal immobile. Other methods can be used to reduce stress to the animals and allow the animal mobility. One method is the use of an Elizabethan collar. This is a 4- to 5-cm wide strip of plastic or metal that fits around the neck of the animals. This prevents the animal from being able to turn its head to gain access to its back. Consideration should be given to the use of this method as the collar also might prevent the animal from being able to properly eat from a feed jar. A second method is to wrap the animal with gauze and then with plastic wrap. Care should be taken when wrapping the animal to ensure that the wrapping is not too tight. This type of covered exposure might affect the absorption of the test compound. Other types of harness, collars, and acrylic chambers can be used and the appropriate technique should be chosen based on the intended length of exposure and the efficiency with which the technique can be performed by the technicians.

Rectal Route

The rectal route is not a routinely used method of administration in toxicology. However, administration by this route is sometimes required to support drugs given rectally by suppository. For dosing, the animal is held by the base of the tail and a stainless steel, ball-tipped gavage needle (5 cm) or vinyl tube (6 cm) attached to a syringe is inserted into the rectum. Care must be taken not to damage the rectum when inserting the needle. The syringe should be held lightly; the weight of the needle and syringe propel the needle. The animal can either be awake or anesthetized. If animals are awake, excretion of the unabsorbed test compound might occur. Methods to control excretion have included ligation of the rectum (Nishihata et al. 1984) or various types of septums that are tied or glued in place (DeBoer et al. 1980; Iwamoto and Watanabe 1985). Anesthetized animals can be placed on an inclined board to retard expulsion.

An important factor in rectally dosing the rat is that the depth of deposition of the test compound will affect the rate of absorption and should be standardized. Drugs subject to extensive first-pass metabolism, such as propranolol and lidocaine, have been found to be much more bioavailable when injected close to the anus rather than in the upper areas of the rectum (DeBoer et al. 1980; Iwamoto and Watanabe 1985). The reason for this difference in bioavailability appears to be that the veinus return in the upper rectal area is through the upper rectal vein that feeds back into the portal circulation and then into the liver. The veinus return in the lower rectum is through the lower hemorrhoidal veins and is not connected to the portal system, but goes directly to the inferior vena cava (Iwamoto and Watanabe 1985).

Intranasal Route

With the increasing number of drugs being delivered nasally, methods have been developed to support this route. For administration in unanesthetized animals, the appropriate volume of test material is drawn into the tip of a pipette or other appropriate dosing implement. The tip of the dosing implement is placed directly over but not into the nostril to be dosed and the test material is instilled into the nostril. The animal will aspirate the test material into the nasal passage. This

can be repeated for the opposite nostril, or the opposite nostril can be used as a control treatment. In the event that the animal sneezes or the test compound is otherwise expelled, the nostril should be retreated.

Inhalation Route

Owing to the complexities and equipment involved in generating, maintaining, and measuring appropriate atmospheres, the inhalation study is one of the most technically difficult to perform. This section is not written to provide a complete discussion of the skills necessary to perform an inhalation study, but will deal with general considerations about the three major steps in exposing rats by the inhalation route: generation of the test atmosphere, exposure of the test animals, and measurement and characterization of the test atmosphere.

Generation of Test Atmosphere

Test atmospheres that are commonly generated include gases, vapors, aerosols, and dusts. Gases are the easiest atmospheres to generate as they can be metered into an airstream, mixed to an appropriate concentration, and then introduced into the test chamber. Vapors are the gas phase of a liquid or solid that has been heated. These can be passed at an appropriate concentration to the exposure chamber in nitrogen or compressed air. Considerations with vapors involve avoiding chemical modification during the heating process and avoiding condensation. Aerosols (stable suspensions of fine solid or liquid particles and dusts [solid particles in air, but not necessarily in a stable suspension]) are more difficult to generate. A variety of generators have been developed to nebulize or disperse the particles into the air. Once generated, aerosols and dusts are mixed to appropriate concentrations and introduced into the exposure chamber. Considerations involved in aerosol and dust generation include particle size, size distribution, and particle shape, as these parameters will primarily determine where in the respiratory tract the particles will be deposited and the rate at which they will be absorbed. As discussed earlier, rats are obligate nasal breathers. As a result of this characteristic and their smaller nasal passages, particulates above 1 micron are essentially excluded from rodent lungs.

Exposure of Test Animals

Exposure of animals can be performed using several different chamber designs. The most common three are whole body, nose-only, and head-only. The following design features are common to these chambers: They are made of nonreactive material and have provisions for easy access to and viewing of the animals; they are designed to ensure a uniform flow of test material to the animals; and they have a number of ports for monitoring test compound concentrations, chamber temperature, humidity, and pressure. The advantages of head- and nose-only chambers are that they require much less compound, compound exposure is limited to the nose or to the head, and the animals are more accessible for physiological measurements. A major disadvantage is that the animals must be restrained in tubes and, therefore, exposure times must be limited to minimize stress and possible overheating. Advantages of the whole body chamber are that loading and unloading are much less labor intensive than other designs and the animals are maintained in cages, allowing longer exposure times. Disadvantages of whole body chambers are that aerosols and dusts deposit on the fur and can be eaten or absorbed dermally and the size of chamber requires a much larger amount of test compound.

In the exposure process, animals are placed into chambers and exposed for a predetermined amount of time to the test atmosphere. Generally, exposures in head- and nose-only chambers are limited to a set number of hours per day, whereas whole body exposures can be continuous, and even chronic. Dosages in inhalation studies are generally stated on the basis of the period of time

at a stated atmosphere concentration. Estimations of actual dosage received are complex and are based on the physiology and anatomy of the rat and several characteristics of the atmosphere.

Measurement and Characterization of the Test Atmosphere

An important part of the process is documenting the atmosphere to which the animals were exposed. Measurements are made to determine characteristics, including the concentration of the atmosphere, homogeneity of distribution, and, in aerosol and dust studies, the size of the particles generated. Samples should be drawn from a number of sites within the chamber to determine homogeneity. Gases and vapors can be analyzed using chromatography or spectrophotometry. Aerosols and dusts are collected and measured using methods such as sedimentation, filtration, centrifugation, impaction, thermal or electrostatic precipitation, or by optics.

Intratracheal Administration

An alternative to inhalation administration is to instill the test compound into the trachea of the animal. Techniques have been developed to instill the test compound into the trachea safely and repeatedly. To perform the procedure, the animal must first be anesthetized, preferably with a gas anesthesia such as isoflurane. Once anesthetized, a speculum is inserted into the mouth and passed into the trachea. A syringe and needle with a 5-cm piece of tubing is used to instill the test compound. The tubing is then passed over the speculum and the test compound is administered. Volumes should be limited to 2 ml or less of test compound.

Data Collection Techniques

Observations and Physical Examinations

Rats are routinely monitored during toxicology studies as an assessment of their general health and to define the effects of the test article. In acute and subchronic studies, animals might be observed frequently in an effort to define short-term pharmacologic changes induced by the test compound that might become apparent at peak blood levels. Specifically in acute toxicology studies, clinical observations will help to establish an MTD. In chronic studies, these observations are critical in tracking tumor development and for determining animals in extremis, which should be euthanized for humane reasons and to prevent autolysis and tissue loss. Arnold et al. (1977) contains a useful description of a clinical assessment program for chronic studies.

Daily observations are performed first thing in the morning and last thing before leaving in the afternoon to assess the health of the animals and identify animals that might be in extremis. In this observation, behavioral status, respiratory signs, skin, eyes, and excretory products are noted. Care should be taken to disturb the animal as little as possible, as any disturbance could induce stress and affect the animal's behavior. The animal should be picked up and examined more closely if abnormalities are detected. Special attention should be paid to the amount of feces present, because a decrease in fecal output might be the first sign of a watering system malfunction. In acute studies or where pharmacological effects are expected, animals might be examined continuously or at peak plasma levels.

A more thorough physical examination should be done weekly. Each animal is taken from its cage and placed on an examination table where respiration, behavior, general appearance, and locomotion are observed. The technician should then pick up the animal, examine its body orifices, skin, and coat, and perform a palpation of the trunk and limbs to check for tumors. The detection and tracking of the size and fate of masses (potential tumors) is essential for carcinogenicity studies.

Animals that have experienced severe weight loss over the previous week, a progressive decline in weight over several weeks, or other severe clinical signs should be observed more frequently

and marked for possible euthanasia. Body and feeder weights can be measured as part of the physical examination or as a separate function. Performance of these operations as a part of physical examinations will minimize animal handling and potential stress. If the operations are combined, it is important that the physical examinations be done completely and not rushed.

Neurobehavioral Examination

Neurobehavioral examinations are included in toxicology studies to assess the behavioral and neurological effects of test compounds. These examinations, which might be done as part of acute or repeat dose toxicity studies or studies specifically designed to assess just the neurobehavioral effects, typically involve screens consisting of an abbreviated functional observational battery and some measure of locomotor activity. The EPA has written guidelines on the design and conduct of these studies (OECD 1995, 1997). To meet EPA guidelines, the screen is performed prior to the start of treatment, then periodically during the course of treatment. It can be performed as a separate study, on satellite groups of animals in conjunction with the main study, or on animals in the main portion of the toxicology study. Recently, the ICH has adopted guidelines requiring neurobehavioral assessment of all new pharmaceuticals prior to initiation of Phase I clinical trials. To meet the ICH guidelines, a more formal functional observational battery should be conducted and can be conducted on main study animals or as a separate study (ICH 2000). Where initial screens indicate the possibility of a test compound-related change, a more specialized series of tests can be performed to assess the nature of the effect and the extent of the central nervous system involvement. These secondary tests evaluate motor and sensory function as well as cognitive ability. Examples of secondary tests include sensory-evoked potential experiments and schedule-controlled behavior studies. Descriptions of these secondary tests can be found in Annau (1986).

Functional Observational Battery

The typical functional observation battery (FOB) includes observation of home-cage and open-field activity as well as measurements of reflexive, physiological, and neuromuscular function (Moser et al. 1988; Moser and Ross 1996) as outlined in table 3.14. Observations and measurements that have become standard for an FOB evaluation are given in table 3.15. The order of measurement should be consistent, progressing from the least interactive to the most interactive measurements. Home-cage observations are made first. Assessments of posture, clonic movements, tonic movements, and palpebral closure (if the animal's eyes can be seen) are taken prior to removing the animal from its cage. However, it might be necessary to pull the cage from the cage bank or remove the cage cover to see the animal's eyes. The animals are then transferred to the open field. During the transfer, certain physical observations are made. The technician removes the animal from the cage. The technician holds the animal and notes increased or decreased body tone as well as such observations as bite marks, soiled fur appearance, missing toenails, emaciation (shallow stomach, prominent spinal vertebrae), or death. In addition, observations of lacrimation, palpebral closure, piloerection, exophthalmus, and salivation are also made. The animal is then placed in the open field apparatus for a set period of time. Measurements of rearing, urination, and defecation are made immediately at the end of the assessment period. Assessment of clonic movements, tonic movements, gait, mobility score, arousal, respiration, stereotypic behavior, and bizarre behavior can be made immediately after the time period has ended or the technician can continue to observe the animal for a longer period of time to allow for more accurate assessment. When the open-field assessment has been completed, the animal is removed from the open field apparatus for the approach response, touch response, click response, tail pinch response, pupil response, righting reflex, thermal response, hindlimb splay, and grip strength measurements. After completion of the manipulative assessments, the physiological evaluations are completed.

Table 3.14 Functional Observational Battery in Rats

	Number of Animals	
	Males	**Females**
Control	10	10
Low dose	10	10
Mid dose	10	10
High dose	10	10

Dosing: The test article will be administered by the required route.

Functional Observational Battery: FOB evaluations will be conducted prior to dosing, at the estimated time of peak effect, and 24 hr postdose. The evaluations are as follows:

Home Cage Observations:

Assessments of posture, clonic movements, tonic movements, and palpebral closure (if the animal's eyes can be seen) are taken prior to removing the animal from its cage.

Handling Observations:

Observations of ease of removal, handling reactivity, lacrimation, palpebral closure, piloerection, and salivation are made on removal of the animal from the home cage.

Open-Field Observations:

The animal is placed in the center of an open-field testing box (measuring 20" $\times$ 20" $\times$ 8"). Clean absorbent paper can be used to cover the bottom of the box if required by protocol. Using a stopwatch, the animal's stay in the box is timed for 3 min. Measurements of rearing, urination, and defecation are made immediately at the end of the 3 min. Assessment of clonic movements, tonic movements, gait, mobility score, arousal, vocalization, respiration, stereotypic behavior, and bizarre behavior can be made immediately after the 3 min have ended or the technician can continue to observe the animal for a longer period of time to allow for more accurate assessment.

Sensorimotor Observations:

The approach response, touch response, click response, and tail-pinch response (stimulus reactivity tests) are performed while the animal is in the open-field apparatus, after the 3-min time period is over and all other measurements have been recorded. The animal is removed from the open-field apparatus for the pupil response, righting reflex, thermal response, hindlimb splay, and grip strength measurements.

Physiological Evaluations:

The animal's body weight and rectal temperature are measured and recorded.

Clinical Examination: Following each FOB assessment (additional observations will be conducted prior to dosing for locomotor activity animals, if requested)

Table 3.15 Functional Observation Battery

Home Cage	Open Field	Manipulative	Physiological and Neuromuscular
Posture	Rearing	Ease of removal from cage	Body weight
Clonic movements	Urination	Handling reactivity	Body temperature
Tonic movements	Defecation	Lacrimation	Hindlimb extensor strength
Palpebral closure	Clonic movements	Palpebral closure	Grip strength
	Tonic movements	Piloerection	Hindlimb splay
	Gait	Exophthalmus	
	Mobility	Salivation	
	Ataxia	Approach response	
	Arousal	Touch response	
	Vocalizations	Click response	
	Respiration	Tail pinch response	
	Stereotypy	Pupil response	
	Bizarre behavior	Eye blink response	
		Forelimb extension	
		Hindlimb extension	
		Righting reflex	
		Thermal response	

Source: From Haggerty (1989) and Moser (1989).

Procedures used during the performance of an FOB must be standardized because some observations made have a subjective component. If at all possible, a single observer should be used throughout a single study. If this is not possible, a single observer should conduct all assessments of an animal. In addition, technicians should be blinded to the treatment conditions for each animal.

Locomotor Activity

Methods used for recording motor activity include direct observation and automated techniques such as photocell devices and mechanical measurements (Macphail et al. 1989). In direct observations, the observer can make quantitative measurements of the frequency, duration, or sequencing of various motor components of behavior or qualitative records of the presence or absence of certain components of activity. Photocell devices record the number of times an animal interrupts a beam in specially designed chambers. In mechanical chambers, the animal's movements result in a vertical or horizontal displacement of the chamber; records are kept of the chamber's movements. There are advantages and disadvantages of each technique. In direct observation, a record can be made of behavior, such as convulsions, that might not be observed when using the photocell or mechanical methods. A disadvantage of the direct observation method is that the animal might be influenced by the presence of the observer. Advantages to the photocell and mechanical methods are that the data are captured electronically, the observer does not have to be present, and the computer system can graphically present the data in the form of lines crossed or a map of the activity.

To make activity determinations, an animal or group of animals is put into an observation or recording chamber and activity is recorded for a specific period of time. Because activity will normally decline over the course of the session, the length of the observation period is important. The EPA guideline specifies that activity should approach asymptotic levels by the last 20% of the session. Haggerty (1989) used a 15-min recording session, accumulating data over three 5-min intervals. Because a large number of environmental conditions can affect motor activity (e.g., sound level, cage design, lighting, temperature, and humidity, or odors), it is important to minimize variations in the test environment.

Cardiovascular Parameters

Examinations of the cardiovascular system can be scheduled into toxicology studies or performed when the cardiovascular system is a suspected target of the test compound. The ICH has adapted guidelines requiring cardiovascular safety assessment of all new pharmaceuticals prior to initiation of Phase I clinical trials. This guideline recommends that this assessment be conducted in a nonrodent species, but cardiovascular assessment can be performed for screening purposes or for additional support data.

Electrocardiography

Although the dog has traditionally been the species of choice in toxicology studies of effects of ECG, research with the rat has progressed and increased over the years. Detweiler (1981) provides an excellent review of the use of electrocardiography in toxicology studies in the rat. This section presents a general discussion on the aspects of the recording methods and interpretation of the ECG in the rat.

Recording Methods

Restraint. One of the disadvantages of traditional methods of studying ECGs in rats is that it is difficult to keep the animals still while recording. It is important that the animal remain in a constant position during the procedure using skin leads to avoid muscular artifact in the ECG. Various forms

of restraint have been tested, each of which requires acclimation to the procedure prior to evaluation. These methods included restraining the rat in a supine position using rubber gloves (Hundley et al. 1945), pinning the animal to a board, boards with clamps, and plastic tubes with slits on either side that allow for placement of the electrodes (Zbinden et al. 1980). Various forms of anesthesia have also been evaluated.

No conclusion has been reached about the best method of restraint. The basic concerns are that manual methods and physical restraint require acclimation to allow the animal to become accustomed to the procedure and tracings can be reasonable free of muscular artifact. Also, varying pressures of clamps or handling during the restraint could affect the results. The use of anesthesia has been shown to produce changes in the ECG and there is a possibility of drug interactions between the anesthetic and the test compound.

Position. The most common positions are the prone or ventral recumbancy position when animals are awake and the supine or dorsal recumbancy position when anesthetized. Beinfield and Lehr (1956) compared the positions and concluded that the prone position produced and increased R wave and it avoided unfavorable cardiac rotation and an undesirable variation in the projection of the special QRS loop.

Tethered. Robineau (1988) developed an electrode system that can be implanted subcutaneously a few days prior to recording. The device has a disconnect that is exteriorized between the scapula. The advantage of this system is that a cable can be connected to the plug and the ECG can be taken in unrestrained rats. The disadvantage of this method is that this method should only be used for short-term studies as the implant provides a source for infection in the animal.

Telemetry. Recently, techniques have been developed for monitoring cardiovascular parameters via telemetry in the rat (Ichimaru and Kuwaki 1998; Kramer et al. 1995; Kuwahara et al. 1994). Totally implantable battery-operated systems have been developed that can monitor several physiological parameters including ECG. The implants are available with ECG leads that can be positioned in the Lead II configuration for monitoring ECG continuously for extended periods of time in a freely moving animal. As the leads are attached subcutaneously to the musculature, the signal is of a higher quality than skin leads. Also, the animal is not affected by the observer, as the animal can be monitored remotely. One of the disadvantages is that these implants are designed to monitor only a single lead.

Leads. Most investigators use Einthoven's bipolar limb lead system, lead I (right and left foreleg), lead II (right foreleg and left hindleg), and lead III (left foreleg and left hindleg), with and without the augmented unipolar limb leads aVR (right foreleg), aVL (left foreleg), and aVF (one of the hindlegs). Because foreleg position can alter the scalar ECG wave amplitudes, investigators must standardize foreleg positions during recording. When implanting telemetry leads, the leads are placed on the right clavicle and the most caudal rib on the left side in a modified lead II configuration.

In the past various types of leads have been used to connect the ECG wires. These included the use of hypodermic needles inserted under the skin, small-gauge insulated copper wires wrapped around the shaved distal portion of the limbs, or alligator clips. In addition, platinum-tipped pin electrodes are commercially available that provide a good quality signal with limited discomfort to the animal.

ECG Waveform

A diagrammatic example of a normal rat ECG is illustrated in figure 3.6. The major points to notice about the rat ECG are that the conventional waves of the mammalian ECG (P, QRS, and T) are all identifiable in the rat ECG, there is no isoelectric line during the electrocardiographic

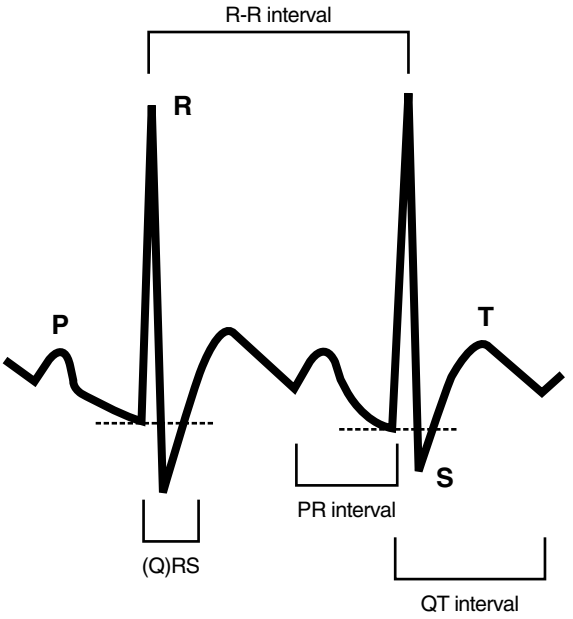

Figure 3.6 Diagrammatic representation of rat ECG waveform with intervals indicated. (From Driscoll [1981], used with permission.)

complex, and there is no ST segment. The duration of the standard intervals evaluated in the ECG of the rat are as follows: P, 10–20; PR Interval 35–50; QRS complex 12–25 and QT interval 38–80 msec (Detweiler 1981). The duration of the intervals is related to the heart rate: As the heart rate increases, the intervals shorten and as the heart rate slows, the intervals prolong. These intervals can also be affected by administration of test compounds and are the basis for the requirement to assess the affects of a test compound on cardiac function. Specifically, prolongation of the QT interval has been correlated with a phenomenon called Torsade de Pointe, or sudden cardiac death. Several classes of compounds, such as antihistamine and Ca2+ channel blockers have been shown to prolong the QT interval (Gras and Llenas 1999). Each wave of the ECG represents either a depolarization or repolarization of the atria and ventricles of the heart. For example, the P wave is an electrical representation of the depolarization of the right atria and the T wave is an electrical representation of the repolarization of the left ventricle. Spear (1982) provides a more in-depth discussion of the waveforms and the electrophysiology of the heart. Several computerized systems have been developed that are capable of recognizing the independent waveforms and measuring the intervals. Caution should be used when using these systems as they are typically programmed to recognize a normal ECG and the presence of arrhythmias might be missed or an interval might not be measured correctly.

Heart Rate

The heart rate can be calculated from standard limb lead ECGs by measuring the distance between the two peaks of the R wave. This distance is then divided by the chart speed (i.e., 50 mm/sec) to calculate the RR interval in seconds. This is then divided into 60 sec to calculate the heart rate in beats per minute. Using the following formulas, the heart rate of an RR interval of 10 mm measured on a chart printed at 50 mm/sec = 300 beats per minute.

$$\text{RR interval (mm/beat)/chart speed (mm/sec)} = \text{RR interval (sec/beat)}$$

$$\text{(60 sec/min)/RR interval (sec/beat)} = \text{heart rate (beats/min)}$$

Many environmental factors can affect the heart rate of the rat, such as excessive manipulation, technicians with whom the animal is not familiar, new environments, and so on. Therefore, when evaluating ECG using a restrained method, it is important to acclimate the animal to the test procedures prior to starting. Detweiler (1981), in a review of the literature, found that published heart rates for rats varied between 250 and 750 beats per minute. Awake, restrained adult rats had heart rates from 330 to 600 beats per minute and well-acclimated restrained adult animals had heart rates from 250 to 350 beats per minute. Heart rate in unrestrained telemetrized animals has been reported to be between 225 and 350 beats per minute (Guiol et al. 1992).

Blood Pressure

During the conduct of a toxicology study it might be necessary to monitor blood pressure. This can be done using either indirect or direct methods. Caution should be used when using indirect methods as the values obtained can be variable. The direct method involves the implantation of an arterial cannula for measurement of blood pressure. This method is more reliable, but has a limited period of time during which it can remain patent. It is recommended that for definitive assessment of the hemodynamic effects of a test compound, a nonrodent species such as a dog or a nonhuman primate be used.

Indirect Measurement

Indirect methods of blood pressure measurement detect systolic blood pressures by the occlusion of arterial inflow of blood and the subsequent detection of the pressure at which the first arterial pulsation occurs. The two places where indirect measurements can be made on the rat are the tail and the hindpaw.

Tail Cuff Method. The tail cuff method monitors pressures in the ventral caudal artery. In this method, the animal is put into a restrainer that allows for free access to the tail. An inflatable cuff is then placed around the base of the tail and the pressure is increased until flow stops. The pressure is then slowly released until flow resumes. The cuff pressure at the time when flow resumes is the systolic blood pressure. Various methods have been used to determine when this occurs.

Because the caudal pulse is rather weak, preheating of the animals in boxes at temperatures of 30°C to 42°C for periods up to 10 min might be necessary to dilate the caudal artery. This technique should be used with caution, as previously discussed changes in body temperature can have widespread effects on the animal and can produce unexpected test compound effects.

The placement and width of the tail cuff is important. There is a gradient in pressure along the caudal artery that amounts to 4.5 mmHg/cm. For this reason, the cuff should be placed close to the base of the tail and this should be standardized. If multiple readings will be done over time, marking the placement of the cuff with an indelible marker will help to standardize placement. In addition, variation in the width of the rubber tubing can be a source of error. Bunag (1973) found that the most accurate readings were given by a 15-mm cuff; shorter cuffs gave falsely elevated readings and longer cuffs gave low readings.

Hindpaw Method. Measurement of blood pressure in the hindpaw does not measure the pressure in a specific vessel. In this method, the animal is placed in a restrainer, as in the tail cuff method. A pressure cuff is placed around the ankle to occlude blood flow and blood pressure is measured as the cuff pressure is released and blood flow returns. As in the tail cuff method, several techniques have been used to determine the return of blood flow. These include visual observation (Griffith 1934–1935), photoelectric cell (Kersten et al. 1947), and oximeter (Korol and McShane 1963). The advantage of the photoelectric and oximeter methods is that they do not require preheating to dilate the vessels of the hindpaw. The oximeter method measures mean arterial pressure rather than systolic

pressure. None of the indirect measurements are able to evaluate the complete hemodynamic cycle. They evaluate only systolic or mean arterial pressure, but not systolic, diastolic, and mean arterial pressure together.

Direct Measurement

The direct measurement techniques involve the cannulation of an artery with the blood pressure being determined with a manometer or transducer connected to the free end of the cannula. This is true for types of direct measurement including telemetry. Surgery and cannula placement utilize similar techniques as those previously described for placement of a venous catheter. In short, the artery is isolated and a small incision is made in the femoral artery. The catheter is then inserted into the hole and passed into the abdominal aorta. The carotid artery can also be used for this procedure, but care should be taken not to be insert the catheter too far as the catheter might be passed into the left ventricle of the heart. If this occurs, the blood pressure waveform will change in appearance. The left ventricular waveform has a similar systolic pressure as an arterial blood pressure waveform. However, the diastolic pressure is much different. If the catheter has been placed in the left ventricle, the diastolic pressure will be 0 mmHg or slightly negative. If this occurs the catheter should be backed out into the aortic arch.

Where chronic use is desired, cannulas are typically run subcutaneously and exteriorized between the scapula or at the back of the head. A carotid artery catheter can be expected to remain patent for 3 to 5 weeks (Andrews et al. 1978; Ross 1977), whereas an abdominal aorta catheter might remain patent for several months. Care should be taken when using arterial catheters for long periods of time as fibrin deposits can build up on the catheter or clots can form in the catheters. The risk exists that these deposits or clots could be expelled during the flushing of the catheter or during normal movement of the animal. These clots or deposits, once free, can occlude other vessels downstream and in the case of a carotid catheter could cause a stroke.

Blood Collection Techniques

Blood samples are routinely collected in safety studies to determine (a) direct test compound effects on the blood or bone marrow; (b) effects on other organs as indicated by the contents of the blood, for instance, leakage enzymes such as aspartate amintransferase; and (c) blood levels of the test compound or its metabolites. A variety of techniques have been described for the collection of blood from the rat. The choice of a specific technique might depend on factors such as (a) the volume to be collected, (b) if the animal is to survive the procedure, (c) the frequency with which samples will need to be collected, (d) whether anesthetics can be used, (e) likelihood of the animal surviving the procedure, and (f) the impact of organ damage resulting from the procedure. An adult rat has a blood volume of about 50 ml/kg; approximately 10% of the total blood volume can be collected from a rat in a single draw without adversely affecting hematology parameters. For longer term studies, 10 ml per kg per 2 weeks is a reliable guideline for volume of blood drawn. If these volumes are exceeded, hematocrit and red cell mass could be reduced on evaluation (Diehl et al. 2001).

Technique, anesthetic used for blood draws, and treatment of the animals (i.e., fasted or not fasted) should be standardized throughout a study if repeated samples are being taken. The technique, anesthetic, and handling of the animal might produce effects of the hematology or clinical chemistry parameters to be evaluated.

Retro-Orbital Plexus

The retro-orbital plexus is a commonly used site for periodic sampling during the course of a study. This method has been shown to be reliable for the repeated collection of blood samples.

Collection from this site should always be conducted under anesthesia to reduce pain and stress to the animal. Light anesthesia with a mixture of carbon dioxide and oxygen will minimize struggling of the animal and will help to ensure a quick collection with little injury to the animal.

Blood is collected using a microcapillary tube or the fine end of a Pasteur pipet. The tube is inserted into the orbit of the eye at an anterior angle formed by the lids and the nicitating membrane. A short thrust past the eyeball will make the tube enter the slightly resistant horny membrane of the sinus. The tube can be rotated slightly as it is inserted. Once the sinus has been punctured, blood will fill the tube. Once the tube is filled the blood can be allowed to drip out of the end of the tube into an appropriate collection tube. If the flow stops, the tube can be pulled out or advanced slightly to reestablish flow.

In the hands of an experienced technician, there is minimal risk to the animal. Studies have shown that repeated collection of blood from the retro-orbital sinus can produce histological and behavioral changes that might require an animal to be removed from the study (McGee and Maronpot 1979; van Herck et al. 1998). Several serious side effects of this collection method have been documented. These include retro-orbital hemorrhage, corneal ulceration, keratitis, pannus formation, damage to the optic nerve, and fracture of the orbital bones. When designing the study, the method of blood collection should be taken into consideration. If an important endpoint of the study is ophthamological examinations, this method should not be used. In addition, anesthetization of the animals might affect other study endpoints.

Tail

A tail vein bleed offers a visible target and is of minimal risk to the animal. Blood will flow faster if the tail has been warmed, causing vasodilation. This can be accomplished by dipping the tail in warm water (40°C–45°C), placing the animal in a warming cabinet for 5 to 10 min, or by warming the tail with a heat lamp. This method does not require that the animal be anesthetized, but the animal should be restrained such that the tail is held immobile. Several methods can be employed to collect blood from the tail. These include clipping the end of the tail off, vein puncture, or artery puncture.

Tail Clip. For this method, the animal can be lightly anesthetized or placed in a restraint tube. To collect blood, 2 to 3 mm of the distal part of the tail is amputated with sharp scissors or a scalpel blade. Three to 4 ml of blood can be collected in 20 to 30 sec from a 200- to 250-g rat. When collection has been completed, the cut surface can be cauterized with a hot spatula or glass rod. This method produces a reliable volume of blood, but should not be used for repeated sampling over extended periods of time.

Veinupuncture. Animals should be restrained in a holder that allows complete access to the tail. The tail should then be cleaned and pressure applied to the base of the tail, causing the vein to dilate. The vein should be punctured using a 21-gauge needle and the blood can either be slowly withdrawn into a syringe through the needle or the needle can be removed and the tail allowed to bleed freely. The use of a butterfly needle might help facilitate the collection of blood, as it is less likely to be dislodged if the tail moves. Collection of blood from the tail vein typically yields samples of between 0.5 and 1.0 ml. On completion of sample collection the needle should be withdrawn and pressure applied to the tail to stop the bleeding (Frank et al. 1991).

Arterial Puncture. This method is conducted in a similar manner to a veinupuncture in that the animal is placed in an appropriate restraint tube and the tail is then cleaned. A 21-gauge needle is then inserted into the artery in the midventral surface of the tail close to the distal end. The blood can then be withdrawn into a syringe or allowed to flow freely into a collection tube. The animals' blood pressure will ensure that the blood continues to flow until pressure is applied to the wound.

Cardiac Puncture

This method should always be performed under general anesthesia and should not be used as a survival collection technique. This technique offers a rapid method for collection of a large volume of blood from the rat. It is possible to collect between 5 and 7 ml from a 300- to 350-g rat using this technique and it is possible to exsanguinate the animal using this method.

To collect blood using this method the animal should be anesthetized with a combination of carbon dioxide and oxygen or carbon dioxide alone. The animal is then placed in a dorsal recumbancy and the heart is located. The heart can be located by placing the index finger over the fourth and fifth left ribs and the thumb on the right side of the thorax. The collection needle (25–26 gauge, 1–2 cm long) should be inserted at a 45º angle into the heart. Once the needle is introduced, the syringe should be aspirated slightly to produce a vacuum. The needle is then advanced until blood is obtained.

Abdominal Aorta and Vena Cava

Collection from the abdominal aorta offers a convenient way to exsanguinate an animal and obtain a maximal amount of blood. To perform the procedure, the animal is anesthetized and the aorta exposed by dissection. A section of the aorta distal to the diaphragm is then exposed and the proximal end is clamped. The aorta is then cut, the distal end is placed in a collection tube, and the clamp is released. This method allows for collection of a maximal amount of blood in a short period of time. This method should only be used as a terminal procedure. Alternatively, the aorta can be accessed using a needle or a butterfly needle and samples can then be collected into a syringe.

Winsett et al. (1985) described a method of repeated sampling from the vena cava of conscious rats. An assistant holds the animal while the operator grasps the animal just below the last rib. The needle is inserted 1 cm to the right of the spinous process of the first lumbar vertebra at a 45º angle until the needle touches the bone. The needle is then slightly withdrawn and then advanced at a slightly shallower angle to miss the bone and access the vena cava. The maneuver of first identifying the bone is essential to the procedure. This procedure can be used for repeated collection, but care should be taken to ensure the animal does not struggle during the collection. If the animal struggles the needle could lacerate the vena cava, causing the death of the animal.

Jugular Vein

The jugular vein provides a means for chronic blood sampling that is of low risk to the animals' health. This method does not require the use of anesthesia and can be accomplished through the proper restraint and positioning of the animal.

The unanesthetized method requires two technicians to perform the collection. The animal is placed on a restraint board in a dorsal recumbancy. The forelimbs are tied down to the board and one technician holds the hindlimbs of the animal. The second technician grasps the animal's head and turns it down and away from the desired collection site, right or left jugular. The needle (21 gauge) is then inserted into the middle of the triangle formed by the neck, shoulder, and clavicle, parallel to the body. As the needle is inserted the syringe is aspirated until blood is observed in the syringe. The collection is then completed and the site is held off for approximately 30 sec. It is important that the technician holding the hindlimbs of the animal continually observe the respiratory rate of the animal. If the head is turned too far or is held in the wrong position for too long, the animal might go into respiratory distress. This method can be used for repeated collections with a high level of success.

Alternatively, the animal can be anesthetized and the jugular vein can be surgically exposed. The animal should be prepared using standard aseptic technique and the ventral neck should be shaved. The jugular vein can be exposed by incision of the skin and dissection of the subcutaneous

tissues. A needle (20 gauge) can then be inserted into the vein through the pectoral muscle and directed toward the head. Inserting the needle through the muscle will help to stabilize the needle during the collection. Once the needle is in place, the blood should flow freely with little to no aspiration. Care should be taken when aspirating the syringe, as too much pressure will cause the vein to collapse. Once the collection is complete, the incision can be closed with a wound clip. This method can be used as an alternative if the animal does not require serial bleeds or as a method to replace a terminal bleed. If the procedure is used for a terminal bleed, strict aseptic technique is not necessary.

Proximal Saphenous and Metatarsal Vein

A small amount of blood (0.1–0.2 mL) can be collected from animals at minimal risk to their health utilizing the proximal saphenous and metatarsal veins. No anesthesia is required.

Proximal Saphenous Vein. The inner aspect of the thigh of the hind limb should be shaved free of hair. While one technician holds the animal and compresses the inquinal area to dilate the vein, a second technician creates a longitudinal nick in the vein with a 20-gauge needle or a hematocrit lancet. The blood can then be collected into hepranized capillary tubes. This method works well for repeated sampling of small amounts of blood but would not be appropriate for the blood volumes required for evaluation of clinical pathology parameters.

Metatarsal Vein. This procedure can be conducted with or without an assistant. The animal is restrained and a nick is made in the vessel with a needle. The blood can then be collected into a capillary tube or through the needle into a syringe.

Sublingual Vein

When using the sublingual vein for blood sampling, the animal should be anesthetized and then cradled in the palm of the hand. By holding the animal's head between the thumb and index fingers, the head can be stretched back and the skin of the face pulled backward. This forces the mouth open and the tongue against the palate. The right or left vein should be cut with iris scissors and the animal held such that blood drips into the collection tube. The bleeding can then be stopped by applying pressure with a gauze pad or cotton-tipped applicator.

Decapitation

Decapitation should only be performed by trained technicians with the appropriate equipment. There are several commercially available small animal guillotines that should be used to perform this technique. This technique is appropriate when a maximal blood volume is desired and contamination of the sample is not considered to be an issue. To perform this method, the head is first removed and the animal is held over the collection vessel and arterial and venous blood is allowed to drain from the body.

Cannulation

Although the blood collection methods previously described will provide sufficient volumes and quality of sample for the majority of toxicology studies, specific protocols might require blood to be samples from animals that have been subjected to a minimum of handling or from specific sites within the body of the animal. Cannulation of a specific artery or vein will typically meet this requirement, though in rodents, the usable life span of the cannula is limited by the length of time the cannula remains patent. Yoburn et al. (1984) compared jugular, carotid, and femoral cannulas

for long-term sampling of blood. They found the femoral artery cannula was preferable in terms of patency and postsurgical weight loss. Collection from a cannula is the same regardless of implant site. The cannula is typically exteriorized between the animal's scapula and a stilet is inserted into the end of the cannula. For the purpose of collection, the stilet is removed and a needle is inserted into the cannula with a syringe attached. The heparin lock is then drawn out of the cannula until blood is observed in the syringe. The syringe is then removed from the needle and a new syringe is used to collect the sample. The collection syringe is removed from the needle and replaced with a syringe filled with the desired solution for locking the cannula, typically a heparin dextrose solution. Once the cannula is flushed and locked with the heparin solution the stilet is replaced. Using the appropriate technique for flushing and locking the cannula is key to maintaining the patency of the cannula.

Jugular Vein. The cannulation procedure is the same as previously described for infusion techniques. The cannula have been found to remain patent for variable periods of time and the length of time the cannula remains patent is directly related to the skill of the technician collecting the samples. It is important to remember that a cannula placed in the venous system can easily develop clots if not flushed and locked properly. Various methods of anchoring the cannula to the rat's back or head have been developed for ease of sampling or as a connection point for continuous infusion. Each of these methods has been developed such that the exteriorized cannula is positioned such that the animal cannot gain access to it.

Inferior Vena Cava. The inferior vena cava appears to provide a site for long-lived cannulas. The cannula can be surgically placed either directly into the vena cava through abdominal surgery or can be advanced into the vena cava from the femoral vein. Either way, the cannula has been shown to remain patent for months (Kaufman 1980). In either implantation, the cannula is then tunneled subcutaneously and exteriorized between the scapula in the same manner as the jugular vein cannula.

Abdominal Aorta. The most common method for placing a cannula in the abdominal aorta is via the femoral artery. The cannula is inserted into the femoral artery and advanced to just above the kidneys. The opposite end of the cannula is then tunneled to and exteriorized between the scapula. This is a minor surgical procedure that the animal recovers from easily. Similar methods are employed when collecting blood from the arterial catheter as those used for the venous catheter. Caution should be taken when removing the stylet from the cannula, as a cannula in the arterial system is under pressure. If not properly prepared, the animal could quickly lose a large amount of blood. It is recommended that the technician place a clamp on the cannula prior to removal of the stylet. Once the blood collection is complete, the cannula should be thoroughly flushed with saline prior to locking the cannula. The tip of an arterial cannula is positioned such that it is against the flow of blood. Due to this, clots and fibrin deposits develop easily on the end of the cannula, limiting its usable life.

Subcutaneous Ports. An exteriorized cannula is a source of contamination and infection, and is subject to destruction by the animal or other animals if group housed. Several types of subcutaneous ports have been developed for implantation along with the cannula. These ports are designed such that test materials cab be injected or infused through them or blood can be collected from the port. In most cases, the port is implanted subcutaneously on the dorsal side of the animal. As with exteriorized cannula, the skill of the technician accessing, collecting samples, flushing, and locking the port directly affects the useful life of the port.

Urine Collection

Urine is generally collected in toxicology studies to assess kidney function. The most common method used for urine collection is a stainless steel commercially available cage. The cage is designed

such that the urine and feces are separated by a cone-shaped device. The urine drains off the collecting walls into a tube and the feces fall into an inverted cone. Food and water are made available in such a way that the urine will not be contaminated. Although this type of cage produces urine of acceptable quality for normal urinalysis, the sample might be contaminated with hair or feces. Other methods for urine collection in the rat include cystocentesis, which involves a needle stick into the bladder, and cannulation of the bladder.

Necropsy

The necropsy is the link between antemortem findings and histological observation. It is an essential portion of the toxicology study and because a necropsy will involve the processing of a large number of animals, it is important that the procedure is well planned (Black 1986). At a prenecropsy meeting involving the patholgist, prosectors, and the study director, necropsy responsibilities can be discussed. Additionally, the study director can summarize clinical findings and potential target organs. The prosectors should be familiar with the protocol and amendments for the study involving the animals being necropsied. The protocol should clearly state which tissues are to be collected and weighed and how the tissue should be preserved. During the necropsy, devices such as checklists or prelabeled compartmentalized trays should be present to ensure that all required organs are taken and weighed. In recent years commercially available computer software has been developed to assist in the collection and weighing of tissues at necropsy. This is an electronic copy of the checklist, but also provides a method for recording observations in a consistent manner. Copies of the last clinical observation should be present at the necropsy so the prosectors are alerted to lesions that might be present and require special attention. Palpation records are particularly important at carcinogenicity study necropsies to ensure that all masses detected at the last examination are confirmed and collected.

The necropsy will involve a check of animal identification and sex, an external examination of the animal, an in situ examination of all tissues and organs (prior to dissection), and the collecting and weighing of the required tissues.

Summary

In summary, there are several advantages to the use of the rat in toxicology studies. Because of its widespread use in many fields of biology, there is a large historical database of information about the anatomy and needs of the species. This knowledge, along with information about the species' metabolism and response to toxicants, has shown the rat to be generally a good model for the prediction of the human response to toxicants. Rats have a lifespan of 24 to 30 months, which is convenient for chronic toxicity and carcinogenicity studies in which animals need to be exposed for the majority of their lifetime. The short gestation time and large litter size make the rat a good model for reproductive studies. The development of specific pathogen-free rats and improvements in husbandry have eliminated most of the disease outbreaks that might have introduced variability into a study. The lack of an emetic response allows for the testing of higher dosages of compound that could cause vomiting in other species. The small size of the rat is useful in that large numbers can be housed economically. The size is also useful in that smaller amounts of test compound are required to gain maximal exposure.

The relatively small size of the rat is also one of its major disadvantages. The amount of blood that can be taken from the animal is limited, thus limiting the number of parameters that can be investigated or the number of toxicokinetic samples that can be collected from a single animal. This problem can be overcome by adding additional animals in interim sacrifice groups or by collecting toxicokinetic samples from cohorts of animals at different times. In most cases it is recommended that toxicokinetic samples be collected from satellite animals and not from the main study animals being used for evaluation of toxicity. However, an increased number of animals

means increased work in the conduct of the study. The small size and relatively active nature of the rats makes some procedures, such as IV dosing or collection of electrocardiograms, difficult. These issues have been overcome with the use of suitable restrainers or, in some cases, anesthesia. The rat has been used successfully in toxicology research for close to a century and will continue to be used for the foreseeable future.

PATHOLOGY

The literature available on the toxiccologic pathology of the laboratory rat continues to outpace that of all other model species combined. It is thus not possible for this section to reproduce that literature. Rather, the desire is to provide an overview of the key elements that most commonly influence the design, conduct, and interpretation of toxicity studies and then to give access to the relevant literature for those who need more information or detail. There are now a number of excellent texts addressing the toxicologic pathology of the rat, and the interested reader is directed to them for a more in-depth review (Greaves 2000; Haschek and Rousseaux 1998; Haschek et al. 2002).

Any discussion of rat toxicologic histopathology must first start with differences due to age, strain, and sex. Our appreciation of the influence of these factors continues to evolve, particulary in the area of carcinogenesis (Chandra and Frith 1992; Nakazawa et al. 2001; Poteracki and Walsh 1998; Tennekes et al. 2004). It must always be kept in mind that the selection of strain (generally Sprague-Dawley, Fischer 344, or Wistar, although others such as the Long Evans do see use) is a feature of experimental design to be carefully considered. Most contract research organizations (CROs) have a favorite strain, and all too frequently choice of the CRO leads to default choice of strain. Before the initiation of any study there are three inputs from the laboratory animal professional and pathologist that must be considered for the optimal outcome of the study. First, the source of test animals must be carefully selected and all individual shipments carefully screened to ensure that only healthy rats with the desired characteristics (e.g., age, sex, strain) are used. This screening process should include a period of acclimation and health surveillance after the rats have been received to the facility (although 2 weeks is generally desirable, 7 days is adequate).

Second, the objective of the study and the degree of variability in the pathology of the animals must be considered in the design of the study, as should any factors such as the known time course of a response (Haschek et al. 2002). Sufficient animals need to be used so that an effect can reasonably be detected (if present) as an increase above the existing background levels of morphological variation. In other words, the study design must be adequate to allow development of a pathological response and yet also include sampling (collection of blood and tissues) at appropriate time points so that the effects of age or other confounding processes do not obscure results.

Finally, the necropsy must be designed to be both efficient and effective, and the involved personnel must have adequate training and experience with the type of animals being utilized.

Necropsy

Except for pilot or dose-ranging studies, unexpected mortalities in rodent toxicity studies should be limited to rare accidental deaths associated with procedures (e.g., dosing mistakes in intubation studies) and deaths due to unexpected toxicity. Otherwise animals should live to the time of scheduled termination, providing for optimal tissue collection and fixation and proper correlation of clinical signs, clinical chemistry, and pathology findings. Because moribund or dead animals frequently have treatment-induced or important spontaneous lesions, they must receive complete necropsies. Rats should be observed a minimum of twice daily to identify moribund individuals. If there is a significant chance that death could occur before the next observation, the animal should

be sacrificed. Little is gained by attempting to have the animal live a few days longer, considering the risk of loss of data due to premature death and autolysis.

Dead animals should be refrigerated immediately on discovery and necropsied as soon as possible thereafter. Refrigeration will significantly delay autolysis (for up to 8 hr [Kupp and Strolle 1979]). Dead animals should not be frozen, as ice crystals created during the freezing and thawing process will damage or destroy cellular integrity and severely limit or impair any interpretation of histopathology. For scheduled necropsies, animals should generally be fasted overnight to provide for the natural emptying of the GI tract and for a standard physiological baseline for evaluation of clinical pathology parameters.

Clinical observation records of the animal should be available at the time of necropsy, enabling the prosector to focus on target sites of disease and decreasing the chance of missing important lesions. The necropsy technique might have to be modified to suit the clinical signs. For example, the standard necropsy calls for collecting a piece of lumbar spinal cord. If paralysis or paresis was noted clinically, the entire spinal cord and possibly the brachial or lumbar plexuses would need to be examined.

A standard necropsy procedure should be followed. After reviewing the clinical records, one should palpate the animal thoroughly and examine all external orifices, eyes, and skin. Essentially, a physical examination is performed before the necropsy. The actual necropsy should follow a standardized format. This provides increased efficiency, optimal tissue accountability, and improved fixation. A thorough necropsy is probably the first essential step to assure eventual quality in pathology. General methods for rat necropsies and guides to gross anatomy have been published (Bohensky 1986; Chiasson 1988; Feldman and Seely 1988; NCI 1976; Reuber 1977). If special considerations dictate or alterations in which tissues are to be collected are needed (usually seen as the addition of tissue sections or changes in preservatives used) these must be discussed thoroughly with the necropsy staff before initiation of the procedures.

Table 3.16 provides a list of the tissues that would be collected from a rat at necropsy, along with an indication of which should normally be weighed to provide an indication of potential morphological indications of treatment-related effects.

Organ weights can be extremely useful if accurately determined and when considered in the proper context. They can help to identify subcellular changes (e.g., enzyme induction in the liver), target organs (although they are more useful for some organs than others; see Gad et al. 1984), and

Table 3.16 Tissues Collected and Weighed at Necropsy

Skin	Liver[a]
Mammary gland	Pancreas
Lymph node(s)	Spleen[a]
Salivary gland	Kidneys[a]
Sternum/costochondral junction	Adrenal gland[a]
Femur (including bone barrow)	Urinary bladder
Muscle (PSOAS)	Seminal vesicle
Thymus[a]	Prostate[a]
Trachea	Testes[a]
Lung[a]	Ovary[a]
Heart[a]	Uterus[a]
Thyroid gland (with parathyroid)[a]	Vagina
Tongue	Brain
Esophagus	Pituitary gland[a]
Stomach	Spinal cord
Duodenum	Eyes
Jejunum	Lacrimal gland
Ileum	Any additional tissues or organs with "abnormal findings" or deemed appropriate
Colon	

[a] Tissues to be weighed at necropsy.

they can assist in identifying hormonal influences in some target organs. The downside is that improper tissue handling by the prosector can lead to artifactual tissue damage and organ weights might not correlate with other findings (in which case a weight of evidence and nature of the data warrant consideration).

If tissues, lesions, or observations are missed at necropsy, they will never be recovered. Accurate observations must be recorded and descriptions should include the location, size, color, and consistency of the lesion. Whenever possible, lesions should be collected and fixed with some adjacent normal tissue. Equipment for gross photography should be available to document significant study findings. Not all gross lesions must be photographed; however, representative toxic lesions should be recorded on film.

Routine tissues should be fixed in approximately 10 volumes of neutral buffered formalin. Excellent fixation of lungs can be obtained by inserting a 14- to 18-gauge blunt needle into the lower trachea and inflating the lungs to their original size with formalin. The stomach should be opened and examined carefully for ulcers, erosions, and so on. The small intestine of rodents can be opened and examined or flushed with formalin and examined by transillumination. If the latter is done, the gut should be opened and examined at the time of tissue trimming. The bladder should also be inflated with formalin and examined by transillumination, followed by an open examination when trimmed for embedding. The pituitary is one tissue most often lost in rodent necropsies. This can be minimized by leaving the organ attached to the basisphenoid bone. The bone and attached pituitary can be placed in a special tissue capsule until embedding. After removal of the eyes, brain, pituitary, tongue, and mandible, the nasal turbinates can be fixed by flushing from the nasopharynx to the nares with formalin followed by immersion in formalin. Tissue sections should have a maximum thickness of 0.5 cm for good fixation, and should be fixed for a minimum of 48 hr before trimming and processing.

Clinical Pathology

Clinical pathology comprises both hematology and clinical chemistry. These are extremely powerful tools for both assessing specific target organ toxicities during the in-life phase and providing correlative information for understanding both disease processes and the relevance of anatomical findings.

Laboratory determination of various clinical chemistry parameters in the rat is not particularly difficult, especially with the recent development of many automated analyzers. Most of these instruments require from 1.5 to 50.0 µl of serum or plasma and are preprogrammed to perform up to 20 different clinical chemical tests. The primary challenge of the clinical pathology laboratory is to validate equipment or procedures designed for use with human serum, or blood, and modify these into reliable methodology for the evaluation of organ function and toxicity in rats. Toxicologists must be aware of a seemingly infinite number of variables that impact the interpretation of clinical pathology. Controllable sources of variation can be divided into at least three general categories: (a) variation related to the physiological status of the animal and its environmental condition, (b) variation related to sampling, and (c) variation related to analytical instrumentation and methodology.

Several authors have addressed the effect of these factors on many of the common clinical chemistry procedures. Variations related to the physiological status of the rat and its environment include disease status (Cotchin and Roe 1967; Deb and Hart 1956), age (Kozma et al. 1969; Vondruska and Greco 1973; Weisse et al. 1974), sex (Kozma 1967; Weisse et al. 1974), husbandry, nutritional condition, and degree of hydration. Variations related to sampling collection include method of collection, anesthetic used, the time of day the sample is collected, restraint technique, anticoagulant used, hemolysis, sample processing and storage, and site of sampling. Blood sampling techniques are described and discussed elsewhere in this chapter.

In the rat as in other species, prompt separation of serum from cells is critical. Hemolyzed or transparent serum that has remained in contact with erythrocytes can have falsely elevated values for potassium, lactic dehydrogenase, and total protein, less consistently and to a lesser degree for phosphorus, and sometimes for bilirubin. Prolonged contact of serum with erythrocytes reduces serum glucose. Centrifugation and prompt removal of a serum from the clot should occur at intervals not exceeding 30 min after collection to yield reliable data.

Table 3.17 and table 3.18 summarize literature values for clinical chemistries and hematological values of common laboratory rats. One notable variation in hematologies for different strains of rats is the life span of red blood cells (Derelanko 1987), which suggests that other functional aspects of the hematological system might vary between strains to the point that they can influence interpretation. All such tabular summaries of normal or control values (e.g., Leonard and Ruben 1986; Loeb and Quimby 1999; Mitruka and Rawnsley 1977), however, should be considered critically and used only as general guidance. Current control values for a particular strain and CRO are generally much more meaningful for comparison than literature values.

Common Diseases

Common diseases that occur in modern caesarean-derived (CD), SPF rats used in toxicology laboratories can be viral, bacterial, parasitic, or rickettsial. Outbreaks of infectious disease are

Table 3.17 Clinical Chemistry Values

	Male		Female		
	M	SD	M	SD	Range
Bilirubin (mg/dl)	0.35	0.02	0.24	0.07	0.00–0.55
Cholesterol (mg/dl)	28.3	10.2	24.7	9.62	10–54
Creatinine (mg/dl)	0.46	0.13	0.49	0.12	0.20–0.80
Glucose (mg/dl)	78.0	14	71	16	50–135
Urea nitrogen (mg/dl)	15.5	4.44	13.8	4.15	5–29
Uric acid (mg/dl)	1.99	0.25	1.79	0.24	1.20–7.5
Sodium (mEq/l)	147	2.65	146	2.50	143–156
Potassium (mEq/l)	5.82	0.11	6.70	0.12	5.40–7
Chloride (mEq/l)	102	0.85	101	0.90	100–110
Bicarbonate (mEq/l)	24	3.80	20.8	3.60	12.6–32
Phosphorous (mg/dl)	7.56	1.51	8.26	1.14	3.11–11
Calcium (mg/dl)	12.2	0.75	10.6	0.89	7.2–13.9
Magnesium (mg/dl)	3.12	0.41	2.60	0.21	2.24–3.81
Amylase (Somogyi units/dl)	245	32	196	34	128–313
Alkaline phosphatase (IU/l)	81.4	14.8	93.9	17.3	56.8–128
Acid phosphatase GIULIA	39	4.30	37.5	3.70	28.9–47.6
Alanine transaminase (SGPT) (IU/l)	25.2	2.05	22.5	2.50	1.5–30.2
Aspartate transaminase (SGOT) (IU/l)	62.5	8.40	64.0	6.50	45.7–80.8
Creatin phosphokinase (CPK) (IU/l)	5.60	1.30	6.80	2.40	0.84–11.6
Lactic dehydrogenase (LDH) (IU/l)	92.5	13.9	90	14.5	61.0–121
Serum total protein (g/dl)	7.61	0.50	7.52	0.32	4.70–8.15
Albumin (g/dl)	3.73	0.53	3.62	0.52	2.70–5.10
(%)	49	7.10	48.1	7.40	33.3–63.8
α_1-Globulin (g/dl)	1.03	0.22	0.89	0.25	0.39–1.60
(%)	13.5	2.20	11.9	3.80	4.30–21.1
α_2-Globulin (g/dl)	0.71	0.14	1.40	0.32	0.20–2.10
(%)	9.3	1.80	8.60	2.70	3.20–14.7
β-Globulin (g/dl)	1.07	0.35	1.31	0.26	0.35–2.00
(%)	14.1	4.70	17.4	3.60	5.70–26.8
γ-Globulin (g/dl)	1.05	0.21	1.18	0.21	0.62–1.60
(%)	13.8	2.70	14	2.80	10–19.8
Albumin/globulin	0.96	0.24	0.93	0.25	0.72–1.21

Table 3.18 Hematology Values of Common Rat Strains

Test	Unit	Long-Evans (Blu:LE)	Wistar/Lewis Albino	Osborne-Mendel	Fischer Inbred Strain 344/Cr
Erythrocytes (RBC)	($\times 10^6$/mm^3)	5.98–8.30	7.20–9.60	6.26–8.96	6.68–9.15
Hemoglobin	(g/dl)	13.1–16.7	12–17.5	14.30–17.7	13.4–17.2
MCV	(v^3)	52–69	57–65	52–66	54–67.5
MCH	(vvg)	18.5–23.5	14.6–21.3	18.8–23.3	17–21.8
MCHC	(%)	32–38.5	26–38	32–42	26–35.5
Hematocrit (PCV)	(ml%)	39–48	42.5–49.4	39.4–46.2	46–52.5
Leukocyte (WBC)	($\times 10^3$/mm^3)	3.30–7.90	5–8.96	6.23–12.6	5.35–11.2
Neutrophils	(%)	5.50–35.5	9–34	4.5–23.5	11.5–41.6
Basophils	(%)	0	0–1.50	0	0
Lymphocytes	(%)	60–93.5	65–84.5	72–94	43–79.5
Monocytes	(%)	0–5.50	0–5	0.50–3.50	0–2
Eosinophils	(%)	0–1.50	0–2.50	0–1	0–4
Platelets	($\times 10^3$/mm^3)	140–460	160–470	145–450	150–450

becoming less common in well-managed laboratory animal facilities but sporadic outbreaks and subclinical infections do occur. It behooves the laboratory animal professional to maintain high operating standards to minimize the impact of infectious diseases in toxicity studies. Infectious diseases can produce lesions and functional defects that might be difficult to distinguish from those of target organ toxicity. Infectious agents might produce lesions at the site of entry equivalent to local toxicity, and lesions equivalent to systemic toxicity. Either local or systemic effects can predominate, or the disease can be expressed as a combination of both. Infectious skin diseases are uncommon in most laboratory animals, and the two most frequently affected portals of entry are the respiratory and digestive tracts. In rats, infections of the respiratory tract are more frequent.

Viral Pneumonitis

Viral pneumonitis is a subclinical respiratory infection producing a lung lesion that is a focal alveolitis, which can be observed in any phase of the inflammatory process from acute to chronic and is accompanied by prominent perivascular leukocyte foci, usually lymphoid, with occasional eosinophils. The alveolitis is generally of little clinical significance, but its presence can seriously confound the interpretation of inhalation toxicity studies, particularly of some of the low-grade focal dust-induced lesions. Hyperplasia of the mandibular lymph node is also common in infected rats, and probably represents an immune reaction to viral infection. The causal organism could be the pneumonia virus of mice (PVM).

Sialodacryoadenitis

Sialodacryoadenitis (SDA) is a frequent epizootic disease in rat colonies with a high morbidity and negligible mortality. It was first recognized in rats in 1961 (Innes and Stanton 1961). SDA is a generic term for infection with several serotypes of a coronavirus. These serotypes have a spectrum of virulence, primarily infecting the upper respiratory tract with variable infection of the glands around the head and neck.

Respiratory tract lesions include rhinitis and focal interstitial pneumonitis, but this is generally subclinical. In contrast, infection of the glands often produces striking clinical symptoms. The most striking clinical features are red staining around the eyes due to infection of the Harderian gland, and swelling of the ventral neck region associated with infection of the submaxillary salivary gland. The swollen neck (“rat mumps”) rapidly subsides and the rats appear normal within about a week. Thus, SDA is a disease dominated by acute local effects.

The main finding at necropsy, depending on the stage of the disease, is either a swollen or shrunken salivary gland. Histologically, the gland progressively shows various combinations of degeneration, inflammation, and regeneration, but it is quickly restored to normal. The Harderian gland shows a similar cycle of disease, but squamous metaplasia is very prominent in the proliferative repair phase. In some animals, there are ophthalmic lesions, including reddish discharge, cloudy cornea, and corneal ulceration. Microscopically, the changes in the Harderian and submaxillary salivary glands can be dramatic: There is necrosis, intense inflammation, and often marked squamous metaplasia of the ductal epithelium.

Lesions in the eye associated with SDA consist of keratoconjunctivitis, corneal ulcers, and synechiae of the iris and ciliary body. They are largely resolved by 10 weeks; however, megaloglobus persisted in 6% of the rats in one study (Lai et al. 1978). Such severe alterations of ocular structures make infected rats unsuitable for research in which the eye is the target organ. Apparently, this disease is widespread in rat colonies, invading the ear canal, and usually has both sebaceous and squamous differentiation.

Sendai Virus Infection

The Sendai virus is a paramyxovirus that is enzootic in many modern rat colonies. Enzootic infections generally affect weanlings as maternal milk-transmitted immunity wanes. The pups develop a respiratory disease that is generally subclinical. At necropsy, many rats appear normal, but some have small red foci scattered over the surface of the lung. Histologically, there are acute necrotizing inflammatory lesions in the mucosal epithelium of the nose, trachea, and pulmonary airways, the last of these often extending into the alveoli. Most of the airway mucosal lesions repair rapidly and completely, but focal fibrosis and scarring can occur in terminal airways and associated alveoli. In a general, however, Sendai infection can be considered an acute transient infection of the rat respiratory tract with little or no residual effects.

Although it is widely recognized that Sendai virus is a respiratory pathogen of mice that produces pneumonia and death, its role in naturally occurring respiratory diseases of rats is much less clear. In one report, a Sendai virus epidemic occurred in an aging rat colony paralleling an outbreak in a mouse colony at the same institute (Burek et al. 1977). The epidemic was characterized by seroconversion, but increased mortality and clinical signs were not observed. During this period, however, rats coming to necropsy had distinct microscopic pulmonary lesions not found before the outbreak. Three types of lesions were found: perivascular cuffing of pulmonary vessels by plasma cells and lymphocytes; multifocal interstitial pulmonary infiltrates of macrophages, lymphocytes, and neutrophils; and hyperplasia of peribronchial lympholid tissue.

Corynebacterium kutscheri *Infection*

Corynebacterium kutscheri is an example of a bacterial disease dominated mainly by acute local effects in the respiratory tract. The infection is usually latent or inapparent, but could become activated to produce acute suppurative pulmonary lesions that are either fatal or resolve by fibrosis and the formation of granulomas. Activation of this disease is usually associated with factors that lower host resistance. Administration of chemicals could be such a factor, either through immunosuppression or the general stress of toxicity. As a result, a pathologist could be presented with a pattern of minor lesions in controls and a dose-related increase in lung lesions in treated groups. This apparent "pulmonary toxicity" is another example of the complex interaction between different causal agents that can occur in animal models.

Pinworms

Pinworms are parasitic nematodes that are very common and are visible living free in the lumen of the large intestine without producing any obvious lesions. They are common in many species and are a good example of an asymptomatic local infection.

Mycoplasmosis

Mycoplasmosis tends to be tissue and host specific, and in the rat the disease primarily affects the respiratory tract and to a lesser extent the female reproductive tract. The respiratory disease begins in early life, and progresses as a chronic condition; therefore respiratory mycoplasmosis in the rat is commonly termed chronic respiratory disease (CRD). CRD has been the major health problem affecting the laboratory rat. When endemic in a colony, it is generally the life-limiting factor. This disease has been eliminated from most modern colonies, but is still occasionally encountered as a highly contagious, chronic enzootic disease of the respiratory tract.

Mycoplasmosis often begins with inner ear involvement, and only later is pulmonary involvement seen (Kohn 1979). The most characteristic clinical sign in the young rat is snuffling and wheezing during the first 3 months of life. This snuffling is associated with inflammation in the upper respiratory tract, notably rhinitis, and with increasing severity or age it progressively affects the larynx, trachea, and lungs to produce the bronchiectatic abscess characteristic of the older rat. After mycoplasmal colonization small amounts of purulent exudate appear in the airway lumen. This is followed by hyperplasia and increased mucus production in the respiratory epithelium and by lymphoid infiltration and proliferation. The hallmarks of chronic infection are thus exudate, epithelial hyperplasia, squamous metaplasia, and lymphoid cell accumulation. The infection might also affect the middle ear, and subsequent labyrinthitis could produce clinical symptoms such as circling.

Like *C. kutscheri* infection, the disease pattern can be altered by extraneous factors such as ammonia levels in the animal room as well as by interaction with other infectious agents such as the Sendai virus, and of course by effects related to test article administration. Any stress such as crowding or experimental procedure will cause increased mortality and decreased life span.

Gross pathological findings include areas of red to gray consolidation of the lung, often containing abscesses. In a majority of rats, the bullae of the ears contain a green-yellow exudate. The most spectacular pathological changes are seen in the old rat, and in protracted cases, the lung might be converted to a mass of bronchiectatic tissue and abscesses. Microscopically, there is a severe chronic purulent bronchopneumonia with bronchiectasis and marked peribronchial lymphoid hyperplasia. The histopathological findings in advanced stages are fairly typical of chronic disease, consisting of a combination of chronic inflammation and reparative processes.

Although numerous agents have been proposed as the cause of CRD, *Mycoplasma pulmonis* appears to be the main, and in many cases the sole, agent responsible. The organism can continue to be isolated from the pneumonic lung, and rats with more severe respiratory involvement are more infectious for other rats. Although high complement fixation antibody titers are present and might serve as an aid in diagnosis, there is no correlation between circulating antibody and the presence of the organism. This suggests that circulating antibody does not play a role in protection against the mycoplasma infection. Occasionally, *Mycoplasma arthritidis* can be a secondary invader in CRD of rats. Common nonneoplastic lesions are summarized in table 3.19, and changes in incidences in age at termination are presented in table 3.20.

Histopathology of the Rat

Although there are several ways that one might organize an overview of the microscopic pathology of the laboratory rat, a good (and fairly common) approach is to compare the patterns commonly found in both young and aged animals, as these form very different backgrounds to evaluate (or detect) any toxicological response against secondary patterns of concern that might complicate or confound determinations of causality, such as a response to generalized stress that is not uncommonly seen at high doses, and these then can also be considered. In all cases, reference can be made to some standard glossary for use in the description of findings (e.g., Greaves and Faccini 1992).

Table 3.19 Example of a Pattern of Pathology in Young Rats

Organ	Diagnosis	% of Incidence	
		Male	Female
Skin	Alopecia/fur loss	5	9
	Dermatitis/sore	4	4
Tail	Dermatitis/sore	5	3
	Other lesions	3	3
Esophagus	Myositis	2	6
Eye	Periorbititis	10	11
	Other lesions	6	8
Heart	Leukocyte foci	7	2
Kidney	Leukocyte foci	12	7
	Hyaline droplets	15	0
	Tubular regeneration	32	4
	Mineralization	0	6
	Hydronephrosis	10	10
Liver	Leukocyte foci	80	80
Lung	Leukocyte foci	52	56
	Pneumonitis	20	18
	Foamy histiocytes	20	16
	Mandibular lymph node hyperplasia	40	26
	Large intestine nematodes	8	3
Pituitary	Cyst	3	3
Stomach	Erosions and ulcerations	10	10[a]
Thyroid	Ectopic thymus	2	3
Prostate	Leukocyte foci	14	—
Testis	Atrophy	2	—[b]
Thymus	Atrophy	—	—[b]
Uterus	Distension	—	14

[a] Seems to be increasing in recent years (1988 onward).
[b] Commonly found associated with nonspecific stress at higher dose levels.
Source: Adapted from Glaister (1986).

Table 3.20 Incidence (%) of Morbidity and Mortality in Sprague-Dawley Rats Due to the Major Groups of Nonneoplastic and Neoplastic Causes

Cause	Study Interval (Weeks)					
	Male			Female		
	52–78	79–104	>104	52–78	79–104	>104
Nonneoplasia						
Degeneration	8	8	12	2	1	0
Inflammation	8	8	8	2	2	2
Other conditions	2	2	8	1	1	5
Uncertain/multifactorial	14	19	8	2	2	0
Neoplasia						
Pituitary	40	30	21	45	41	45
Subcutis	19	13	16	1	2	2
Mammary	0	0	0	33	43	32
Other tumors	9	20	27	14	8	14

Note: Coleman et al. (1977) includes extensive incidence tables on male Fischer 344 rats.
Source: Adapted from Glaister (1986).

Pathology of Young Rats

Strain-to-strain variations for patterns of age-related lesions are recognized in rats. Although the patterns associated with old animals are generally of more interest, there are also common spontaneous lesions in young animals. Control rats rarely die in short-term studies and neoplasia

is also rare, so significant treatment-related incidence of tumor after 6 months or less of treatment is very striking. The observed pattern of pathology is, therefore, of nonneoplastic lesions and is usually presented in tabular form, such as in table 3.19. Only lesions occurring with an incidence greater than 1% are listed. A toxicologist presented with such a table should try to understand what the diagnoses and their frequencies mean in terms of the general background of pathology in control animals. Among the 40 organs commonly evaluated, only a minority has lesions, and relatively few lesions have incidence rates greater than 10%. Thus, the majority of organs of a young rat are either normal or rarely show histopathological changes. There are numerous subpatterns within this general overall pattern of infrequent lesions. The observations presented in incidence summary tables, such as table 3.19, are usually listed on an organ-by-organ basis, but lesions within any particular organ can represent degenerations, inflammations, or proliferations; they might be trivial or severe; and they could be related to a variety of causes. It is the association between cause and effect that is the main interest in toxicological pathology and, in young animals, it is convenient to describe the pathology in terms of causation rather than in terms of lesions within any one organ.

Rats are commonly housed in small groups, often in metal cages with wire mesh floors. A variety of minor external inflammatory lesions are encountered owing to attrition between rats and between rats and their cages. Fur loss and dermatitis are typical changes seen on the skin and on the tail in group-housed rats. Lesions on the feet and ears might also be seen. These lesions are usually dismissed as insignificant, but differences between control and treated groups might occur in certain circumstances, such as a decrease in the incidence of fight wounds in rats given tranquilizers.

Some lesions are associated with the method of administration of the chemical or with the removal of blood samples for analysis. Rats might struggle when gavaged and the esophagus can be traumatized by the cannula. This bruising appears histologically as a focal myositis with evidence of acute inflammation or with healing and repair. The usual appearance is one of basophilic regenerating myofibers accompanied by a few leukocytes. Although the oral route is the most common method of test article administration, equivalent inflammatory lesions such as phlebitis or myositis might be common following IV and intramuscular administration. It should always be kept in mind that the point of introduction of a drug or test substance into the body (e.g., the vasculature immediately in the region of a catheter) could have some unique pathology associated with experiencing the highest concentration of an administered agent. This pathology might be associated more with physio-chemical factors than with actual drug toxicity. This holds true for any parenterally, nasally, or topically administered agents, and sometimes for agents administered orally.

Hair Fragments in the Lung

Another lesion sometimes associated with IV injections is hair fragments in the lung. These fragments lodge in pulmonary vessels as hair emboli after their introduction into a peripheral vein during venipuncture. Blood sampling by orbital sinus puncture is another common procedure, and this can cause a variety of inflammatory changes around the eye and orbit, designated by the general term periorbitis. Repeated sampling or poor technique can produce other lesions such as degeneration of the optic nerve (optic neuropathy). The severity and incidence of these procedure-related lesions are variable depending on the nature and frequency of the procedure and on the skill of the technician. Lesions might be uncommon and minor in short-term, well-conducted studies, or at the other extreme might be fatal if the esophagus or eyeball is accidentally punctured.

Congenital or Developmental Defects

The thymus has the same embryological origin as the parathyroid and during its migration toward the base of the heart, small nests of tissue descending from the third and fourth branchial pouch might remain adjacent to or be embedded in the parathyroid or thyroid. These can be quite

striking histologically, but they are of trivial significance. Similarly, pituitary cysts are usually trivial. They are derived from remnants of the upgrowth of the craniopharyngeal (Rathke's) pouch, which develops into the adenohypophysis. They vary in site and appearance, but they are usually seen in the pars distalis as small colloid-filled cysts lined by ciliated epithelium. Hydronephrosis (dilation of the renal pelvis) can be caused by urinary tract obstruction, but is more commonly due to developmental or congenital defects. It tends to be unilateral, of little significance, and associated with normal renal function. Severe bilateral and fatal cases might occasionally occur, but care must be taken to exclude obstruction of the lower urinary tract in these instances.

Background Changes

A variety of minor inflammations, degenerations, and proliferations of diverse or uncertain cause can be encountered in the young rat. They are grouped together as miscellaneous "background" changes and include (see table 3.19) hyaline droplets, tubular regeneration, and mineralization in the kidney; foamy histiocytes in the lung; testicular atrophy; and uterine distension. One also sees leukocyte foci in the liver and lung.

Renal Hyaline Droplets. Dense eosinophilic hyaline droplets of variable size are seen in the cytoplasm of proximal tubular cells in both man and experimental animals. The kidney is not only the major route of elimination for many agents, but also an important site of uptake and catabolism of low molecular weight proteins such as albumin, immunoglobin light chains, parathyroid hormone, and glucagons. The main pathway for the extraction of proteins from the circulation is by glomerular filtration. The amount of protein reaching the urinary space is dependent on glomerular filtration rate, plasma concentration and the physico-chemical characteristics of the protein and the degree of uptake is inversely proportional to the molecular size of the protein. The majority of protein within the tubular fluid is taken up into the proximal tubule by endocytosis. Absorbed proteins within endocytotic vacuoles are transported to regions of the tubular cell rich in lysosomes where fusion takes place and hydrolysis to amino acids occurs. Amino acids are then returned to the circulation. Disturbance of this balance, either by increased filtered loads of proteins or their decreased catabolism, can result in the accumulation of protein in the form of hyaline droplets in renal tubular lysosomes.

Hyaline droplets are particularly prominent in the proximal renal tubules of untreated male rats and male rats are liable to show increased droplet formation after treatment with a wide range of drugs and chemicals. This predisposition appears to be due mainly to the presence of a specific protein, α_{2u} globulin, which is the major normal urinary protein in the male rat, synthesized by the liver and under synergistic control by testosterone and corticosterone (Alden 1985). Although humans excrete proteins of a similar nature, they are found in only trace amounts (Hard 1995). This protein has a molecular weight of 18,000 to 20,000 Daltons and it is freely filtered by the glomerulus and reabsorbed by the proximal tubular cell. Immunocytochemical study has shown its location to be mainly in the S2 segment of the proximal tubule in the male rat. Moreover, the male rat might have a lower capacity of renal protein handling than females because protein tracer studies using ovalbumin have shown that the male rat proximal tubule has a lower rate of reabsorption and catabolism of protein ultrafiltrate than female mice.

It should be noted that other proteins are also linked to the formation of hyaline droplets spontaneously in rodents. One example is the increase in hyaline droplets reported in rats and mice suffering from histiocytic sarcoma that immunocytochemistry showed contained lysozyme but not increased amounts of α_{2u} globulin or other proteins such as α_1 antitrypsin or immunoglobulin (Hard and Snowden 1991).

Similar morphological changes can be observed in the male rat following the administration of pharmaceutical agents and can also be the result of binding of drug with lysosomal protein constituents in the proximal tubule, which impairs their catabolism. This mechanism was suggested

to explain the hyaline droplet accumulation found in the S1 and S2 segments of proximal tubules in male rats treated with the antihelminthic, levamisol, the antimalarial BW 58c and an anti-inflammatory 540c. These histopathological features were different from the myeloid bodies found in the renal tubules following treatment with aminoglycosides and other lysosomotropic agents.

Small cytoplasmic droplets reflecting the accumulation of dense secondary lysosomes are reported in the cytoplasm of collecting of the renal papilla rats treated with carbonic anhydrase inhibitors (Owen et al. 1993; Owen et al. 1994). As these effects were ameliorated by administration of potassium, it was argued that their development might be related to depletion of renal medullary potassium.

Although injection or infusion of normal proteins might simply increase the number of hyaline droplets with identical morphology to those found spontaneously, it should be kept in mind that even normal proteins have differing nephrotoxic potential. For instance, perfusion of rats with human immunoglobulin k light chain of molecular weight of between 20,000 and 50,000 Daltons was associated with droplet formation and acute tubular injury, whereas albumin was devoid of nephrotoxicity under similar circumstances. Although the reason for this difference is unclear, physicochemical properties are presumably important. It is of interest to note that k immunoglobulin light chains are also capable of inducing acute tubular injury in humans.

Renal hyaline droplets are common findings in the kidneys of the male rat. These appear as bright eosinophilic globules of various sizes in the cytoplasm of the proximal tubular epithelium. The glomerulus of the male rat is "leaky" to low molecular weight proteins, and these droplets represent protein resorbed by the proximal tubule cells and sequestered in lysosomes prior to recycling into the blood. Although these droplets are common and are generally considered trivial, like many other background observations, the severity of the change occasionally increases in a dose-related manner. The cause of such an increase in hyaline droplets is related to the interaction of the test article or a metabolite with a protein, α_2 microglobulin, and interference with the recycling of the globulin by renal tubules. In some cases, the increase in hyaline droplets is accompanied by focal tubular degeneration or tubular necrosis. Thus, a common change might be accentuated to a sufficient extent in treated animals to be variably interpreted as evidence of nephrotoxicity. The kidneys of females are usually normal.

Renal Tubular Regeneration. Renal tubular regeneration is another common but minor finding in male rat kidneys. Synonyms are "blue tubules," tubular atrophy, and tubular basophilia. The affected tubules stand out against the generally eosinophilic cortex because of their basophilia. On closer inspection, they appear as slightly shrunken tubules with cuboidal basophilic epithelium and sometimes a thickened basement membrane. The response of tissues to injury is limited, and tubular regeneration is one sequel to nephrotoxicity. This otherwise trivial lesion might therefore assume importance if it confounds the interpretation of no-effect levels in studies of nephrotoxic chemicals.

Renal Tubular Mineralization. Renal mineralization, also termed nephrocalcinosis, is a common minor renal lesion seen mainly in females. The mineral can be found under or in the pelvic epithelium, or more commonly in the tubules at the corticomedullary junction. The corticomedullary mineralization usually appears between weaning and sexual maturity, and the etiology appears to be multifactorial, involving dietary and endocrine factors. Manipulation of dietary components in nutritional studies on certain food or protein substitutes might increase the incidence and severity of mineralization. This is another example of synergism between the specific substance of interest and background factors, and it might be difficult to establish no-effect levels if there is a high incidence of mineralization in controls.

Foamy Histiocytes. In the lung, the main observation of interest is foamy histiocytes. These are also called foam cells or termed lipidosis. The condition is characterized by accumulations of plump pale cells in the alveolar lumen, often located subpleurally so that they appear at necropsy as

pinpoint gray spots on the surface of the lung. The foamy appearance of these alveolar macrophages is due to the uptake of surfactant released from type II alveolar cells. This condition can be considered an uptake, storage, and recycling process analogous to the hyaline droplets seen in the male rat kidney and, similarly, might become enhanced in response to certain treatments. This enhancement is seen most commonly in response to inhaled particles such as silica and in association with phospholipidosis-inducing compounds such as cationic amphiphilic drugs. A high control incidence of foam cells could interfere with the determination of no-effect levels with these compounds.

Testicular Atrophy. Testicular atrophy is usually minor, unilateral, and affects the subcapsular tubules. Occasionally, it may be severe and diffuse, and appear at necropsy as a small, watery, blue testis. Histologically, the affected tubules are small and show partial or complete lack of germ cells, leaving only Sertoli cells. This condition can have many causes, including congenital origin, obstructive lesions, and physical restraint as in nose-only inhalation studies. It could also result from treatment with chemicals and hormones, and a high background incidence in controls might hinder the evaluation of these substances. It is not uncommon to see significant incidences of testicular and thymic atrophy in high-dose group animals attributable to nonspecific stress rather than any specific target organ toxicity. In these cases, however, the animals also generally have reduced food consumption and reduced absolute body weight.

Leukocyte Inflammatory Cell Foci in the Liver and Lung. Leukocyte inflammatory cell foci are frequently encountered in certain tissues, especially the liver and lung, and to a lesser extent the prostate. They might be related to enzootic viral infections such as PVM or Sendai, but the relationship is difficult to establish. The foci are usually minor and multifocal, and usually comprise mononuclear leukocytes in various admixtures, but mainly lymphoid. These foci appear histologically as small basophilic cellular aggregates scattered across the plane of section. Other terms used are round cell foci, lymphoid foci, inflammatory foci, and similar nonspecific terms. This tendency to use nonspecific terms is quite common among toxicological pathologists because of the frequent misunderstanding of the more specific terms when crossing species lines or scientific disciplines. Thus, leukocytes in the liver might occasionally surround a necrotic liver cell and could justify the term hepatitis. However, a medical pathologist or a toxicologist unfamiliar with laboratory animal pathology might interpret such a diagnosis as a serious condition.

In terms of the two basic scales for assession lesions—incidence and severity pathology—findings in the young rat can be considered to be generally infrequent, and when present, of minor biological significance. Their main importance is in the two ways in which they can complicate the interpretation of treatment-related responses. In the first place, they might mimic the effect of treatment. For example, renal tubular regeneration might complicate the assessment of no-effect levels of nephrotoxins. Second, the incidence or severity of background pathology might be enhanced in a dose-related manner. The enhancement might be of endogenous processes such as the lysosomal cycling of pulmonary surfactant and renal protein, or it might be of processes initiated by exogenous agents such as infections. In either case, the precise causal relationship of treatment to the observed response might be difficult to unravel.

Pathology of Old Rats

The pathology of the aged rat is an important confounding factor in long-term bioassays for chronic toxicity and carcinogenicity (see Chandra and Frith 1992; Nakazawa et al. 2001). In contrast to the pattern of infrequent and generally minor lesions seen in young rats, the pattern seen in aging animals during chronic studies is one of an increasing incidence and severity of lesions culminating in death. Although lesions related to husbandry, procedures, development, and infections still occur, these are relatively minor compared with the main age-associated lesions and, therefore, the pattern

of pathology will be classified in a different way. The three main items of interest in chronic rodent studies are morbidity and mortality, nonneoplastic lesions, and neoplasms. These will vary from strain to strain (more so than the patterns associated with very young animals), but a basic pattern is still present. The two main parameters of interest in analysis of morbidity (illness) and mortality (death) are the rate at which they occur and the causation. These data give the pathologist and toxicologist an excellent opportunity to overview some of the major background characteristics of the rat strain. One or both of these parameters might be affected by treatment, but must also always be considered in study design and conduct. The status of changes in body weight (are the animals gaining, losing, or stable) is an important indicator of general health status for a group of rats.

Age at Which to Terminate Animals

A key issue in rodent carcinogenicity studies is the age at which to terminate the animals. As such a study progresses, the rise in the background level of tumors makes it more and more difficult to clearly partition treatment-effect tumors from age-effect tumors. Swenberg (Solleveld et al. 1984; Swenberg 1985) has made the point that the incidence of many tumor types has increased from 100% to 500% when control rat results from 2-year studies (rats 110–116 weeks of age) were compared to those from life-span studies (140–146 weeks of age). If such an increase in age (25%) can result in such extreme increases in spontaneous tumors, what is the effect on interpretation of incidence rates seen in concurrent treatment groups? This is especially the case if, as Salsburg (1980) has suggested, any biologically active treatment will result in a shift in the patterns of neoplastic lesions occurring in aging animals. The current practice is to interpret tumor incidence on an independent site-by-site basis (on the assumption that what happens at each tissue site is independent of what happens elsewhere), and no allowance or factoring is made for the fact that what might be occurring in animals over their life span (as expressed by tumor incidence levels at an advanced age) is merely a shifting of patterns from one tumor site to another. In other words, commonly the "significantly" increased incidence of liver tumors is focused on, whereas the just as statistically significant decrease in kidney tumors compared to controls is ignored. Clearly, we should not be trying to analyze tumor data from rats that are advancing into senescence in the same manner that we do the data from those that lack these confounding factors. Where should a cutoff point be? This is a problem, but clearly Cameron's data (Cameron et al. 1985) suggest that the growth curves of 9,385 B6C3F1 mice and 10,023 F344 rats from control groups in NCI/NTP studies show consistent patterns of decline in body weights from these animals starting at the ages (in weeks) shown in table 3.21.

Table 3.21 Ages (in Weeks) at Which Body Weights Begin to Decline

	Males	Females
B6C3F1 mice	96	101
Fischer 344 rats	91	106
Sprague-Dawley rats	112	104

The existence of similar data for tumor incidences (unfortunately not available from NCI/NTP studies) would certainly improve our confidence in selecting cutoff points for age, but ages given in table 3.21 merit consideration as termination points.

Patterns of Lesions

The patterns of lesions are equally as important as the rates and incidences at which they occur, as these can also be altered by treatment and might provide essential insight into the causal relationship. Such patterns can be altered without any change in survival rates.

In rats, the patterns of neoplasia associated with deaths on carcinogenicity studies are sex specific. Approximately 80% of unscheduled deaths in females are attributable to tumors in two organs, the pituitary and mammary gland, and this pattern is constant throughout the aging period. Other lethal tumors considered on an individual organ basis account for less than 5% of morbidity and mortality, and a similar case holds for various types of nonneoplastic lesions. Pituitary tumors cause illness and death because of their critical position at the base of the brain, and relatively small tumors can be fatal. Mammary tumors are not critically located. These subcutaneous tumors can exceed 100 g without causing any apparent ill health unless they ulcerate or impede the animal's movement.

The patterns of mortality associated with neoplastic lesions in males are more diverse. Two main tumor types, pituitary tumors and fibrous tumors of the subcutis, account for many losses, but the pattern is not as prevalent in females, in which several other tumor types occur. The incidence of these other lethal tumors tends to increase with time, but rarely exceeds 5% if they are considered on an individual tissue basis. Two tumors predominate, liver tumors and various endocrine tumors, accompanied with time by a slowly increasing range of low-frequency tumor types such as bone tumors.

About one-third of unscheduled necropsies in males are not associated with any large, ulcerated, or critically situated neoplasms, and morbidity and mortality in these cases has to be attributed to nonneoplastic causes. In many cases, there might be no major morphological lesion to account for illness, or there might be two or more conditions of equivalent biological impact and the cause has to be stated as uncertain or multifactorial. In other cases, there might be major degenerative lesions in the kidney, nervous system, or heart or inflammation in the skin, appendages, or genitourinary tract, and these together with other obvious lesions can be attributed unequivocally as the cause of the animal's demise. The pattern of these nonneoplastic causes of death has a reasonable constancy with time, except that the uncertainty tends to decrease as the major degenerations associated with old age become more prominent. In some strains, particularly those fed high-protein diets ad libitum, kidney disease can be the major cause of morbidity and mortality in males.

Analysis of the incidence and causes of morbidity and mortality is not a common practice in long-term studies, but has much to commend it in assessing the biological significance of lesions. Changes in mortality rate or in the major patterns of causation are easily detected and are more likely to be relevant in risk assessment than any minor increases in the incidence of microscopic or small tumors found in groups of animals dying from other causes. The survival pattern in most strains of rat also suggests that assay sensitivity is unlikely to be increased by extending a study beyond 2 years. As already pointed out, 2 years is approximately the 50% survival point and the patterns of causation after this point are relatively constant. These patterns are dominated by three or four major entities, and the sudden appearance of a biologically and statistically significant increase in other lesions in the remaining rats could be difficult to detect. Similar considerations apply to many other strains of rat. The majority of morbidity and mortality is usually dominated by a small number of nonneoplastic and neoplastic conditions throughout the aging period.

The common morphological lesions can be grouped into degenerative, inflammatory, and proliferative lesions, the later overlapping to some extent with neoplasms (table 3.22). Most other lesions reported in aging control rats are of low incidence and of minor biological significance, and are not discussed. The biological significance of a lesion is equally as important as frequency and the common lesions in table 3.22 can be divided into two main groups on this basis. For example, degenerations of the kidney and nervous system can be lethal, whereas those in the liver and reproductive organs are usually incidental findings at necropsy. The main emphasis in this section is on the clinically significant lesions and on lesions that cause problems in data analysis.

Nonneoplastic Lesions

Nonneoplastic proliferations are a particular problem in analysis. The main use of long-term studies in rats is as a model bioassay for carcinogenicity. Indeed there is a shift so that the rat might

Table 3.22 Common Nonneoplastic Lesions in Aging Sprague-Dawley Rats

Morphological Lesion	Clinical Condition
Degeneration	
Kidney	Glomerulonephropathy
Nerve	Radiculoneuropathy
Testis	Atrophy
Ovary	Atrophy/cyst
Liver	Steatosis
	Microcystic degeneration
	Telangiectasis
Inflammation	
Foot	Pododermatitis/arthritis
Tail	Dermatitis/folliculitis
Pancreas	Pancreatitis
Proliferation	
Liver	Biliary proliferation
	Altered cell foci/nodules
Adrenal	Altered cell foci/nodules
Mammary	Hyperplasia

be the only species utilized for lifetime oncogenicity bioassays for many compounds. Nonneoplastic proliferations are seldom of clinical significance, but are extremely important because of the diagnostic difficulties they create in differentiation from preneoplastic lesions and from neoplasms. For example, spontaneous liver tumors are rare, but the liver is a common target organ of carcinogens and, therefore, spontaneous proliferations are important because they mimic preneoplastic changes. However, the presence or absence of preneoplastic proliferative lesions associated with tumors in the same organ can be important in evaluating the carcinogenic potential of xenobiotics. In contrast, endocrine proliferations and tumors are common in rats, and the choice of criteria to differentiate nonneoplastic and neoplastic proliferations can profoundly alter the reported incidence of endocrine tumors in various laboratories. Johnson, in the initial section of this chapter, presents a current overview of tumor incidence rates found in rats in oncogenicity studies.

Kidney

Glomerulonephropathy. Of the degenerations, glomerulonephropathy is probably the major nonneoplastic condition in the aging rat of all strains (Gray 1977), in that it accounts for a significant proportion of morbidity and mortality. The inbred Lewis rat appears to be less susceptible and the inbred Fischer 344 rat more susceptible to the development of chronic nephrosis than many random-bred rat strains (Bolton et al. 1976). Males are more susceptible than females. The severity of renal disease can be profoundly influenced by factors such as genotype and diet, and its presence hinders evaluation of chronic nephrotoxicity.

Glomerulonephropathy has numerous synonyms, including nephropathy, nephrosis, and glomerulonephrosis; theories regarding its pathogenesis are equally numerous. The incidence and severity of disease are greater in males than in females. In advanced cases, the kidneys are enlarged, tan, and irregular in shape at necropsy and the cut surface in severe cases can be grossly cystic. Histologically, both glomerular and tubular changes are present, but the latter are dominant. Some tubules are shrunken with thickened basement membranes accompanied by variable degrees of interstitial fibrosis. Other tubules contain hyaline casts or form large cysts filled with proteinaceous material. Glomerular changes are relatively inconspicuous and are characterized mainly by varying degrees of sclerosis and cystic dilation of Bowman's space. Such severe cases are unusual in 2-year-old rats, and in most instances 50% or more of the kidney can appear reasonably normal.

The kidneys play a major role in fluid and electrolyte homeostasis and are important in the excretion of waste products. However, there is considerable reserve capacity and a rat could survive for an extended period with severely damaged kidneys before dying of renal failure. Because the kidneys play a central role in fluid and electrolyte balance, advanced cases might lead to secondary patterns of pathology (syndromes) in other organs. The two main syndromes affect the cardiovascular system and calcium/phosphorus homeostasis.

Cardiovascular Disease. The kidney receives 25% of the cardiac output and is intimately concerned with blood pressure regulation through the renin-angiotensin system. It is not surprising, therefore, that severe renal disease sometimes results in secondary cardiovascular disease. Myocardial fibrosis, especially in the left ventricle, is a common complication of severe renal disease, and the more advanced cases might show left atrial thrombosis or arteritis. Arteritis might be seen in one or more vessels, including renal vessels, but is not commonly recorded in the pancreatic, mesenteric, and testicular arteries. Advanced involvement of mesenteric vessels can be seen at necropsy as blue tortuous or nodular vessels in the mesentery around the duodenum and pancreas. Histologically the affected vessels show varying degrees of thrombosis, aneurysmal dilation, fibrinoid necrosis, and leukocyte infiltration.

Mineralization Syndrome. The mineralization syndrome is related to the kidney's role in calcium and phosphorus homeostasis via control of tubular resorption, and also via synthesis of the 1,25-dihydroxy derivative of vitamin D3. The pathogenesis of the syndrome is incompletely understood, but renal impairment results in hyperphosphatemia and acidosis. Calcium phosphorus imbalance stimulates parathyroid hyperplasia and there is increased bone resorption in an attempt to correct the hypocalcemia. Severe cases can be identified at necropsy. The parathyroids are grossly enlarged, the bones are thin and brittle, and the aorta is dilated and rigid owing to mineral deposited in the media. This mineralization affects many tissues histologically, and is known as metastatic mineralization in contrast to the focal dystrophic mineralization (nephrocalcinosis) that is a common finding in the young rat.

Nerve

Radiculoneuropathy. Another major degenerative lesion in the aging rat is radiculoneuropathy. This disease might appear clinically as minor ataxia or as hind limb paralysis with urinary incontinence in severe cases. The lesion is predominant in males. Histologically, degeneration is seen in the posterior spinal cord (myelopathy), posterior nerve roots (radiculoneuropathy), and sciatic nerve (peripheral neuropathy) and results in secondary changes in the hind limb muscles (atrophy). Paralysis and urinary incontinence can predispose to infection of the urogenital tract, resulting in inflammation of the prostate (prostatitis), bladder (cystitis), or kidney (pyelonephritis). Inflammation in these organs can be severe and fatal in contrast to the minor lesions in young animals, and illustrate the importance of qualifying terms in diagnoses.

Histological evidence of degeneration of myelin sheaths include vacuoles and avoids within the sheaths, associated with foamy macrophages or in severe cases, cholesterol deposits (clefts) and astrocytic, Schwann cell, or proliferation of fibroblasts.

Radiculoneuropathy is very common in aging males, but it is usually asymptomatic. In contrast, there is a less frequent degeneration of the central nervous system, which can produce obvious neurological symptoms, and the animal has to be removed from the study. This condition, termed encephalopathy, or more descriptively spongiform encephalopathy, consists of a multifocal vacuolation of the gray and white matter, most notable histologically in the cerebral cortex. Minor cases can be easily masked by artifacts, but the brain of old rats showing acute neurological symptoms in the absence of pituitary or brain tumors should be examined carefully for this condition.

Inflammations of biological significance are uncommon in aging rats unless they suffer from enzootic diseases such as mycoplasmosis. Prostatitis and other urogenital tract lesions are occasionally seen in animals with hind limb paralysis, but apart from these, lesions of the hind feet are the only common inflammations likely to make a significant impact on the health of the rat. Foot lesions are basically husbandry related and comprise two entities, pododermatitis and arthritis. Rats are commonly housed on grid or mesh floors and males may reach 1 kg body weight on ad libitum feeding. It is not surprising, therefore, that this combination of high body weight and mesh floors occasionally results in lesions of the feet, particularly in the heavier males. Up to 5% of aging males could be removed from a study because of these foot lesions.

Foot

Pododermatitis. Pododermatitis is a local inflammation of the skin of the foot. It begins initially as a wartlike growth or callus on the foot pad due to constant pressure, and subsequent proliferation of the skin. However, infection, ulceration, and bleeding might subsequently occur. Histologically, the lesion consists of inflamed granulation tissue variably covered by a hyperplastic squamous epithelium.

Arthritis. Arthritis is inflammation of the joint and surrounding tissues. Severe cases appear clinically as grossly swollen, firm, blue feet and hocks and can significantly impair the animal's mobility. The initial lesion is a periarthritis or tenosynovitis, resulting in marked periarticular edema with fibrosis and mononuclear leukocyte infiltration. The metatarsal joint ultimately becomes affected, and in some cases the persistent chronic inflammation can incite a dramatic reactive bony proliferation that results in ankylosis of the involved joint.

Liver

The liver is such a frequent target organ in toxicity studies (in fact, the most common) that a discussion of some of the more common lesions that occur with aging in the rat seems warranted. More than half of aging male Fischer and Sprague-Dawley rats have nonneoplastic hepatic lesions such as bile ductule hyperplasia and focal chronic hepatitis (Cohen et al. 1978). The NTP has its own systemic nomenclature specifically for rat hepatoproliferative lesions (Maronpot et al. 1986).

Steatosis. Steatosis in the liver is a very general term unless qualified further, and includes both focal and diffuse change, the latter being either centrilobular or periportal. The appearance of fat droplets is equally variable and can be either macrocytic or microcytic. The pathogenesis of steatosis might be equally diverse and includes dysfunction in the hepatocyte or imbalance in general lipid homeostatic. The latter is probably more common in aging rats either because of obesity due to ad libitum feeding or due to lipid mobilization in debilitated or clinically ill animals secondary to pituitary tumors or other debilitating lesions. In these cases, diffuse periportal steatosis is common.

Microcystic Degeneration. Microcystic degeneration occurs in the liver, and is also known as spongiosa hepatica. It might be visible at necropsy, but is usually a histological finding characterized by groups of thin-walled cysts containing pale-pink proteinaceous material and occasionally a few erythrocytes. The microcysts are thought to be derived from the fat-storing (Ito) cell in the liver. They are found mainly in males.

Telangiectasis. Telangiectasis is another common liver lesion. It is a vascular lesion, but is probably secondary to or associated with atrophy of the hepatic cords. It is most obvious at necropsy as depressed red foci on the surface of the liver and is particularly conspicuous when the liver is

slightly yellow due to steatosis. Histologically, these foci are groups of dilated sinusoids usually located below the liver capsule. Telangiectasis is also very common in the adrenal cortex.

Biliary Proliferations. Focal proliferations of the bile ducts are common in the liver. They are generally minor histological observations. Biliary proliferation (bile duct hyperplasia) consists of clusters or chains of bile duct-like formations lined by cuboidal or flattened epithelium. The proliferations are generally in the vicinity of the portal triad and can show varying degrees of basement membrane thickening or fibrosis. These structures do not progress to neoplasia, but the more basophilic cellular lesions could possibly mimic early-phase oval cell (ductular) hyperplasia, which is a response sometimes observed in the liver of rats fed carcinogens.

Foci of Cellular Alteration. Foci of cellular alteration, a controversial entity (from an interpretive viewpoint), are not uncommon in the liver of aging rats. It remains unclear whether foci of cellular alteration are preneoplastic or "progress" to adenomas. The focal proliferations consist of groups of hepatocytes that stand out from the normal liver parenchyma because of their arrangement, size, or tinctorial properties. They also demonstrate a variety of biochemical and functional properties such as the inability to store iron and increased rglutamyl-transpeptidase activity, which can be utilized to distinguish foci of alteration from normal hepatocytes. However, not all foci of cellular alteration demonstrate consistent biochemical or functional properties in a given animal.

In routine H&E sections, foci are usually classified into vacuolated (clear), acidophilic, basophilic, or mixed cell foci. Clear X11 foci are characterized by "empty" cytoplasm representing the space occupied by glycogen or occasionally fat. The cells are usually larger than the surrounding hepatocytes. An increased amount of acidophilic cytoplasm is the characteristic feature of acidophilic foci, and the hepatocytes can also have an enlarged nucleus with a prominent nucleolus. In contrast, the hepatocytes forming basophilic foci are smaller than normal and the cytoplasm contains prominent clumps of basophilic granules. Mixed foci, as the name suggests, contain hepatocytes of two or more of the previous types. Foci exceeding the size of a hepatic lobule are sometimes called areas of cellular alteration, and ones larger still that compress the surrounding parenchyma can be referred to as nodules. Basophilic foci are the most common and are encountered mainly in aging females, whereas most in males tend to be acidophilic foci. Overall, these altered cell foci and areas are uncommon in most strains of rat and do not significantly mask the interpretation of hepatocarcinogenic effects (Maronpot et al. 1986).

Hepatic cysts are, however, a common aging change in many rat strains. The lesion consists of cysts of different sizes that are lined by cuboidal or flat, endothelial-like cells. A moderate amount of connective tissue often surrounds the cysts. In the brown Norway rat these lesions are especially common where large multilocular cysts often bulge from the hepatic surface (Squire and Levitt 1975). Occasionally, large foci of endothelial-lined spaces containing eosinophilic amorphous material are found. The pathogenesis of these lesions is not clear, but they might represent a sequela to hepatic necrosis.

Adrenals

Altered Cell Foci. In contrast to the liver, altered cell foci areas and nodules are very common in the adrenal cortex, particularly in females. As in the liver, the lesions can be classified on the basis of staining characteristics into vacuolated, acidophilic, basophilic, and so on, although there are subclasses of each type.

Finely vacuolated foci are found in both sexes and usually affect the zona glomerulosa and other zona fasciculata. Coarsely vacuolated foci are most common in males and usually lie in the central zona fasciculata. The small acidophilic cell focus (sometimes called hyperplastic focus) is often multiple and in the outer zona fasciculata. The lesion of most concern is the large acidophilic

cell focus. These proliferations are common in aging females and can grow to grossly visible nodules of debatable diagnostic classification.

The proliferation of hyperbasophilic pheochromocytes in the adrenal medulla is an example of classification in the other direction. In this case, there is a reasonable continuity in both cytology and incidence to suggest progression from small basophilic foci to large metastasizing proliferations in rats nearing the end of their life span. Similar continuities are seen in the anterior pituitary and in the C-cell population of the thyroid. In all these cases, foci might be considered adenomas even though they are small, noncompressing, and with infrequent mitoses. There is no easy answer to these diagnostic problems, but they are often at the center of debate regarding the conclusions from carcinogenicity studies. It cannot be stressed too frequently that toxicologists and statisticians should appreciate the level of uncertainty that surrounds the classification of microscopic proliferations as hyperplasias or as neoplasias and evaluate the data accordingly.

Heart

Loss and degeneration of myocardial fibers with fibrosis is a common lesion of older rats. It occurs most frequently in the wall of the left ventricle or intraventricular septum and is characterized by areas of fibrosis surrounding myocardial fibers showing loss of striation, fragmentation, and vacuolization. The lesions vary from small foci involving only a few myocardial fibers to extensive areas of fibrosis. The lesion becomes progressively more severe with age (Squire and Levitt 1975). Cartilaginous foci at the base of the aortic valve are quite common in some Sprague-Dawley strains and were once attributed to aging. However, similar cartilaginous foci have been observed in young rats (Hollander 1968).

An unusual, but not uncommon, endomyocardial lesion has been described in several strains of rats. The lesion is characterized by proliferation of undifferentiated mesenchymal cells in the subendocardium and usually involves the left ventricle. The lesion is usually sharply demarcated from the myocardium but in places extends along muscle bundles or vessels. A few lymphocytes and occasional cell debris are found in the lesion. An occasional sarcoma is associated with endomyocardial disease, but it is not known whether it represents a progression of the lesion. The lesion is definitely age associated. It is most common in rats over 30 months of age and is rarely observed in rats less than 27 months of age (Squire and Levitt 1975). Myxomatous degenerative change of heart valves occurs in a majority of old rats.

Testes

Testicular atrophy can appear as a primary condition in aging rats, but it is also secondary to large pituitary tumors and to testicular arteritis. This is an example of a condition that might appear as a single entry on an incidence summary table, but its occurrence could be due to a variety of causes. Toxicologists and statisticians should be aware of the conditions in which pooling data due to different causes is common. Atrophy of the seminiferous tubules ranges from focal unilateral to diffuse bilateral, and severe cases appear grossly as small, blue or brown, sometimes flaccid and watery gonads. Histologically, the tubules are shrunken and the seminiferous epithelium is lost, leaving only Sertoli cells. Interstitial edema is also frequent, and Leydig cell hyperplasia can occur.

Eye

Because the eye can be the target organ of toxicological studies, it is important to be aware of spontaneous lesions to avoid misinterpretation. In a study involving 400 rats up to 3 years of age, retinal lesions were found in more than one-third of the animals (Weisse et al. 1974). The lesions consisted of the loss of nuclei in the outer and inner nuclear layer of the retina, neuronal atrophy, and degeneration and thickening plus increased tortuosity of retinal capillaries. The lesions were

both age and light dependent. The authors stated that although the lighting was not unlike that in other animal facilities, 12-hr light–dark exposures to less than 200 lux m/m^2 of light resulted in retinal lesions that appeared to be directly related to the amount of light exposure. There was retinal damage in 79% of the males and 96% of the females in the top rows of cage racks; 38% of the males and 50% of the females were affected in the bottom row. In a study involving Fischer 344 rats that were exposed to less than 10 to 320 lux m/m^2 of light, there was similar light-related retinal degeneration (Lai et al. 1978). In addition, the gradual loss of photoreceptor cells in rats that were exposed to less than 10 lux m/m^2 suggested to the authors that some cell loss is an age-related change. Peripheral retinal degeneration was unrelated to light intensity or severity of photoreceptor cell loss in other parts of the retina. Both studies indicate that age-related and light-associated retinal lesions are common and must be considered in long-term toxicological studies.

Skin and Appendages

The most common inflammatory lesions of the skin and appendages are inflammation of the hair follicle (folliculitis) in the tail. Nodules or postules are frequent along the tail and are primarily a logistic problem related to GLP rather than a clinical problem. Theoretically, a tail nodule could be a tumor, and occasionally this is the case, but the vast majority are various stages of suppurative folliculitis. Many long-term study protocols state that all gross lesions will be examined histologically and a histopathologist will rapidly become an expert on rat tails (and ears or feet) if this requirement is adhered to strictly. These appendigeal lesions could reasonably be treated in the same way as erect fur or fur loss in the skin, or as roundworms and tapeworms in the intestine. Mechanical abrasion from feeders is another possible cause. The clinical and necropsy data can be regarded as a definitive diagnosis in the vast majority of cases without the need to resort to histopathology.

Two other lesions of the integument pose similar problems. These are small nodules in the subcutis of the preputial region or on the back of males. These can be faithfully recorded week after week during the in-life phase of the study as palpable masses that grow slowly if at all, and some might regress. These two entities are preputial abscesses and squamous (epidermoid inclusion) cysts, respectively. Repeated palpation could aggravate and rupture the lesions and necessitate removal of the animal from the study.

Pancreas

Another minor inflammatory lesion affects the pancreas. The histological appearance of this lesion is quite characteristic, but its status as a degenerative lesion (atrophy, microductular change) or postinflammatory lesion (adenitis, pancreatitis) is ill defined. There is focal loss of acinar epithelial cells, producing a ductular structure often accompanied by a mild interstitial inflammatory response. The lesion might be multifocal, but most of the exocrine pancreas is normal.

Neoplasia

Rats, like mice, develop a wide variety of tumors. Some of the factors known to affect tumor incidence include age at time of necropsy, strain of rat, sex, diet, and diligence with which tumors are sought. Careful macroscopic examination of the animal at necropsy plus multiple histological sections will result in a higher tumor incidence, especially of the smaller tumors. In this review, only some of the more common or controversial tumors are discussed.

Interpretation and Classification of Tumors

The two main criteria used in the classification of proliferations as tumors are morphology and the probability of progression. Morphological criteria include atypical cytology and organization.

Nuclear cytoplasmic ratio, tinctorial properties, anaplasia, and mitotic rate are the main cytological characteristics that distinguish neoplastic cells from normal cells. Organizational atypia includes abnormal growth patterns, compression, or invasion, and abnormal relationships between proliferating cells and blood vessels or other mesenchymal elements. These patterns serve to differentiate neoplasms from nonneoplastic proliferations.

Probability criteria (which incorporate both severity and incidence data) are mathematical assessments of the degree of association between small lesions of debatable classification and large clear-cut neoplasms. Crudely stated, if large, lethal masses are common in an organ, then common microscopic lesions are probably microscopic tumors or at least precursor lesions with a high probability of neoplastic transformation. On the other hand, if microscopic foci are common and gross tumors are rare in old animals, there is a low probability of any biologically significant degree of progression suggestive of neoplastic transformation. Pathologists use all of these criteria in assessing proliferations, but with different degrees of emphasis. This results in highly variable incidence data for certain types of proliferative lesions. The reviewing toxicologist must become familiar with the main problem areas in a given study and evaluate the data in the perspective that application of diagnostic criteria might influence the incidence rates.

If the preceding criteria are applied to the large acidophil proliferations in the adrenal cortex, then on a morphological basis large compressing nodules of cytologically distinct cells, sometimes with frequent mitoses, are common in the female adrenal. On this basis, there could be a 40% or more incidence of cortical tumors in some rat strains. Alternatively, the probable fate of many of these proliferations is to undergo vacuolar degeneration, resulting in a large blood-filled cyst that might thrombose. Further evidence against a neoplastic diagnosis is that large undoubted cortical neoplasms are often composed of small acidophilic cells cytologically distinct from the hypertrophied acidophilic cell of the commonly occurnng nodules. Thus, if diagnosis is based on this cytological discontinuity and the high incidence of degeneration, these nodules would be regarded as hyperplastic rather than neoplastic.

The incidence and types of neoplasia are usually the patterns of pathology of most concern in long-term studies. The pattern of common tumors in the Fischer 344 was presented in detail in table 3.23, and this is reasonably representative of other strains (except that more mammary tumors—up to a 55% incidence—are seen in Sprague-Dawleys). The pattern is dominated by subcutaneous connective tissue tumors, mammary proliferations, and endocrine tumors. Other tumor types occur at incidence rates of less than 10% and in most cases rarely exceed 3%.

The rat represents the most commonly employed animal model in toxicological research and testing. As all other animal species, it has certain background incidences of lesions (particularly of neoplastic lesions) that are characteristic of it and that could confound interpretation of results. These vary from strain to strain, but there are general patterns for the species. Table 3.23 presents a summary of findings of tumor incidences in large groups of control Fischer 344 rats. These incidences show, in some cases, great degrees of variability. Haseman et al. (1989) discussed the major reasons behind such variability.

Pituitary

In several strains, one of the most prevalent spontaneous neoplastic alterations is the pituitary adenoma. Female Fischer 344 rats have been found to develop such tumors at a 30% rate by the time they were 110 weeks old in an investigation involving a total of 1,754 females (Goodman et al. 1979), and most of the pituitary tumors were described as chromophobe adenomas. Carcinomas, on the other hand, consist of cells with anaplastic features or show invasion. Pituitary adenomas have also been observed frequently in other strains such as the Crl:CD(SD)BR strain (Cohen et al. 1978). Females appear to develop pituitary adenomas more often than males, although the incidence of such tumors in male F344 rats has been reported to be 14.7% (Coleman et al. 1977). In Wistar-derived SAG/Rij female rats, adenomas of the pituitary gland were found in 69% out of a total of

Table 3.23 Reported Background Tumor Incidences in Fischer 344 Rats

	Chu (1977)		Fears et al. (1977); Page (1977); Gart et al. (1979)		Goodman et al.[a] (1979)		Chu et al. (1981)		Chandra and Frith (1992)	
Organ/Tissue	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Brain	0.9	0.6	1.3	<0	8.1	0.55	.8	.06	0.3	0.1
Skin/subcutaneous	6.6	3.2	5.7	2.5	6.4	3.0	7.8	3.2	8.2	1.9
Mammary gland	1.4	17.9	0	18.8	1.54	8.5	1.5	20.9	0.5	14.6
Circulatory system	0.4	0.5	<1	<1	3.8	0.27	0.7	0.4	0.4	
Lung/trachea	3.1	1.8	2.4	<1	2.9	2.0	3.0	1.9		0.3
Heart	0.3	0.1	<1	<1	0.2	0.05				
Liver	1.8	3.1	1.2	1.3	1.74	3.9	2.2	1.9	0.9	0.3
Pancreas	0.2	—	<1	<0	0.16	0	0.2	—	6.5	0.1
Stomach	0.3	0.2	<1	<1	0.32	0.2	0.3	0.2		
Intestines	0.3	0.5	<1	<1	0.31	0.36	0.6	0.3		
Kidney	0.4	0.2	<1	<1	0.38	0.16	0.5	0.2	0.8	0.3
Urinary/bladder	0.1	0.2	<1	<1	0.1	0.22	0.1	0.3		0.1
Preputial gland	1.4	1.2	—	—	1.4	1.2	2.4	1.8		
Testis	80.6	N/A	76.2	N/A	80.1	N/A	2.3	N/A	79.5	
Ovary	N/A	0.3	N/A	<1	N/A	0.33	N/A	0.4		0.3
Uterus	N/A	15.6	N/A	16.8	N/A	5.55	N/A	17		14.1
Pituitary	11.5	30.5	10.2	29.5	11.4	0.3	4.7	34.9	70.5	30.3
Adrenal	10.0	4.6	8.7	4.0	9.95	4.58	2.4	5.2	7.3	1.2
Thyroid	7.1	6.5	5.1	5.6	7.16	6.65	8.2	6.8	7.6	6.3
Pancreatic islets	0.8	1.0	3.2	1.3	3.89	1.05	3.9	0.8	6.5	0.1
Body cavities	1.1	0.3	<1	<1	2.51	0.38	2.6	0.4		
Leukemia/lymphoma	11.7	9.1	6.5	5.4	12.3	9.9	9.9	13.4	30.5	20.5
N	1,806	1,765	846	840	1,794	1,754	[b]	[b]	740	740

[a] Gives detailed breakdown of neoplastic and nonneoplastic lesions in aged animals.

[b] Range of averages, six different laboratories.

290 animals that had an average life span of 31 months (Boorman and Hollander 1973). The incidences of spontaneous pituitary tumors in various rat strains were reviewed by Carlton and Gries (1983).

Mammary Gland

Another frequently observed spontaneous neoplasm in the rat is the mammary tumor, the incidence of which varies from 10% to 40% depending on the strain and the age of the animals. Whereas in WAG/Rij rats, pituitary tumors appeared to be the most usual cause of death in animals over 1 year of age, mammary tumors tended to develop later in life and were most often seen in animals that had survived for 2 years (Boorman and Hollander 1973). Moreover, 68% of such rats with mammary tumors simultaneously revealed adenomas of the pituitary gland. Histologically, fibroadenomas and adenocarcinomas represent the most common varieties of benign and malignant mammary tumors.

Lymphoreticular System

Spontaneous tumors of the lymphoreticular system are fairly rare in rats, although a 25% incidence has been reported in Wistar-Furth and Fischer strains (Moloney et al. 1969; Moloney et al. 1970). In contrast to mice, such neoplasms have not been studied widely and are, therefore, not

well classified. They usually progress with secondary involvement of the spleen, liver, lung, and often renal adipose tissue. Generally, tumors of the lymphoid system are not common, but a 25% incidence has been reported in Wistar and Fischer rats (Squire and Goodman 1978). Lymphoreticular cell tumors have not been widely studied and are not well classified. They usually involve the spleen with secondary involvement of liver, lung, and often the renal adipose tissue. Large granular lymphocyte leukemia (previously called mononuclear cell leukemia) is the most common "natural" cause of death in Fischer 344 rats used in chronic toxicity and carcinogenicity studies. From 30% to 50% of aging Fischer 344 rats in control populations die from the primary and secondary effects of this leukemia between 14 and 30 months of age (Losco and Ward 1984).

Thyroid

After many strains have reached 2 years of age, neoplasms of the thyroid gland occur spontaneously at high frequencies. Again in WAG/Rij females, such tumors were found in 40% of 290 examined animals (Boorman and Hollander 1973). In this study, electron microscopy revealed the cells of origin to be parafollicular.

Testes

Spontaneous testicular tumors are not common in the majority of rat strains (Sertoli cell tumors are especially rare in rats). However, a 100% incidence of interstitial (Leydig cell) tumors occurs in male Fischer rats by the time they are 30 months of age. Table 3.23 presents a summary of neoplastic lesions incidences in this strain (the most commonly used in long-term toxicology studies) as reported by various investigators.

Skin/Subcutaneous Tissues

Large subcutaneous masses are a common finding in aging rats and can exceed 100 g in weight. In males, a variety of connective tissue tumors occurs, but they are usually fatty or fibrous, the latter predominating.

Fatty tumors (lipomas) are seen at necropsy as large, smooth, soft, glistening masses and are most easily defined in debilitated animals when the normal subcutaneous adipose tissue is depleted. In obese males, the distinction between small lipomas and large fat deposits is not clear cut. Histologically, the lipoma consists of mature lipocytes, sometimes with small bands of fibrosis.

Large Fibroma. Large, well-differentiated fibromas are the predominant subcutaneous connective tissue in males. Large tumors can impede movement or become ulcerated and the animal has to be removed from the study. They appear grossly as well circumscribed, firm, multinodular masses with a variable appearance on cut surface ranging from uniform white to a mosaic of white, cream-pink, and red areas. The histological appearance can vary widely within different areas of the same tumor. Fibroblasts are elongated cells producing collagen and ground substance and the histological appearance of tumors depends on the arrangement of the cells and the relative proportion of cells and extracellular material. Most tumors contain abundant collagen, but in some areas ground substance might predominate, producing a myxomatous appearance. The more cellular areas might suggest malignant transformation, but the fate of these cells appears to be differentiation rather than progression to fibrosarcoma, as cellular areas are common and metastases from these large subcutaneous masses are virtually nonexistent. Fibrosarcomas do occur, but are not common. Their characteristic histological feature is basophilia due to uniform hypercellularity in contrast to the largely eosinophilic fibroma. Mitotic figures are frequent and some tumors contain bizarre giant cells and multinucleate cells.

Dermal Fibroma. Another frequently encountered, but generally small mass, is the dermal fibroma. This is a distinct entity composed of an irregular mate of coarse collagen fibers similar to those of the normal dermis. Larger nodules extend into the subcutis. Both small and large nodules can contain sufficient adipose tissue to justify the combined diagnosis fibrolipoma. The relationship of this tumor to the large subcutaneous fibroma is uncertain, but it is more likely a separate entity rather than a precursor lesion.

Subcutaneous masses are more common in females than in males. They are frequently multiple and usually mammary in origin. The gross and histological appearance of these tumors is highly variable, but the majority are variants of a single entity; that is, mammary fibroadenoma. The rat has six pairs of mammary glands consisting of milk-secreting epithelium and supporting or contractile stroma. Both epithelial and stromal elements proliferate, hence the diagnosis of fibroadenoma. The degree of proliferation often varies in different parts of the same tumor, resulting in areas that are predominantly fibrous, predominantly epithelial, or mixed fibroepithelial. Diagnostic terms such as adenofibroma or fibroadenoma might be used to reflect the relative proportions of each component, but this division is probably unnecessary. Purely fibrous tumors might be impossible to distinguish from subcutaneous fibromas on one single section, but glandular formations can be found if multiple samples of the mass are examined. For statistical classification it is reasonable to consider fibrous tumors in females as a variant of mammary fibroadenoma. Subcutaneous masses are the tumors most frequently seen as "palpable masses" in the in-life phase of long-term studies, but endocrine tumors are just as frequent in the final pathology phase. This group is dominated by the pituitary adenoma both in incidence and biological significance. Pituitary tumors are frequently visible at necropsy and are a common cause of morbidity and mortality. Thyroid C-cell tumors and adrenal pheochromocytomas are less common and frequently microscopic entities.

Endocrine System

Pituitary Adenoma. The anterior pituitary secretes several hormones and pituicytes fall into three main groups, acidophilic, basophilic, and chromophobe depending on the tinctorial properties of the cytoplasm. In the past, attempts were made to use tinctorial classifications in the diagnosis of pituitary tumors, but this has largely been abandoned in favor of the nonspecific diagnosis of pituitary adenoma or pituitary tumor. More sophisticated investigational techniques such as ultrastructure, immunocytochemistry, and hormone assay suggest that the majority of tumors secrete prolactin and the term prolactinoma is sometimes used. However, in routine H&E sections it is impossible to delineate functional properties and it is inappropriate to use specific terms without any evidence for the functional status of the tumor. Pituitary adenomas are the most common tumors in several strains of laboratory rats (Boorman and Hollander 1973; Cohen et al. 1978; Squire and Levitt 1975). Pituitary tumors range in size from microscopic to macroscopic, raising the question of hyperplasia versus neoplasia. Because large lethal tumors 10 mm or more in diameter are common in this strain, the equally common microscopic lesions of similar cytology but just smaller in terms of size can reasonably be considered in the spectrum of adenomas. The microscopic appearance varies. In females, the cells are generally small to medium in size with relatively little cytoplasm (chromophobes). Dilated vascular channels are frequent and hemosiderin pigment is sometimes found. In males the cytology is much more diverse and bizarre pale eosinophilic cells are often seen. Microscopic tumors might be multicentric, and the larger tumors are nodular masses compressing the brain, often causing hydrocephalus. Most tumors are considered benign even though the cytology might be bizarre. Invasion of the meninges and along vascular channels into the brain is occasionally seen, but metastases are extremely rare.

The large tumors are space occupying and often functional, and frequently result in other histological lesions. In females, acinar hyperplasia of the mammary gland and ovarian atrophy are commonly associated with pituitary neoplasms. Testicular atrophy can occur in males. Other

components of the pituitary syndrome are splenic atrophy with hemosiderosis, squamous hyperplasia of the forestomach, and steatosis in the liver. The clinical syndrome of a thin, neurologically abnormal rat with red tear stains around the eyes is almost pathognomonic of large pituitary tumors.

Thyroid Tumors. Naturally occurring thyroid neoplasms occur with high frequency in many strains of rats, particularly after 2 years of age. These tumors have been shown to originate from C or parafollicular cells and to produce calcitonin (Boorman et al. 1972; Deftos et al. 1976; DeLellis et al. 1979). The lesions begin as diffuse or nodular hyperplasia of C cells. When the cells extend through the basement membrane or have distant metastases, they are called medullary thyroid carcinomas because they are similar to this tumor in humans. Unless one is aware of these tumors they are easily missed because they are often microscopic. They tend to metastasize first to the deep cervical lymph nodes. Their malignant nature is more easily assessed if the lymph nodes are examined microscopically.

The thyroid C-cell proliferations are slightly more of a diagnostic problem than those in the pituitary. A minor degree of diffuse proliferation is common and is usually termed hyperplasia. Focal proliferations range from single perifollicular aggregates to grossly visible masses. Terminology varies, but proliferations occupying large areas of the thyroid are common and the smaller foci could reasonably be considered part of the adenoma spectrum. However, other diagnoses used range from nodular hyperplasia to microscopic carcinoma depending on the diagnostic criteria applied by different pathologists. The cells usually form large, pale acidophilic nests of round cells, compressing adjacent thyroid follicles. The larger tumors tend to incite fibrous encapsulation and occasionally show focal invasion of the capsule, surrounding tissues, or metastasis to the cervical nodes. These large masses are designated carcinomas, although the cytology and mitotic rate might not markedly differ from the smaller proliferations; hence the tendency for some pathologists to refer to all proliferations as carcinoma.

It has been reported that a variety of chemical carcinogens can also induce zymbal gland tumors in the rat (Ward 1975). Historically, finding tumors at this site in NCI studies performed in the early 1970s has led to inclusion of the tissue on standard necropsy collection lists.

Recent years have seen the availability of some wonderous comparative and descriptive resources for researching tissue-specific pathological lesions (both neoplastic and otherwise) in the rat. Greaves and Faccini (1992), for example, provides an excellent descriptive text.

When evaluating tissues respective to prior or historical findings, however, one must be aware that baselines change over time. For example, the longevity of common strains (particularly the Sprague-Dawley) have tended to decrease since the 1980s. At the same time, the shift in some quarters to restricted (as opposed to ad libitum) feeding in longer term studies has changed control tumor incidence levels relative to past experiments.

METABOLISM

Cytochromes P-450s (CYP450 enzymes) form the single most important metabolic enzyme system in mammals, with many families and subtypes identified (table 3.24). The CYPs for rats were one of the first isolated and characterized (see table 3.25). In fact, one of the oldest known CYP enzymes is rat form $P450_d$, which has been shown to be similar to human counterparts. It is now named CYP1A2 enzyme, known to be conserved throughout the species with typical substrates as aromatic structures, preferably aromatic amines, but also polycyclic aromatic hydrocarbons and other planar structures. This form is inducible by polycyclic aromatic hydrocarbons as 3-methylcholanthrene or by polychlorinated biphenyls. A similar conclusion holds for the CYP2E1, which is in all species known inducible by ethanol, acetone and metabolizes organic solvents, nitrosamines, and several drugs (e.g., paracetamol; see earlier). To conclude, the rat can serve as a readily available model for liver microsomal metabolism dependent on these two CYP forms.

Table 3.24 Cytochrome P-450s (Nelson 1999)

CYP	CYP
1A1	1A2
1B1	2A2
2A3	2B1,2
2B3	2C6
2C7	2C11
2C12	2C13
2C22	2C23
2D2	2D3
2D4, 18	2D5
2E1	2F4
2G1	2J3
3A1,2,23	3A9
3A18	4A1
4A2,3	4A8
4B1	4F1
4F4	4F5
4F6	4F19
5A1	7A18A1
11A1	11B1,3
11B2	17
19	21
24	27A1
27B1	51

Table 3.25 P-450 CYP Specific Metabolic Activities in the Rat

Activity	Sprague-Dawley Rat
7-Ethoxyresorufin *O*-dealkylation	1A1/2
7-Methoxyresorufin *O*-dealkylation	1A1/2
Caffeine 3-demethylation	1A2
Benzphetamine *N*-demethylation	2B
7-Benzoxyresorufin *O*-dealkylation	2B1/2
7-Pentoxyresorufin *O*-dealkylation	2B1/2
Coumarin 7-hydroxylation	2A3
7-Ethoxy-4-trifluoromethylcoumarin deethylation	
Ethoxycoumarin *O*-dealkylation	1A1/2, 2B1/2, 2E1
Tolbutamide methyl-hydroxylation	2B1/2, 2C6
Chlorzoxazone 6-hydroxylation	2E1, 1A1
4-Nitrophenol hydroxylation	2E1
N-Nitrosodimethylamine *N*-demethylation	
Androstenedione 15α-hydroxylation	2A2
Androstenedione 16α/β-hydroxylation	2B1
Dextromethorphan *O*-demethylation	2D1
Dextromethorphan *N*-demethylation	
Testosterone $\rightarrow$ Androstenedione*	2B1, 2C11, 2A2, 2B2, 3A1
Testosterone 2α-hydroxylation	2C11
Testosterone 2β-hydroxylation	3A1, 3A2, 1A1
Testosterone 6β-hydroxylation	3A1, 2C13, 2A2, 3A2, 1A1/2, 2C11
Testosterone 7α-hydroxylation	2A1, 2A2
Testosterone 15α-hydroxylation	
Testosterone 15β-hydroxylation	3A1
Testosterone 16α-hydroxylation	2C11, 2B1/2, 2C7, 2C13
Testosterone 16β-hydroxylation	2B1/2, 3A1
Lauric acid 11-hydroxylation	4A1-3, 2E1
Lauric acid 12-hydroxylation	4A1-3

Unfortunately, the rat is not a good model of metabolism dependent on the most important human CYP, namely, CYP3A4. The rat orthologous CYP3A1 (the main CYP3A form in the rat) is not induced by a typical CYP3A inducer rifamipiemine and, much more important, many prototypical substrates of human CYP3A enzymes as dihydropyridine calcium channel blockers (e.g., nifedipine) are not metabolized by rat CYP3A1 or by other rat CYP3A forms. The most abundant CYP subfamily of rat liver is the CYP2C, having the role of human CYP3A enzymes, supported by the fact that not only the oxidation of dihydropyridines as well as of the aflatoxin B_1, but also hydroxylations of steroids are performed by rat 2C enzymes. Another CYP enzyme important for drug metabolism, CYP2D6, quinidine, does not function well in the case of CYP2D1, but its stereoisomer quinine is a potent inhibitor in this case. Also, one of the marker substrates of CYP2D6, dextromethorphan, is metabolized specifically by another rat CYP2D enzyme, CYP2D2. There are also probably significant differences in mechanism of induction of the CYP enzymes as the rat CYP2D1 enzyme is inducible by 3-methylcholanthrene and phenobarbital; however, the human CYP2D6 is known not to be inducible and 3-methylcholanthrene induces rather human CYP1A2 and phenobarbital is a classical inducer of human CYP3A, CYP2C, and CYP2B forms (Zuber et al. 2002).

In considering xenobiotic metabolism, one should keep in mind the quantitative as well as qualitative differences between species. For example, at a low dosage (0.16 gg/kg), of benzo(a)pyrene, 1.7% will accumulate in the lungs of rats, whereas 7.9% accumulates in hamster lungs. At a high dosage, these percentages become 9.01% and 8.04% for rat and hamster, respectively (Weyland and Bevan 1987). This is an example of a quantitative species-related difference. At the low dose of benzo(a)pyrene, the predominant metabolites in all species were thioether (glutathione) conjugates. In contrast, thioethers remained dominant at a high dose of benzo(a)pyrene in guinea pigs, but there was a considerable shift toward glucuronide formation in rats. This is an example of a more qualitative species-related difference. Studying metabolism in different species does not always mean that an investigator has to sort through a morass of species-related differences. Frequently, more similarities ifn metabolism exist between species than differences. For example, Berman et al. (1984) studied the *in vitro* microsomal metabolism of a-naphthaflavone (ANF) by various species. For the most part, all species produced ANF-5, 6 oxide and ANF-dihydrodiol as the predominant metabolites. In addition, total hydrophobic (solvent extractable) metabolite formation was amazingly close between species, ranging from 2.7 to 3.2 nmol/15 min/mg microsomal protein.

As in all species, the liver in the rat is the main site of xenobiotic metabolism. In general, the liver to body weight ratio in the rat is about 2.5% to 3.2% in fasted rats and about 3.3% to 4.0% in (ad libitum) fed rats owing to the differences in glycogen content. Many of the characteristics of hepatic xenobiotic metabolism of the rat are summarized in table 3.26 and table 3.27.

Microsomal Mixed Function Oxidase (MMFO)

Native concentration of cytochrome P-450 ranges from 0.20 to 1.0 nmol/mg microsomal protein, depending on age, sex, and strain of rat examined. The rat was one of the first animals in which cytochrome P-450 was isolated and studied, and it was one of the first animals for which it was realized that cytochrome P-450 exists as a family of isozymes. As the literature in this field is extensive and complex, the reader is referred to reviews by Lu and West (1980), Nebert et al. (1981), Gonzales (1989), and Okey (1990), or the compilation assembled by Ortiz de Montellano (1986) for more details. Currently, 25 different isozymes of cytochrome P-450 have been isolated and characterized with regard to molecular weight, substrate specificity, inducing agent sensitivity, and site specificity. The isozymic nature of cytochrome P-450 serves as a fundamental explanation for many of the characteristics of the rat MMFO. For example, aromatic hydrocarbon metabolism is high, induced by 3-methylcholanthrene (3-MC) because this agent induces an isozyme (once called cytochrome P-448 in the rat; now known as cytochrome P-450c by most investigators) with high

Table 3.26 Summary of Hepatic Xenobiotic Metabolizing Enzymes in Rats

Enzyme	Concentration or Activity	Comments and References
Cytochrome P-450 (microsomal)	0.20–1.00 nmol/mg/m 10 to 40 nmol/g (est.)	Variability due to differences in sex, age, and strain of rats used. When these variables are controlled, there is generally good agreement between animals (<10% difference). Astrom et al. (1986), Souhaili-el Amri et al. (1986), Chengelis (1988a)
Cytochrome b_5 (microsomal)	0.10–0.40 nmole/mg-m 6–17 nmol/g (est.)	Differences with regard to strain, sex, and age are not as prominent as with cytochrome P-450. Astrom et al. (1986), Souhaili-el Amri et al. (1986), Chengelis (1988a)
NADPH:cytochrome P-450 Reductase (microsomal)	75-200 nmol/min/mg-m	For the more common strains. Fuller et al. (1972), Litterst et al. (1975), Souhaili-el Amri et al. (1986), Chengelis (1988)
MMFO activities		Determined under saturating conditions. Variations with age, sex, and strain. Page and Vesell (1969), Litterst et al. (1975), Astrom et al. (1986), Chengelis (1988), Koster et al. (1989)
Aminopyrene demethyl Aniline hydroxyl	2.3–10.0 nmol/min/mg-m	
P-nitroanisole demethyl	0.3–1.6 nmol/min/mg-m	
Aryl hydrocar hydroxyl	0.3–1.25 nmol/min/mg-m	
Epoxide hydrolase (with styrene oxide)		Sex-, strain-, and age-related differences. Birnbaum and Baird (1979), Astrom et al. (1986), Chengelis (1988), Kizer et al. (1985), Oesch et al. (1988)
Microsomal	2–12 nmol/min/mg-m	
Cystolic	30–46 nmol/min/mg-c	
UDP-glucuronosyl transferase		
1-naphthol	5–40 nmol/min/mg-m	Boutin et al. (1984), Galinsky et al. (1986), Astrom et al. (1987), Chengelis (1988a)
4-nitrophenol	15–30 nmol/min/mg-m	
Glutathione S-transferase		
CDNB	360–1400 nmol/min/mg-c	Gregus et al. (1985), Galinsky et al. (1986), Chengelis (1988a)
4-nitrobenzyl chloride	110–380 nmol/min/mg-c	

Table 3.27 Rat Liver Microsomes (36)

P-450 Enzyme	Specific Content (pmol/mg Protein)	Percentage of Total Spectral P-450
Total P450		
CYP4A[a]	300	3.0
CYP3A2	146	14.6
CYP2C[b]	650	65.0
CYP1A1, 1A2	12	1.2*
CYP2E1	79	7.9*
CYP2A1, 2A2	54	5.4
CYP2B1, 2B2	19	1.9

[a] Sum of CYP4A1 and 4A2.

[b] Sum of CYP2C6, 2C11, 2C12, 2C13.

* The rat is considered a good model system for man for these (Zuber et al. 2002).

affinity for aromatic hydrocarbons. Also, the well-described differences in MMFO between male and female rats are apparently due to different isozymes of cytochrome P-450 (Kamataki et al. 1985). Hence, we continually refer to the isozymic nature of rat cytochrome P-450 in the ensuing discussions on age, sex, and hormonal effects on MMFO.

The enzyme NADPH:cytochrome C reductase (also known as NADPH:cytochrome P-450 reductase) is the other main enzymic component of the MMFO. It has also been isolated, purified,

and well characterized in the rat. Unlike cytochrome P-450, it does not exist as a family of isozymes. Only one enzyme exists and different isozymes are not induced by different agents. In fact, in the rat, phenobarbital and pregnenolone-16acarbonitrile (PCN)-type inducing agents induce increases in both cytochrome P-450 and the reductase, whereas 3-MC-type agents induce cytochrome P-450, but not the reductase in rats (Lu et al. 1972). The more effective inducer of the reductase rather than of cytochrome P-450 is PCN, and phenobarbital is the better inducer of cytochrome P-450. There has been recurring debate as to whether the reductases of cytochrome P-450 are the rate-limiting step in MMFO activity, but it is generally believed the activity of the reductase is the rate-limiting step in MMFO activity (Mannering 1971). Miwa et al. (1978) reported that incorporation of previously isolated reductase into freshly prepared micromal preparations resulted in rate enhancements that depended on the substrate examined and whether the micromes were isolated from induced animals.

The activities of the MMFO with several model substrates are also summarized in table 3.28. Across rodent species, the rat does not have either the highest concentrations of cytochrome P-450 or levels of MMFO activity. This was highlighted by Kato (1979). Souhaili-el Amri et al. (1986) also noted that the male rat had less cytochrome P-450 than either the guinea pig or the male rabbit. In addition, when maximal reaction rates were compared, the rat had the highest activities with only one substrate, benzo(a)pyrene. The guinea pig had the highest activities with four of the substrates (aminopyrine, benzphetamine, p-nitroanisole, and 7-ethoxycoumarin). This can have obvious toxicological consequences, such as with the higher sensitivity of rats as opposed to other rodents to chlorofenvinphos (an organophosphate insecticide) being due to a lower rate of metabolism in the rat. One should not assume, therefore, that the rat has the highest rates of metabolism for the chemical under study. In fact, there are several instances in which a nonrodent, such as the dog (Duignan et al. 1987) has higher rates of oxidation for a substrate than the rat.

The previous paragraphs stressed quantitative differences between the rat MMFO and those of other species. There are also qualitative differences. The rat has a high tendency to hydroxylate aromatic structures (e.g., benzene to phenol). The rat has a considerable ability to produce "phenolic" metabolites from chemicals like benzo(a)pyrene and aniline. Take the often-cited example of the metabolism of amphetamine. The major pathway in the rat is the formation of 4-hydroxyamphetamine, whereas in other species the major pathway is through oxidative deamination to benzoic acid derivatives. There are exceptions. The rat, for example, lacks the ability to metabolize warfarin to 7-hydroxywarfarin as other species do. Also, unlike many other species, the rat lacks the ability to oxidize the terminal nitrogen in aliphatic amines (Caldwell 1981). N-octylamine is, in fact, a very good inhibitor of cytochrome P-450-dependent MMFO activity in the rat. Similarly, the rat produces relatively few deaminated metabolites in the metabolism of amphetamine.

Not all reactions catalyzed by the MMFO (in the rat as well as other species) are oxidative in nature. Several reductive pathways of toxocological importance are also catalyzed by this system. For example, the carcinogenic activation of azo dyes by rat liver preparations has been extensively explored, with p-dimethylaminoazobenzene (DAB) being the prototypical agent. As reviewed by Zbaida et al. (1989), N-demethylation and N-oxidation reactions are involved in the activation of DAB to ultimate carcinogens, whereas detoxification is associated with C-oxidation and reductive cleavage of the azo bond. Their work clearly demonstrates the MMFO is involved in the reductive as well as the oxidative reactions. The rat is obviously a good model for the study of these reactions. Interestingly, azoreductase activity is highly inducible by G-naphthaflavone (a "3-Mctype" inducing agent that is commonly used because, unlike 3-MC, it is not carcinogenic). In the case of azo dyes, induction of carcinogen-specific cytochrome P-450 leads to decreases in carcinogenic activation.

The MMFO of the rat has been shown to have definite stereospecificity. Trager and colleagues (Bush and Trager 1985; Heimark and Trager 1985) have published a very elegant series of studies on the metabolism of warfarin, demonstrating stereospecificity, both in terms of preferred substrate (R over S) and major metabolite formed (8-hydroxy vs. 6-hydroxy) in rats. Cook et al. (1982)

Table 3.28 Ratios Between Species for the Comparisons Rat–Human and Mouse–Human for the *In Vitro* Activity of the Respective Enzymes in the Liver, Respiratory Tract, Kidneys, and GI Tract

	Rat–Human (Liver)			Rat–Human (Respiratory Tract)			Rat–Human (Kidney)			Rat–Human (GI Tract)		
Enzyme	**GM**	**GSD**	***n***	**GM**	**GSD**	***n***	**GM**	**GSD**	***n***	**GM**	**GSD**	***n***
Alcohol dehydrogenase	1,11	2,14	10	—	—	0	—	—	0	—	—	0
Aldehde dehydrogenase	1,83	2,6	5	—	—	0	—	—	0	—	—	0
Aldeyde oxidase	2,16	2,98	10	—	—	0	—	—	0	1,70	—	1
Carboxylesterase	2,15	1,40	3	3,09	4,16	5	—	—	0	—	—	0
Cytochrome P-450 monooxygenase	0,93	7,13	81	16,60	8,91	6	—	—	0	—	—	0
Epoxide hydrolase mEH	0,41	2,44	38	0,51	1,57	10	0,68	—	1	—	—	0
sEM	0,28	2,48	18	0,61	3,08	5	0,008	—	1	—	—	0
Flavin-dependent monooxygenase	3,24	3,26	13	7,89	—	1	1,14	1,71	4	—	—	0
Gluthione-S-transferase	3,94	3,73	29	1,09	3,41	3	4,03	5,53	3	—	—	0
Monoamine oxidase	0,43	—	1	—	—	0	0,28	1,72	4	—	—	0
Methyl transferase	—	—	0	—	—	0	—	—	0	—	—	0
N-acetyltransferase	1,46	2,91	3	—	—	0	—	—	0	—	—	0
NAD(P)H:Quinone oxidoreductase	34,4	6,51	5	24,8	1,15	3	0,85	4,19	2	8,55	1,02	2
Prostaglandin-H-synthase	—	—	0	—	—	0	—	—	0	—	—	0
Sulfotransferase	2,65	1,46	4	0,34	—	1	0,06	—	1	0,03	—	1
Thiosulfate sulfurtransferase	1,10	—	1	12,90	—	1	—	—	0	—	—	0
UDP-glucuronosyltransferase	0,87	8,19	22	—	—	0	—	—	0	—	—	0
Xanthine oxoidoreductase	4,89	5,83	8	2,81	—	1	23,1	2,36	3	25,1	—	1

Note: GM = geometric mean; GSD = geometric standard deviation; *n* = number of data points.

*Source:*From Griem et al. (2002).

examined the stereospecificity of disopyramide metabolism and observed *in vivo* that the (S)-enantiomer was more extensively metabolized. In addition, the major metabolite with the (S)-enantiomer was the 3-methoxyphenol metabolite, whereas none of this was produced from the (R)-enantionmer (which was largely excreted unchanged) where the predominate metabolite was mono-N-dealkylated disopyramide. Hence, in the rat, optical activity can govern both the rate and the pattern of metabolism. As one can judge based on these two examples, it is difficult to determine a priori which enantiomer will be preferred and it might vary with substrate. In addition, there might well be species differences; the preferred isomer in rats might not be the preferred isomer in humans.

Inhibition (of the MMFO)

The MMFO of the rat is inhibited by a wide variety of chemicals. Discussion here is limited to the four different major classes of inhibition: direct competitive agents, suicide substrates, synthesis inhibitors, and antibodies.

Direct Competitive Agents

The classic competitive inhibitors of the MMFO are represented by agents such as SKF 525-A, metyrapone, and 2,41-dichloro-6-phenyi-phenoxyethylamine (HCI), which avidly bind the catalytic site on cytochrome P-450. Many investigators have used SKF 525-A to block the metabolic activation of many indirect hepatotoxins. Generally, the dose administered is 75 mg/kg (intraperitoneally in saline) 30 to 60 min prior to the administration of the chemical under study. Murray (1989) reviewed these mechanisms of reversible inhibition associated with the imidazole or quinoline drugs. Ketoconazole, for example, is the prototypical imidazole antifungal agent that inhibits cytochrome P-450-mediated reactions in both the liver and the adrenal gland. In theory, any substrate for the MMFO can competitively inhibit the metabolism of any other substrate, depending on the affinity constants, dosages, and isozymes of cytochrome P-450 involved. Such interactions are, in fact, a major concern in polypharmacy and the rat provides an excellent model for the study of these types of interactions.

Suicide Substrates

In contrast to the reversible inhibitors just discussed, the suicide substrates are irreversible substrates that are not necessarily distinctive on the basis of affinity or low turnover number. On oxidation by the MMFO, they form reactive metabolites that subsequently irreversibly bind to and denature cytochrome P-450. Substrate specificity can be used to selectively inhibit specific cytochrome P-450 isozymes. Chloramphenicol (300 mg/kg given intraperitoneally in 0.5 ml propylene glycol) can lead to the destruction of only three of eight of the major cytochrome P-450 isozymes as determined by their methodology. These included the major phenobarbitol inducible isozymes, whereas the isozymes induced by 8-NF, PCN, or clofibrate were unaffected by chloramphenicol treatment. Decker et al. (1989) reported that 7a-thiosteroids specifically deactivate the rat cytochrome P-450 induced by dexamethasone. It is probably only a matter of time before highly specific suicide substrates are available for all known rat cytochrome P-450 species. Arylalkynes inactivate 2B1, 2B4, 2B6, and 2B11 (Roberts et al. 1997).

Synthesis Inhibitors

Chemicals that inhibit cytochrome P-450 synthesis or stimulate its breakdown will also have an inhibitory effect on MMFO activity. Agents that generally block protein synthesis have been used to study mechanisms of induction, but lack specificity to be used as MMFO inhibitors. Generally, agents that inhibit the synthesis of hemoprotein or stimulate hemoprotein deactivation

(by increasing the activity of heme oxygenase) are more acceptable MMFO inhibitors. Cobalt (as the chloride salt) inhibits heme syntheses and has long been used as a tool to inhibit the MMFO-mediated activation of indirect hepatotoxins. The typical treatment regimen is 250 Amol/kg given subcutaneously 48, 36, 24, and 12 hr prior to hepatotoxic challenge (Chengelis 1988d). As discussed by Spaethe and Jollow (1989); however, the use of COC12 as an MMFO inhibitor has several disadvantages in that its effect on cytochrome P-450 is relatively brief and other enzyme systems are affected as well. They discovered that cobaltic protoporphyrin IX can profoundly depress cytochrome P-450 from 3 to 5 weeks. In rats, a single dose (90 Ag/kg sc) led to approximately 90% depletion in cytochrome P-450 1 week posttreatment. This resulted in large decreases in the Vna, of the MMFO toward all typical model substrates but had no effect on flavin-dependent mixed function oxidase (FMFO), UDP-glucuronosyl transferase, PAPS-sulfotransferase, or glutathione S-transferase activities. Hence, Co-protoporphyrin would make an excellent inhibitor to use as a tool for studying the involvement of the MMFO in the metabolism or toxicity of a drug or chemical in the rat.

Antibodies

Antibodies to cytochrome P-450 represent a fourth class of cytochrome P-450 inhibitor. They have the disadvantage of being useful only for *in vitro* broken cell studies. The specificity of antibodies can be used to discern or confirm the specific isozyme of cytochrome P-450 involved in a reaction. As discussed elsewhere, antibodies to NADPH:cytochrome c reductase can be used to discriminate between MMFO- and FMFO-catalyzed metabolic transformations.

There are a variety of miscellaneous other agents that inhibit the MMFO by mechanisms that do not involve direct interaction with cytochrome P-450. These include agents such as menadione, which is a substrate for, and therefore competes with cytochrome P-450 for, NADPH:cytochrome c reductase (Utley and Mehendale 1989). In fact, it is the reduction of menadione by NADPH:cytochrome c reductase that is believed to be responsible for menadione cytotoxicity (Utley and Mehendale 1989). Ethanol has also been repeatedly shown to inhibit MMFO activity, but the mechanism is the subject of debate. There is little doubt, however, that at high concentrations (80–100 mM) ethanol inhibits the MMFO. Both menadione and ethanol have pharmacological and toxicological properties that limit their usefulness *in vivo* as MMFO inhibitors. For example, ethanol is not an MMFO-potent inhibitor and, regardless of the underlying mechanism, would be a poor choice to use as an *in vivo* MMFO inhibitor because of the CNS depression that would be caused at the dosages required.

Induction (of the MMFO)

The fact that the activity of the MMFO with different substrates responds differently to various inducing agents played a key role in the elucidation of the molecular biology of cytochrome P-450. Induction (or hepatic enzyme induction) refers to the process whereby treatment of an animal with a chemical results in an increased amount of endoplasmic reticulum, accompanied by an increased activity of the MMFO. Induction will almost always result in an increased amount of microsomal protein, and this alone will cause increased MMFO activity. In the strictest sense of the word, however, induction will also result in increased specific activity (i.e., increased Vma, on a milligram protein basis). The term *induction* had come to be applied loosely to both situations. In the rat, enzyme induction is almost always accompanied by increases in liver weight. In fact, the rat is a highly inducible species, and subchronic treatment with almost any organic chemical could cause increases in liver weight. There are generally three classes of inducing agents, each named for the prototypical agent: phenobarbital, 3-methylcholanthrene (3MC), and pregnenalone-16-carbonitrile (PCN). They are differentiable on the basis of the classes of cytochrome P-450 isozymes they induce as well as the exact mechanisms of induction (as reviewed by Gonzales 1989). Some agents

are more specific than others. Mixtures of polychlorinated hydrocarbons (e.g., Aroclor 1254) induce both phenobarbital and 3-MC-type activities. It is for this reason that hepatic preparations used in the Ames Salmonella assay for mutagenicity are taken from rats treated with Aroclor 1254. Other enyzmes could also be affected. Phenobarbital, for example, will also induce increases in UDP-glucuronosyl and glutathione S-transferases in the rat. With phenobarbital, good induction (approximate doubling of cytochrome P-450 with about 30% increases in liver weight) is obtained with three dosages of 80 mg/kg (intraperitoneally in saline with the pH corrected by HCl) over a 3-day period. For 3-MC-type induction, current practice is to use one dose of 100 mg/kg (intraperitoneally in corn oil) 6-naphthaflavone (6-NF, which is not carcinogenic) followed by a 3-day rest period. Induction with PCN is usually affected by giving 25 to 50 mg/kg intraperitoneally, for 3 to 4 days. Induction can provide a convenient tool for studying metabolism of a specific chemical in the rat. For example, Decker et al. (1989) reported that spironolactone caused slight losses of cytochrome P-450 in naive rats, but will cause much more profound losses in rats pretreated with dexamethasone (a PCN-type agent that causes increases in cytochrome P-450p). This allowed for more thorough study of this phenomenon: the "suicide substrate" destruction of hepatic cytochrome P-450 by spironolactone.

What are the implications of microsomal enzyme induction? The rat is a highly inducible species, and teleologically, this might explain why it tends to have lower baseline activity than other less inducible rodent species, such as the hamster. It is a rare chemical that (if given in a high enough dosage for a long enough period of time) will not have some inductive effect in the rat. Increases in liver weight with centrilobular hepatocyte hypertrophy (due to proliferation of the smooth endoplasmic reticulum) are a common finding in toxicity studies using the rat. This is a reversible, adaptive response and should not be considered (of and by itself) evidence of toxicity.

Alterations of intrinsic hormone metabolism can be another implication of induction. In the rat, a frequent example of this is increased metabolism and clearance of thyroid hormones (T3 and T4). Although not cytochrome P-450 mediated (rather these reactions are primarily catalyzed by UDP-glucuronosyl transferase, discussed later), they are still inducible. The increased clearance of thyroid hormones, a primary effect of microsomal enzyme induction, results in increased synthesis and release of the pituitary hormone TSH. Hence, an increased size and weight, and sometimes a frank increase in thyroid tumors in long-term studies, could be a secondary effect of hepatic microsomal enzyme induction.

Induction can be accompanied by other physiological changes that might have little to do with increases in xenobiotic metabolism. Induction of MMFO in the rat often causes concomitant changes in plasma proteins. Typical clinical chemical panels in toxicity studies can result in the increased production of various microglobulins by the liver (Makarananda et al. 1987). Hence, MMFO induction in the rat might be accompanied by slight increases in serum proteins associated with an increase in the globulin fraction. Such changes are of little, if any, toxicological importance. As discussed by Makarananada et al. (1987), however, chemical-induced liver damage will also cause changes in the components of the globulin fraction, albeit different ones than those changed by induction. One should not, therefore, jump to a conclusion concerning an increase in the globulin fraction without examining the liver histologically or examining plasma proteins by electrophoresis.

Another implication with regard to induction is that the rat is more inducible than most species. For example, Astrom et al. (1986) compared the effects of 3-MC induction in the rat, hamster, guinea pig, and two strains of mice. In rats, 3-MC produced a threefold increase in cytochrome P-450 and a hundredfold increase in 7-ethoxyresorufin deethylation activity. In hamsters and guinea pigs, the same treatment induced only 1.5- to 2.5-fold increases in cytochrome P-450 and five- to sevenfold increases in 7-ethoxyresorufin deethylation activity, respectively. In mice, the responses were strain dependent: 3-MC did not induce increases in DBA/2 mice, but did induce increases in cytochrome P-450 and MMFO activity in C57bl/6 mice. Hence, one cannot always extrapolate from the results obtained in rats to other species. In fact, it is likely that a weak inducer in rats will have little inductive effect in other species.

Another implication of induction in the rat is that increases in cytochrome P-450 are not always accompanied by increases in MMFO activity. The inducing agent might cause a change in the isozymic spectrum of cytochrome P-450 such that the activity of the MMFO toward some substrates might actually decrease. For example, Astrom et al. (1986) reported that 3-MC induction resulted in decreases in the rate of aminopyrine metabolism *in vitro*. Rhodes and Houston (1983) reported that phenobarbital caused a slight decrease in the formation of 14C-CO2 from 14C-antipyrene *in vivo* in rats, whereas P-NF caused a 50% increase. Ioannides et al. (1981) demonstrated 3-MC induction in rats enhanced subsequent benzo(a)pyrene mutagenicity in the Ames assay, whereas phenobarbital decreased it. The opposite occurred with 2-AAF induction. Thus, the type of induction (i.e., the isozyme of cytochrome P-450) can have important implications with regard to the toxicity of other agents in the rat. For example, as discussed by Lesca et al. (1984), different mutagenic agents are preferentially activated by different cytochrome P-450 isozymes: Ethidium bromide is preferentially activated by 3-MC-inducible MMFO activity. A single 80 mg/kg treatment of rats with 3-MC caused over a hundredfold increase in the number of revertant colonies in the Ames assay versus the response obtained with liver enzyme preparations obtained from control rats. Phenobarbital and PCN, in contrast, caused only modest (2.5%–3.5%) increases. Phenobarbital, however, did induce hundredfold increases in response in the Ames assay to cyclophosphamide, whereas 3-MC caused no increase in this response. Aroclor caused increases in the mutagenic activity of both ethidium bromide and cyclophosphamide, indicative of the broad spectrum of isozymes induced by this agent. Lesca et al. (1984) recommended using changes in responses in the Ames assay to known mutagens to determine the nature of cytochrome P-450 induced by new chemical entities in the rat.

Rats are common species used for carcinogenicity testing. What are the potential implications of chronic exposure of rats to an inducing agent? Kurata et al. (1989) studied the long-term effects of phenobarbital on the liver and the MMFO. The greatest degree of induction was achieved with 7 weeks of treatment. MMFO activity and cytochrome P-450 concentrations fell off slightly thereafter, but remained at a plateau 2.5- to 3.5-fold greater than control levels for the remainder of the study. Hence, induction has a finite effect: The liver cannot hypertrophy indefinitely. In pharmacological terms, rats accommodate to induction but do not become tachyphylactic. Many chemicals induce increases in their own metabolism. In a chronic toxicity study, plasma levels of the parent test article often decrease, whereas levels of metabolites increase during the test period. This can result in changes in toxicity depending on whether the parent or the metabolite is most responsible for toxicity.

Other than expected pharmacokinetic consequences, does induction have any other implications? Lubet et al. (1989) explored the relationship between induction and hepatocellular tumor promotion. They suggested that potent inducing agents of cytochrome P-450b (e.g., phenobarbital, DDT) are all potent liver tumor promoters. Structural analogs (e.g., hexobarbital) that are not potent inducing agents are also poor liver tumor promoters. In addition, hamsters, in which cytochrome P-450 is not inducible, are resistant to hepatocellular tumor promotion. Hence, in chronic toxicity studies in the rat with inducing agents, promotion of naturally occurring "background" hepatic neoplasias is a real possibility such that an nongenotoxic chemical could appear to be a hepatic carcinogen.

Most of the implications of induction in the rat have to do with the specific isozymes of cytochrome P-450 induced. Therefore, if in the testing of a chemical, one has reason to believe that it is an inducing agent (gross increases in liver weight, centrilobular hypertrophy, increases in the grossly measurable cytochrome P-450), then identification of the specific isozyme of cytochrome P-450 induced would aid in determining the implications of such findings. This can be accomplished without isolating and characterizing microsomal proteins by immunoelectrophoresis (Thomas et al. 1983; Thomas et al. 1984) by simply looking at shifts in metabolic patterns in crude microsomal preparations. Wood et al. (1983) used changes in *in vitro* testosterone metabolism to discriminate or categorize types of induction. For example, a shift in *in vitro* metabolized spectrum that includes a large increase in 16p-testosterone formation would be

indicative of cytochrome P-450a, or phenobarbital-type induction in the rat. 7-ethoxyresorufin is a specific substrate for 3-MC-inducible cytochrome P-450b and Iwasaki et al. (1986) reported that 3-MC treatment (50 mg/kg intraperitoneally for 3 days) increased 7-ethoxyresorufin deethylation 32-fold (from 0.05 to 1.6 nmol/min/nmol. These increases will appear even larger when corrected for increases in microsomal protein. Thus, the estimation of changes in the metabolic rate or metabolite profile for a handful of well-studied model MMFO substrates can provide useful metabolic and toxicological information.

In summary, the phenomenon of microsomal enzyme induction has been well studied in the rat. Different chemical classes have somewhat different specific effects but, in general, most of the implications of microsomal induction are due to the increases in specific isozymes of cytochrome P-450. The rat is a highly inducible species, but there are age-, sex-, and strain-related quantitative differences. Given that, and also given the implications induction might have for the metabolism and toxicity of a chemical in the rat, the careful investigator should consider inspection of the type of induction related to a specific chemical under study. The technology for doing so is available and easily adaptable.

Microsomal induction studies generally involve biochemical analyses conducted at a single time point following treatment with the suspect inducing agent. One needs to be cautious in the design and interpretation of such studies, however, as different isozymes have time courses of response to different inducing agents. For example, Parkinson, Thomas, Ryan, Reik, et al. (1983) examined the time course of the response of Long-Evans rats to a single dose of Aroclor 1254 (500 mg/kg intraperitoneally). Cytochrome P-450c was maximally induced 2 days after treatment and remained constant. In contrast, cytochrome P-450a was not maximally induced until 9 days after treatment. Other isozymes had still different patterns. All isozymes started to decline 15 days following treatment. Hence, if one is concerned about the nature of the hepatic induction affected by a poorly characterized chemical in the rat, several time points should be examined.

Sex-Related Differences

The hepatic MMFO is under a variety of hormonal controls that are responsible for the well-reported differences in male and female rats. In fact, sex-related differences have been most frequently and convincingly demonstrated in the rat as opposed to other species. Hormonal control of drug oxidation is very complex but has been most well characterized in the rat. This effort dates back to the late 1950s and early 1960s, when it was first noticed that there was a sex difference in drug metabolism, with males generally having higher activity than females. For example, Kato et al. (1964) demonstrated that male Wistar rats had consistently higher *in vitro* rates of microsomal aniline, aminopyrine, and strychnine metabolism. Furner et al. (1969) reported that regardless of the strain of rats examined, male rats generally had higher rates of microsomal metabolism with aniline, aminopyrine, ethylmorphine, and other model substrates. The extent of this difference varied depending on age, strain, and substrate examined. These earlier works were among the first to also note that the sex-related difference were less apparent in sexually immature rats. This has also been confirmed repeatedly and is discussed in greater detail later (under “Age-Related Changes”).

Ariyoshi et al. (1981) demonstrated in Wistar rats that there was no difference in cytochrome P-450 content between males and females at 30 days of age, but there were large differences at 300 days. Tht fact that the sex-related differences in xenobiotic metabolism were less apparent in sexually immature rats led to many experiments exploring the hormonal control of MMFO activity. The work in this area has been reviewed in detail by Skett (1988).

In general, there are substantial data suggesting that the androgenic and estrogenic hormones exert different and competing modulating effects on MMFO activity. For example, early work demonstrated that castration reduced activity in males to female levels and testosterone supplementation “remasculinzed” drug metabolism in rats. The differences are due to differences in the total amount of as well as the spectrum of cytochrome P-450 isozymes induced. Kato and coworkers

(Kamataki et al. 1985) have identified two distinct (by immunological methods and by electrophoretic mobility) forms of cytochrome P-450 (termed cytochrome P-450-male and P-450-female) that are specific to the respective sexes in Fischer 344 rats. Cytochrome P-450-male is associated with a higher rate of MMFO activity with several model substrates (e.g., 7-propoxycoumarin, aniline, and benzphetamine) than cytochrome P-450-female. High levels of cytochrome P-450-male are induced by testosterone: Castration leads to decreases in this isozyme. In contrast, cytochrome P-450-female is present only in female rats and the levels are attenuated after oophorectomy and exogenous testosterone administration. Other hormonal effects on MMFO activity have been reported. Growth hormone (a pituitary hormone) has been shown to have a feminizing effect on xenobiotic metabolism in the rat (Skett 1988), also presumably due to differences in expression of cytochrome P-450 isozymes. Waxman et al. (1985), for example, have described a distinct isozyme of cytochrome P-450 (termed P-450 2c(&)/UT-A) that is responsible for testosterone 16a-hydroxylation activity in liver microsomes from male rats. Castration of male rats at birth led to diminished amounts (to levels found in female rats) of both this cytochrome P-450 isozyme and 16a-hydroxylation activity.

Although hormonal effects on MMFO activity are generally attributed to control and expression of specific cytochrome P-450 isozymes, other actions are also involved. Waxman et al. (1989) demonstrated that hypothesectomy resulted in elevated amounts of several cytochrome P-450 isozymes and decreases in others, but there was little correlation between the changes in cytochrome P-450 and changes in MMFO activity. They demonstrated that this was due to decreases (26%–30% of control) in NADPH:cytochrome P-450 reductase activity and was restored by exogenous T4 treatment, but not by other hormones. They concluded that the reductase is subject to hormonal controls that are distinct from those for cytochrome P-450. This observation is also consistent with the observation that reductase activity is also the rate-limiting step in MMFO reactions.

In summary, the well-described sex-related differences in MMFO activity (toward xenobiotics and endogenous steroid hormones) in the rat (with males generally having higher activity than females) are due to the implicit hormonal differences that result in the expression of different isozymes of cytochrome P-450 and total NADPH:reductase activity. Hence, it is not at all uncommon for a chemical to have different pharmacokinetic behavior in male versus female rats, or for there to be quantitative differences in chemical toxicity between the two sexes of this species. In addition, as the use of surgically altered rats in pharmacological experiments is not uncommon, these potential effects that the resulting hormone changes could have on drug metabolism should be kept in mind.

Strain-Related Differences

Strain-related differences in MMFO activity and inducibility have long been recognized, as Conney (1967) mentioned in an early review on the subject of microsomal drug metabolism. In their classic article, Page and Vesell (1969) compared native and induced rates of *in vitro* (with S-9 fractions) oxidation of ethylmorphine and aniline in 10 different strains of rat. They noted several strain-related differences. For example, baseline aniline hydroxylation activity in males varied from 6.9 (Wistar) to 20.2 nmol/min/mg (Long-Evans). Interestingly, these baseline differences in activity existed despite the fact that cytochrome P-450 content did not differ substantially between strains. Following induction by phenobarbital, activity tripled in Wistar rats but increased only 50% in Long-Evans rats. More recently, Koster et al. (1989) examined strain-related differences in the MMFO of 14 highly inbred strains that had originated from four different European facilities, and identified considerable strain-related variations. Kai et al. (1988) determined that Sprague-Dawley rats had lower amounts of cytochrome P-450 as well as lower MMFO activity with the model substrates aniline and aminopyrine than Wistar rats. The Sprague-Dawley rat, however, was more inducible with malotilate than the Wistar rat. Augustine and Zemaitis (1989) also demonstrated that there were significant differences in MMFO activity among Wistar, Sprague-Dawley, and Fischer

344 rats. Jackson and Li (1987) reported that citral was a more potent inducer in Long-Evans than in Wistar rats.

There are also strain-related differences in the metabolism of endogenous substrates. Shefer et al. (1972), for example, compared testosterone 7a-hydroxylation activity in Charles River Sprague-Dawley (CD-1) and Wistar rats. Activity was 3.59 and 2.36 nmol/min/mg (approximately 50% difference), respectively. Following phenobarbital treatment, there was essentially no change in this enzyme activity in Sprague-Dawley rats, but a sixfold increase in Wistar rats. Although there are exceptions, three generalizations tend to emerge from the literature with regard to strain-related differences in MMFO activity in rats: (a) In all strains, males have higher activity than females. Quantitatively, this difference is greater in the Fischer 344 and Long-Evans strains than the Sprague-Dawley and Wistar strains. (b) Baseline (native) activities in Wistar rats tend to be lower than those of the Fischer 344 and Long-Evans strains, and the Sprague-Dawley strain tends to be intermediate. (c) The Wistar strain tends to show the greatest inductive response to phenobarbital-type inducers.

Strain-related differences are an important consideration in the design and interpretation of toxicity studies, but they can also be used as specific experimental tools. The Lewis and DA rats are closely related strains that differ in that the DA rat has poor hepatic debrisoquine 4-hydroxylation activity (Kahn et al. 1985). Tucker et al. (1980) made interesting use of strain-related differences in rats to study the metabolism and toxicity of carbon disulfide. In their hands, the extent of CS2-mediated hepatic hydropic degeneration varied considerably in four different inbred strains of rats, and was accompanied by hepatic necrosis in the most susceptible strain. There was also a good correlation between the extent of hepatic damage and the CS2-mediated loss of cytochrome P-450 (CS2 being a suicide substrate). Hence, although the exact mechanism is unclear, these results demonstrate the loss of cytochrome P-450 is involved in the mechanism of CS2-mediated hepatotoxicity. These examples demonstrate the utility of using strain-related differences for studying the relationship between metabolism and toxicity of a drug or chemical.

Celier and Cresteil (1989) have recently described an interesting strain-related phenomenon involving the Gunn rat. As discussed elsewhere (see "Conjugation Reactions") this strain has a well-characterized lack of UDP-glucuronosyl transferase activity. Celier and colleagues further demonstrated that the Gunn rat also does not respond to 3-MC induction with increases in total cytochrome P-450, but does develop increases in MMFO activity with substrates such as 7-ethoxyresorufin, generally associated with this type of induction. Immunoquantification methods demonstrated that 3-MC-treated male (a different pattern was seen in females) Gunn rats had increases in cytochrome P-450c and P-450d, but with an equivalent decrease in other isozymes. A more usual inductive pattern would have been an increase in specific isozymes while the levels of others remained constant. Interestingly, whereas 3-MC has been shown to induce UDP-glucuronosyl transferase (as well as cytochrome P-450) in other rat strains, it does not induce increases in Gunn rats. The Gunn rat provides an interesting example of a sex difference nested within a strain difference in inductive response. This highlights the need to be very cautious in interpreting the implication of microsomal induction in the rat.

The Zucker rat is a strain with a marked tendency to develop obesity and is frequently used as a model to study the physiological consequences of this condition. This strain is not commonly used to study toxicological problems, but does provide an interesting example of strain-related differences that can be tied to phenotypic expression. Brouwer et al. (1984) compared the inductive response of lean versus obese Zucker rats to Sprague-Dawley rats and found that phenobarbital treatment caused similar increases in Sprague-Dawley and lean Zucker rats; that is, increases in antipyrine clearance, liver weight, microsomal protein, and cytochrome P-450 content. The obese Zucker rat, however, failed to respond to phenobarbital treatment with increases in any of these parameters.

Another implication in considering strain-related differences is illustrated by the case of pregnenolone-16a-carbonitrile. There has been some debate on whether or not pregnenolone-16a-car-

bonitrile induced increases in benzo(a)pyrene metabolism in the rat. This disagreement resulted from strain-, age-, and sex-related differences, as reported by Gorski et al. (1985). They demonstrated that PCN did induce increases in benzo(a)pyrene metabolism in 1-month-old rats regardless of strain (Long-Evans, Sprague-Dawley, Wistar, or Holtzman). Differences began to develop as the rats matured. In male (but not female) Long-Evans rats, for example, inducibility of benzo(a)pyrene metabolism with PCN decreased with age. Hence, the age, sex, and strain of rat used as well as the model substrate and assay techniques can all influence the apparent inductive response in the rat. It is not unusual to see this type of disagreement in the literature. This example also underscores the need to pay close attention to experimental details, such as the sex, strain, and age of rats used when one needs to replicate the work of another investigator.

Although strain-related differences in metabolism might often be the basis for the differences in toxicity, this is not always the case. For example, acetaminophen is nephrotoxic in Fischer 344 rats but not in Sprague-Dawley rats. Tarloff et al. (1989) extensively explored the metabolism and pharmacokinetics of acetaminophen in age- and sex-matched rats for these two strains and could not identify any differences to account for the strain-related differences in toxicity. Further, Newton et al. (1983) were unable to identify any specific differences in renal xenobiotic metabolism that could provide an explanation for the difference in acetaminophen nephrotoxicity in these two strains of rat. Elsewhere, Plummer et al. (1987) were unable to identify any metabolic differences between Fischer 344 and DA rats that could account for the strain-related differences in sensitivity to aflatoxin. In other studies (Kahn et al. 1987), this group was also unable to demonstrate differences between DA and Fischer 344 rats with regard to phenacetin meta ISM.

Age-Related

Age-related changes in xenobiotic metabolism, particularly that affected by the MMFO, have been studied in the rat for quite some time. In general, these studies fall into two categories: those on postnatal or perinatal changes, and those on senescent changes. Perinatal changes are discussed first.

Kato et al. (1964) claimed (quoting earlier works by Fouts and Jondorf) that "It is a well known fact that in newborn animals there is a deficiency in certain drug metabolizing enzymes." They demonstrated (using female Sprague-Dawley rats) that the *in vitro* microsomal metabolism of five different model substrates was quite low in neonatal rats (approximately 5% of the activities observed in 30-day-old rats), but these activities increased gradually and steadily to a peak in 30-day-old rats and declined thereafter. They also demonstrated that there were age-related changes in NADPH:cytochrome P-450 reductase that paralleled the changes in MMFO activity.

Nearly 10 years after the publication by Kato et al. (1964), Muller et al. (1973) published on age-related changes in cytochrome P-450 in male Wistar rats; the concentrations in 10-day-old rats were less than half of those observed in adult (30- to 60-day-old) rats. Barbital was a poor inducer in young rats. This paper also noted that changes in MMFO with age did not correlate well with the amount of cytochrome P-450, but did not mention the earlier work by Kato. The additional literature in this area through 1980 was summarized by Klinger (1982). Although he questioned whether the activity of NADPH:cytochrome P-450 reductase is the rate-limiting step in all MMFO activities, his review clearly indicates that neonatal rats have low MMFO activity that might be due to a combination of factors: low amounts of cytochrome P-450 (not always seen by all investigators), the low activity of NADPH: cytochrome P-450 reductase, and the isozymic character of the cytochrome P-450 present.

Depending on the model substrate, there are different time courses of MMFO development and responsiveness to inducing agents. Devasagayam et al. (1983), for example, reported that female Wistar rats at 1 day of age have 25% of the cytochrome P-450 and 20% of the NADPH:cytochrome P-450 reductase as those at 75 days of age. Although there are exceptions, in general, the sex-dependent differences in MMFO activity are not apparent in neonatal rats and tend to develop concurrently with sexual maturity. Waxman and colleagues (1985) demonstrated that this was because different

gonadal hormones control the levels of different cytochrome P-450 isozymes. For example, both males and females are born with very low testosterone 16a-hydroxylating ability. This increases rapidly in males, but remains low in females even after sexual maturation (4–6 weeks of age).

The quantitative and qualitative responsiveness of neonatal and perinatal rats to inducing agents is still a matter of some debate. In contrast to the aforementioned results with barbital, Pyykko (1983) reported that perinatal (3-day-old) rats are as responsive to the inducing effects of toluene as adults. In toxicological research, experiments that require the treatment of neonatal rats are probably quite rare. The inducibility of 2-day-old rats might be of intellectual interest, but the implications in developmental and reproductive toxicity are not clear. In contrast to inducibility, the fact that neonatal rats have a low metabolic capacity has definite toxicological implications. Depending on the toxicological mechanism of action of the chemical, neonatal rats will respond differently than adult rats. For example, CC14 requires metabolic activation for hepatotoxicity, and neonatal rats are refractory to CC14-induced hepatic damage.

The activity of MMFO in rats has also been shown to change with advancing age. Kato et al. (1964) also discussed changes in senescence. In general, MMFO activity appears to decrease as rats age. For example, Kato et al. (1964) noted that cardosopil metabolism peaked at 30 days of age and declined progressively thereafter. The rates of plasma clearance were equivalent in 15- versus 250-day-old rats. Baird et al. (1975) reported that there were age-related (30- to 900-day-old) changes in CFN male rats in that although the concentrations of cytochrome P-450 did not decline appreciably, *in vitro* microsomal metabolism of zoxazolamine did, and this decline seemed to reflect an age-related decline in NADPH:cytochrome P-450 activity. Rikans and Notley (1981, 1982a, 1982b) examined age-related changes in male Fischer 344 rats, and found that microsomal cytochrome P-450, cytochrome b5, and NADPH:cytochrome c reductase decreased in middle-aged (14–15 month) and old (24–25 months) rats compared to young rats (3–5 months). Changes in MMFO activity were variable (sometimes increasing, sometimes decreasing with age) depending on substrate. Schmucker and Wang (1980a) reported the levels of cytochrome P-450 and NADPH:cytochrome c reductase were highest in middle-aged (16 months) rats, with marked decreases in older rats (27 months). The MMFO activities tended to be lowest in older rats (Schmucker and Wang 1980b). Additionally, they reported that the microsomal cholesterol–phospholipid ratio increased in senescent rats. The resulting change in membrane fluidity was thought to change the interaction between the reductase and cytochrome P-450 (Schmucker et al. 1984).

Although age-related changes in MMFO activity in Fischer 344 rats have been extensively studied, relatively few reports on age-related changes in Sprague-Dawley rats have been published. Chengelis (1988d) reported a rather extensive investigation of age-related changes in MMFO activity in male and female Sprague-Dawley rats. In a pattern similar to that observed in Fischer 344 rats, cytochrome P-450 peaked (on a gram liver basis) in rats at 39 weeks of age. NADPH:cytochrome P-450 reductase activity also peaked at 39 months of age and declined thereafter. In senescent rats (greater than 7–8 weeks of age), there were apparent differences between sexes with regard to the levels of the components of the MMFO and MMFO activity. Interestingly, there was little correlation between MMFO activity and the amounts of cytochrome P-450. For example, the activity toward p-nitroanisole was lowest in 39-week-old rats (when cytochrome P-450 was highest). Aniline hydroxylase was lowest in 104-week-old rats. These results suggest that there are not only age-related changes in total cytochrome P-450, but also age-related changes in the isozymic spectrum. This would be consistent with the earlier findings by Kamataki et al. (1985), who reported that there were definite age-related changes in male Fischer 344 rats. Benzo(a)pyrene hydroxylation and 7-propoxycoumarin dealkylation activities peaked at 6 months of age in males, but not in females, where activities remained constant throughout adult life. In males, there was a drop in MMFO activities after 24 months of age to female levels. For benzo(a)pyrene hydroxylation, for example, peak *in vitro* activity was approximately 350 pmol/min/mg microsomal protein in 6-month-old male rats and approximately 90 pmol/min/mg in both sexes at 24 months of age. These changes directly reflected the amounts of cytochrome P-450-male, as well as the appearance of

cytochrome P-450-female in senescent male rats. Hence, although there are quantitative and qualitative exceptions, depending on strain, sex, and age of the rats, as well as the substrate of interest MMFO activity in rats tends to be highest in middle-aged rats (9–14 months) and lowest in senescent rats (24 months). Age-related changes are due to a combination of changes in total cytochrome P-450, the spectrum of cytochrome P-450 isozymes, NADPH:cytochrome P-450 reductase, and microsomal lipid content. A further complication is that microsomal protein will also change with age (Chengelis 1988b). Because of this variability, one should not base too many conclusions on these generalizations without specific examination.

Given there are age-related changes in cytochrome P-450 in rats, can there also be age-related changes in response to inducing agents? Age-related changes in the sensitivity of rats to inducing agents have been discussed since the late 1960s. The literature in this field is mixed. For example, Kato and Takanaka (1968a) reported that phenobarbital (60 mg/kg intraperitoneally for 3 days) caused much larger increases in 40-day-old than 300-day-old rats; 23% versus 8% in microsomal protein, 249% versus 52% in NADPH:cytochrome c reductase, 259 versus 60% in cytochrome P-450, and 713% versus 173% in aminopyrine N-demethylation activity. Kao and Hudson (1980) compared and contrasted the inducing effect of phenobarbital (80 mg/kg intraperitoneally for 4 days) versus 3-naphthaflavone (80 mg/kg intraperitoneally for 4 days) in young (10 weeks) versus old (100 weeks) male Fischer 344 rats. As expected, the baseline amount of MMFO components as well as the MMFO activities examined were higher in young adult rats than senescent rats, but both agents caused comparable degrees of increase in both age groups. They concluded that young and old rats responded to inducing agents in relatively the same quantitative fashion. In contrast, Rikans and Notley (1982a, 1982b) reported that age affected the response to different inducing agents differently. They concluded that phenobarbital induced increases to approximately the same extent in old versus young rats, whereas G-naphthaflavone induced larger increases in older rats. They further suggested that age-related differences in sensitivity to induction are due to differences in the inducibility of specific cytochrome P-450 isozymes. This latter conclusion is consistent with the work of Sun et al. (1986), who examined the levels of six different forms of cytochrome P-450 as a function of both age and inducing agents using immunochemical techniques. They noted that phenobarbital induced a different pattern of isozymes in 2- versus 52-week-old rats. Sitar and Desai (1983) reported that male Sprague-Dawley rats lost responsiveness with age to phenobarbital but not 13-naphthaflavone. McMartin et al. (1980) observed the same pattern in Sprague-Dawley rats. In contrast, Baird et al. (1975) reported no decline in responsiveness of male CFN rats to phenobarbital. Birnbaum and Baird (1978) examined the effectiveness of three different inducing agents (phenobarbital, 3-MC, and PCN) in Fischer 344 rats. They observed that inducibility of microsomal benzphetamine demethylation and aniline hydroxylation decreased with age, whereas inducibility of pnitroanisole demethylation increased with age.

Thus, despite the lack of uniformity in the literature, age clearly plays a role in the response of rats to inducing agents. The extent that age plays depends on the strain and sex of the rats used, the class of inducing agent, and the specific MMFO parameter examined. As a broad generality, most rats lose responsiveness to phenobarbital induction as they age.

What are the implications of age-related changes in MMFO activity? One should expect changes in pharmacokinetic behavior of a given drug or chemical in rats. Kapetanovic et al. (1982), for example, reported that the plasma half-life was longer and plasma clearance rate lower in old (32–34 months) rats than in young (3–4 months) rats. If toxicity of a chemical depends on metabolic activation (e.g., an indirect hepatotoxin) there could be age-related changes in sensitivity. Rikans (1984) examined age-related changes in response to various hepatotoxins and reported that old (24–25 months) rats were more sensitive to ethyl alcohol but more sensitive to bromobenzene than middle age (14–15 months) rats. The sensitivity of rats in acute toxicity testing varies considerably with age, and this might be due in part to age-related differences in metabolism. Hence, one should always consider the age of the rats in attempting to reproduce an experimental result.

Other Influences on MMFO Activity

Age, sex, and the strain of rats used all play modulating roles in xenobiotic metabolism. Husbandary and other external events have also been shown to influence xenobiotic metabolism. The feeding regimen is a good example: Gram et al. (1970) reported almost 20 years ago that 72 hr of starvation elicited increases in cytochrome P-450 and MMFO activity. This is consistent with the authors' experience that fasting increases the sensitivity of the rat to many indirect hepatotoxins. Radzialowski and Bousquet (1968) reported that MMFO activity followed a diurnal cycle that tended to mirror the diurnal cycles in plasma corticosterone levels. Hence, the time of day a rat is dosed can affect the subsequent metabolism and pharmacokinetic behavior of the drug or chemical under investigation. In another classic work, Fuller et al. (1972) demonstrated that cold exposure (4°C for 4 days) stimulated hepatic drug metabolism in the rat. Hence, environmental conditions can also influence MMFO activity in the rat. Room conditions in a toxicity study should be carefully controlled and monitored to avoid temperature extremes that could alter rates of test article metabolism.

Peroxisomal Proliferation

There is a class of chemicals that induce not only increases in cytochrome P-450, but other changes as well; namely, those that induce peroxisomal proliferation (Hawkins et al. 1987). In general, these fall into two classes: hypolipidimic drugs related in structure to clofibrate and plasticizers related in structure to di(2-ethylhexeyl)phthalate. These agents cause hepatomegaly, proliferation of smooth endoplasmic reticulum with increases in MMFO activity, increases in peroxisomal number with associated enzyme level changes, and changes in mitochondrial number and function with increases in certain enzyme levels. This phenomenon has been most extensively studied by Reddy and Lalwani (1983) in rats and mice. The phenomenon is diagnosed microscopically by increased content of peroxisomes or biochemically by measuring the activity of peroxisomalspecific enzymes, such as catalase or camatine-acetyl transferase. They also, as reported by Oesch et al. (1988), cause large increases in cytosolic epoxide hydrolase. For example, these investigators have reported 1 week of treatment with clofibrate (200 mg/kg/r day, dietary admixture) will cause a 50% increase in liver relative organ weights (from 4.1%–6.3%), and an eightfold increase in both palmatyol-CoA transferase activity (from 11.4–90.0 nmol/min/mg) and c-EH activity (38–312 pmol/min/mg protein) in rats.

Bezofibrate is a hyperlipidemic drug that is a potent peroxisomal-proliferating agent. Halvorsen (1983) reported that administration of bezofibrate (by dietary admixture, 12.4 /μmol/g feed for 9 days) caused 60% increases in liver weight and total hepatic protein, and a 2.4-fold increase in camitine palmatoyl transferase activity in male Wistar rats. Watanabe et al. (1989) demonstrated that a longer dosing period (100 mg/kg for 13 weeks) can have even more dramatic effects (e.g., a 35.8-fold increase in camatine acyltransferase). Increases were seen in other species, but (except for mice) nowhere near the levels of increases seen in rats.

The safety of peroxisomal-proliferating agents is an issue because these agents are also liver carcinogens in rats and mice. Rats and mice are the most inducible and other species are less responsive. It would appear that rats are good models to determine if an agent causes peroxisomal proliferation, but poor models to determine if such agents are genotoxic carcinogens. These agents will almost always uniformly (100% response) cause hepatocellular tumors at the MTD in rats. As these tumors are probably a result of the peroxisomal proliferation, the relevance of these findings to nonresponsive species is questionable. The potential carcinogenicity of peroxisomal-proliferating agents might be better assessed in a species such as the guinea pig, which Oesch et al. (1988) showed to respond poorly to peroxisomal-proliferating agents.

The mechanism by which peroxisomal-proliferating agents cause hepatic carcinomas in rodents is a subject of some debate. One theory holds that the increase in peroxisomal number leads to an

increase in the generation of reactive oxygen species that can damage DNA. The oxidative stress hypothesis has come under some question because there is little evidence to suggest that oxidative injury in and of itself is sufficient to cause genotoxic changes. An alternative explanation is that peroxisomal-proliferating agents also have promoting activity. Cattley and Popp (1989) have studied this hypothesis using WY-14643 as a model peroxisomal-proliferating agent and compared its promoting activity with that of phenobarbital. Both agents were very effective (100% response in 4–5 weeks) in promoting altered hepatic foci in diethylnitrosamine-initiated rats, but with qualitative differences. In contrast to phenobarbital, WY-14643 promoted foci were larger, did not stain for r-glutamyl transpeptidase, and were highly ATPase deficient. The authors concluded that these differences were evidence that WY-14643 is a tumor promoter (as well as a peroxisomal-proliferating agent), but the mechanism of promotion differs from that of phenobarbital. In fact, the mechanisms of WY-14643 promotion and peroxisomal proliferation might be distinct. Whether these findings can be generalized to all peroxisomal-proliferating agents remains to be established and the role of peroxisomal proliferation in carcinogenicity needs to be more thoroughly examined. The rat is probably the species of choice for such investigations.

Flavine-Dependent Mixed Function Oxidase (FMFO)

Not all the microsomal mixed function oxidase activity is cytochrome P-450 dependent. There is a separate flavin-dependent mixed function oxidase (FMFO) that is different and distinct from the cytochrome P-450-dependent MMFO. Dannan and Guengerich (1982) clearly identified with immunochemical techniques the presence of this enzyme in various rat tissues, including the rat liver. The literature on this enzyme has been extensively reviewed by Ziegler (1988). There are species- and organ-related isozymes, but the presence of more than one different isozyme in the same organ has not yet been demonstrated. There are currently no data to suggest that this enzyme is inducible in the strictest sense of the word (i.e., causes increases in specific activity). The literature is inconsistent with regard to sex-related differences. Ziegler reported that, as with cytochrome P-450-dependent activity, there is a greater activity in males than in female rats (approximately 5:1), whereas Dannan and Guengerich (1982) reported nearly equivalent activity in male and female rats. Also in rats, the highest amounts of activity are found in the liver, although in female rats nearly equivalent amounts (on a gram tissue basis) are found in the liver and kidney (Dannan and Guengerich 1982).

N-octylamine (an inhibitor of cytochrome P-450-dependent MMFO) is an allosteric activator of the hog liver FMFO (the preparation traditionally used to study this enzyme), but not of the rat hepatic FMFO. The FMFO metabolizes chemicals containing thiol, sulfide, thioamide, (secondary and tertiary) amine, hydrazine, and phosphine substituents. This enzyme requires NADPH as a cofactor and is inhibited by both carbon monoxide and SKF 525-A. With these similarities to the MMFO, it can be difficult to distinguish between them and determine which enzyme system is actually playing the dominant role in the metabolism of a specific chemical, especially *in vivo*. In *in vitro* preparations, however, enzyme-specific antibodies can be used to determine the relative contributions of each system. For example, Tynes and Hodgson (1985) used antibodies to the NADPH:cytochrome c reductase to demonstrate that 100% of the thiobenzamide S-oxidation is due to the FMFO.

The implication of such findings is that for the aforementioned classes of chemicals, there might be two competing pathways of oxidative metabolism (in addition to the competing isozymic cytochrome P-450 pathways). For example, Cashman (1989) reported that verapamil is oxidized by both systems in rats; via the FMFO the main metabolite is 3,4-dimethoxystyrene and via the MMFO the main metabolite is N-desmethylverapamil. As 3,4-dimethoxystyrene is the dominant metabolite, the FMFO is the major enzyme involved in verapamil metabolism in the rat. Interestingly, the FMFO is stereoselective for the S(+)-enantiomer of verapamil, which results in steroeoselective first-pass metabolism of verapamil with shifts in the S(+)/R(-) ratio. In general, the FMFO

is probably stereoselective in the metabolism of many chemicals. In studying the metabolism of racemic mixtures of potential FMFO substrates, one should be aware of the possibility of stereoselective first-pass metabolism. As pharmacological properties of optical isomers are frequently different, stereoselective metabolism might cause changes in pharmacological actions that do not follow changes in plasma concentrations of a drug.

Epoxide Hydrolase

Epoxide hydrolase (EH) is an important microsomal enzyme in the study of xenobiotic metabolism and toxicity in the rat. The products of MMFO metabolism of many aromatic and olefinic chemicals are highly reactive arene and alkene oxides. These are, in turn, substrates for EH, which converts them to inactive dihydrodiols. EH is thus truly protective, as many of the endogenous substrates of the MMFO are oxidized to epoxides. It has been the subject of many reviews (see also the introductory chapter) such as those by Oesch (1972) and Seidegard and DePierre (1983). The enzyme has been found in every avian and mammalian species examined, including the rat. Highest activity is present in the liver, testis, kidney, ovary, and lung. Microsomal EH activity is induced in the rat by phenobarbital treatment, but only sparingly induced by 3-MC-type inducing agents. Microsomal EH is not affected by the more common MMFO inhibitors, such as SKF 525-A. In fact, some of the more common MMFO inhibitors, such as metyrapone, are allosteric activators of microsomal EH. Specific inhibitors of microsomal EH include 1,1,1-trichloropropane-2,3-oxide, and cyclohexane oxide. This enzyme also has some interesting substrate conformational specificity. For example, cis-stilbene oxide is hydrated 700 times more rapidly than the trans isomer. Treatment of rats with poor substrates like trans-stilbene oxide (400 mg/kg intraperitoneally in corn oil) was once thought to specifically induce EH. This is true only in a relative sense, as trans-stilbene will cause slight increases in MMFO activity. It, however, causes much larger increases in EH. Few, if any, chemicals induce EH without also increasing MMFO activity to some extent.

If one plans on using inducing agents to study EH, they need to be aware that the time course of the response of cytochrome P-450 and EH are different. For example, Parkinson, Thomas, Ryan, and Levin (1983) demonstrated that following treatment of 4-week-old male Long-Evans rats with Aroclor 1254 (single intraperitoneal injection, 500 mg/kg), peak total cytochrome P-450 increases occurred in 4 days, whereas maximal EH activity increase did not occur until about 10 days. The implication of this finding is that longer pretreatment periods might be required than those normally used in experiments with inducing agents (e.g., 3 days of treatment with phenobarbital tends to be common practice) if one wants assurance that maximal induction of both cytochrome P-450 and EH has occurred.

As might be expected, EH follows a similar pattern of distribution as cytochrome P-450. Thus, the centrilobular hepatocytes contain higher amounts than those of the periportal region (Seidegard and DePierre 1983). Whether or not microsomal EH has more than one isomeric form has been the subject of some debate. It was once held that the enzyme existed as a single form with a molecular weight of 50,000 but the presence of multiple forms cannot be ruled out. Guengerich et al. (1979) demonstrated that there were at least two and possibly three isozymes of EH in rat liver microsomes that could be distinguished on quantitative differences in amino acid content, activities toward different substrates, sensitivities toward different substrates and inducers, and the effectiveness of different allosteric activators. Walz et al. (1983) also identified two hepatic microsomal forms of EH. Despite such evidence, the concept of the multiplicity of EH isozymes has not been as easily digested as the same concept applied to cytochrome P-450. For example, Parkinson, Thmoas, Ryan, Reik, et al. (1983) examined the turnover time of various microsomal proteins. Isozymes of cytochrome P-450 were examined individually, but EH was described in terms of a single enzyme. No mention was made of the aforementioned article by Guengerich et al. (1979), which appeared 4 years previously. Given the broad substrate specificity and the fact that the differences between the isozymes reported by Guengerich et al. (1979) were largely more quanti-

tative than qualitative in nature, the implications of isozymic forms of EH are of more theoretical than practical concern in toxicological and pharmacological testing.

Some strain-related differences in rat hepatic EH have been identified. Oesch et al. (1983) examined microsomal EH activity with styrene 7,8-oxide (a common model substrate) in 22 different rat strains. They found that the activity was highest in Sprague-Dawley and lowest in Fischer 344 rats (4.3 vs. 12.7 nmol/min/mg protein). No qualitative differences were identified to account for these differences; they were due entirely to strain-related differences in the amounts of enzyme synthesized.

The effects of age and sex on microsomal EH have been examined to some extent in the rat. Birnbaum and Baird (1979) studied EH in male CFN rats, and reported that there were age-related increases in young (3 months), middle-age (12 months), and old (27 months) rats. They also noted that induction of EH with phenobarbital also increased with age. Chengelis (1988a) reported on the age-related changes in EH in Sprague-Dawley (Charles River) male and female rats. At 4 weeks of age, rats of both sexes had about the same activity (about 90 nmol/min/gm tissue) but it increases dramatically in males thereafter, peaking (at 340 nmol/min/g) at 78 weeks of age. In females, activity remained relatively constant throughout their lifetime. This could result in age-related shifts in the metabolite profile for a chemical in male but not in female rats. One should be aware, therefore, that the extent of EH activity, and gender-related differences, might depend on the age of the rats studied.

EH was once thought to be solely considered to be a microsomal protein, but recently, a distinct cytosolic isozyme has been identified. The activity of this enzyme (with the same model substrates) is an order of magnitude less than that of the microsomal enzyme, so its biological and toxicological significance is obscure. Levels of this enzyme, however, increase markedly in rats treated with peroxisomal-proliferating agents (Oesch et al. 1988) and might have some utility as a marker for this effect. In contrast, microsomal EH is greatly increased in hepatic hyperplastic nodules and hepatomas (Seidegard and DePierre 1983). Kizer et al. (1985) examined the induction of microsomal EH by known carcinogens, such as 2-acetylaininofluorene, thioacetamide, and aflatoxin. They noted that 3 weeks of feeding with various carcinogens that require metabolic activation resulted in four- to tenfold increases in EH activity in Holtzman rats. They recommended that the induction of EH (direct measurement of the enzyme by ELISA techniques) could be used as part of a preliminary screen for determining the carcinogenic potential of new chemical entities. In our opinion, such data are useful, but only as part of an overall package of short-term tests (including but not limited to genetic toxicology testing).

Conjugation Reactions

Conjugation reactions, or biosynthetic pathways, are those that take the products of MMFO, FMFO, or EH and add an additional functionality to form a new chemical moiety. In the past, these were frequently called phase 11 reactions, whereas the MMFO reactions were called phase I reactions. The oxidation of benzene to phenol is phase I, whereas the formation of phenol sulfate is a phase II reaction. The rat has a complete set of conjugative reactions, including (in ascending order of importance) N-acetylation, amino acid (almost always either glycine or glutamate) conjugation, sulfate conjugation, glutathione conjugation (thio-ether formation), and glucuronic acid conjugation. The reader is referred to Caldwell (1982) for a more complete description of these processes. The preference for a specific conjugation reaction is very much substrate specific. For example, Huckle et al. (1981) examined the *in vivo* metabolism of 3-phenoxybenzoic acid, and found that 5-hydroxy-3-phenoxbenzoic acid sulfate to be the major metabolite (accounting for more than 60% of the radioactivity in the urine). In contrast, Weyland and Bevan (1987) reported that the predominate conjugate metabolites of benzo(a)pyrene were thio-ethers (glutathione conjugates), whereas sulfates accounted for only 10% of the excreted metabolites. Both quantitative and qualitative differences in species also lead to further complications. In contrast to the rat, for example,

the major metabolite of 3-phenoxybenzoic acid in the guinea pig is 3-phenoxybenzoic acid glucuronide (Huckle et al. 1981). The basic apects of the classic conjugation reactions as they apply to the rat are as follows.

Amino Acid Conjugates

In general, amino acid conjugation is more highly developed in larger species, and amino acid conjugates are seldom major metabolites in rats. Amino acid conjugates are also usually quite stable and not involved in further activation reactions.

Acetylation

Although O-acetylation of hydroxyl groups (ester formation) is theoretically possible, in the rat this is apparently very rare, whereas acetylation of free amines to amides is a common reaction in rats. It is catalyzed by the cytosolic enzyme N-acetyl transferase, using acetyl-CoA as the cosubstrate. N-acetyl transferase has gathered most attention with regard to the metabolism (and activation) of carcinogenic aromatic amines and hydrazides. The acetylation of 2-aminofluorine is the first step in the activation of this chemical to an ultimate carcinogen. Interestingly, in comparison to other rodent species, the rat is a relatively poor acetylator. As reported by Lower and Bryan (1973) the hamster, guinea pig, and mouse have approximately 16, 10.5, and 9 times greater (*in vitro*) activity as the rat with 2-aminofluorene as the substrate. Lotlikar et al. (1967) reported the rat has relatively low aromatic amide hydroxylation capabilities; it is not detectable in uninduced rats. Highest activity (both baseline and induced) is found in the hamster. Hence, in the study of aromatic amine metabolism and toxicity, the hamster (not the rat) is the preferred species.

Depending on substituents, hydrazines can have a number of effects. Of most concern, however, are carcinogenicity and hepatotoxicity. Many hydrazines, hydrazones, and hydrazides have caused cancer in laboratory animals (Toth 1979). McKennis et al. (1959) first demonstrated (in rabbits) that acetylhydrazine is the final common reactive metabolite in the hepatotoxicity of both hydrazine and isoniazid. This work was further developed using the rat as a model by Timbrell et al. (1980). Single doses of acetylhydrazine (30 mg/kg) or acetylisoniazid (200 mg/kg) produced liver necrosis in rats. Isoniazid itself, however, is poorly hepatotoxic in the rat, an observation that is consistent with the observation that rats have relatively poor N-acetyltransferase activity. Rats must be pretreated with phenobarbital and then aggressively treated with (100 mg/kg every hour for 6 hr) isoniazid to develop hepatic damage. Thus, rats are apparently poor models to study hydrazine toxicity, but are good models for the study of the metabolism and toxicity of these chemicals once they are acetylated.

Sulfate Conjugates

Sulfate ester formation (e.g., phenol sulfate formation from phenol) is catalyzed by the cytosolic enzyme PAPS-sulfotransferase using 3'-phospho-adenosine-5'-phosphosulfate (PAPS) as the cosubstrate. The reaction has important toxicological involvements. For example, N-sulfo-acetyl-2-aminofluorene is the ultimate carcinogen formed from 2acetyl-aminoflurorene involving a reaction catalyzed by phenolic-specific sulfotransferase. Generally, the availability of sulfate to be activated (PAPS formulation) is the rate-limiting step (Weinshilbourn 1990). Agents that depress PAPS formation also depress sulfate formation (Hjelle et al. 1985) in the rat. Chemicals with free phenolic hydroxyl groups are the most common substrates. Hence, PAPS-sulfotransferase and UDP-glucuronosyl transferase usually compete for the same substrate, and both conjugates are frequently found together. The one that predominates will depend on the specific chemical, dose of parent chemical, and metabolic state of the rat. As a broad generalization, because of Km differences between PAPS-sulfotransferase and UDP glucuronosyl transferases, sulfates tend to

predominate at lower dosages, whereas the percentage of glucuronides increases with increases in dosage (Mulder 1986).

PAPS-sulfotransferases exist as a collection of enzymes, some with broad and some with narrow substrate specificity (Mulder 1986). Steroid hormones are the natural endogenous substrates for a specific class of sulfotransferases. As there are sex-related differences in steroidal hormones, it is not surprising that the rat displays considerable sex-related variation in sulfotransferases involved in steroid metabolism (as reviewed by Mulder 1986). Common aryl sulfotransferase (p-nitrophenol) activity is two to three times higher in the male than female rat (Matsui and Watanabe 1982). As a matter of course, sulfotransferases now tend to be classified as phenol sulfotransferase and steroid sulfotransferases (STs). Sekura and Jakoby (1979) isolated from rats two closely related phenol STs with activity toward a wide variety of xenobiotic phenolic chemicals, such as phenol, 0-naphthol, and p-nitrophenol. Maas et al. (1982) studied phenol sulfotransferase using 3-methoxy-4-hydroxyphenol glycol as the substrate. Whereas there are measurable amounts of activity in the brain and kidney, the activity in the liver is two orders of magnitude greater, and would by far make the greatest contribution to sulfate formation from xenobiotics. Hepatic activity peaks at about 10 weeks of age and is somewhat induced by dexamethasone (1 Amol/kg for 2 days). There are some slight strain-related differences in activity, but generally it falls between 25 and 50 units/mg cytosolic protein. Chengelis (1988a) examined age- and sex-related differences in Sprague-Dawley rats using P-naphthol as a substrate. No consistent sex-related differences were identified; however, males did have greater activity at 12 and 26 weeks of age, and at no point did females have statistically significant greater activity than males. Activity in both sexes peaked at 78 weeks of age. As UDP-glucuronosyl transferase peaks at an earlier time point, the ratio of sulfate to glucuronide conjugates might also change as a function of age. In contrast to Sprague-Dawley rats, at 5 months of age Fischer 344 male rats had twice the *in vitro* activity with acetaminophen (phenol transferase) than females, whereas with glycol lithocholic acid, females had five times the activity of males (Galinsky et al. 1986). Using a relatively small dose (30 mg/kg, which is not hepatotoxic), these authors observed an age-related decrease in the sulfate to glucuronide ratio in male rats. This was confirmed with a higher dose (Galinsky et al. 1986). The reasons for the lack of consistent reportedly sex-related differences for the phenol sulfotransferase are unclear, but are likely due to strain, age, and methodology (e.g., model substrate).

As mentioned, there are clear sex-related differences in the steroid sulfotransferases. Female rat liver cytosol contains at least three sulfotransferases that transform a variety of hydroxymethyl polyaromatics (e.g., 5-hydroxy-methylchrysene) to sulfates that are potent mutagens (Ogura et al. 1990). This is an interesting exception to the "rule" that conjugates are inactive end products. The dominant isozyme in this class had no activity with p-nitrophenol, but was competitively inhibited by dehydroepiandrosterone, but not 2,6-dichloro-4-nitrophenol. Interestingly, according to Ogura et al. (1990), neither hydroxymethylarenes nor the methylarenes are heptocarcinogens in rats because of the high activity of hepatic glutathione S-transferase.

In summary, the rat has an appreciable capability to form organic (ethereal) sulfates. As the rat has a high tendency to form phenolic chemicals when given aromatic-fused ring structures, one can almost always expect sulfate- (and glucuronide-) containing metabolites to be formed in the rat. Activity is classified (by isozymic specificity) as either phenolic or steroid sulfotransferases. Both, however, are capable of activity toward xenobiotics and the products can sometimes be toxic "activated metabolites."

Glucuronide Conjugates

UDP-glucuronosyl transferases are microsomal enzymes that catalyze the formation of glucuronide conjugates using uridine-diphosphoglucuronic acid (UDP-glucuronosyl) as the cosubstrate. In general, glucuronides are usually inactive end products, but this is not always the case. Smith, McDonagh, et al. (1986) have demonstrated that glucuronidation of zomepirac results in a reactive

chemical moiety that binds to macromolecules. Generally, the generation of glucuronic acid is considered the rate-limiting step in rate limiting. Gregus et al. (1988) have demonstrated that treatments that decrease UDP-glucuronate also decrease glucuronide formation. Large numbers of functional groups are capable of being glucuronidated, including hydroxyls, carboxyls, primary amines, and thiols (Burchell and Coughtrie 1989; Caldwell 1982). Substrates containing phenolic hydroxyl groups are the most commonly studied substrates. As with the sulfotransferases, different types of phenolic substrates are used to type or characterize the different isozymes. UDP glucuronosyl transferase (UDPGT) of the rat has been extensively studied. At least eight different isozymes have been isolated and characterized from rat liver (Boutin 1987; Burchell and Coughtrie 1989; Tephly et al. 1988). Known molecular weights vary from 52,000 to 56,000. Isozymes are divided between those that have primary activity against xenobiotics (4-nitrophenol, morphine, phenol, and 4-hychroxyphenol UDPGT) and those that have primary activity against endogenous substrates (17p-hydroxysteroids, 3ahydroxysteroids, bilirubin, and estrone UDPGT). In general, isozymes that prefer xenobiotic substrates have broader substrate specificity than those that prefer endogenous substrates (Chowdhury et al. 1986; Tephly et al. 1988). The activity of this system is particularly important in the rat. Glucuronides are actively transported and excreted in the bile, and the rat has an extremely efficient biliary excretion system. Enterohepatic circulation of xenobiotics and metabolites is always a factor to consider in rats. For example, indomethacin is so highly ulcerogenic in the rat as opposed to the guinea pig (Mariani and Bonanomi 1978) because the enterohepatic circulation of indomethacin results in greater exposure of the intestinal mucosa. Despite the efficiency of this species in biliary excretion, UDPGT activity in the rat is not distinctively high. Astrom et al. (1987) examined the activity of microsomal preparations against 1-naphthol for various species (rat, mouse, guinea pig, and hamster). There were not large differences between species, with mean activity from 5.9 to 12 nmol/mg/min. Guinea pigs had the highest activity. Boutin et al. (1984) examined a much larger number of substrates in five different species. Again, there was nothing either quantitatively or qualitatively distinctive about the activity in rats, and guinea pigs tended to have the highest activity.

In the rat liver, UDPGT is also a highly inducible enzyme, particularly those with isozyme activity toward exogenous substrates. It has been conclusively known since the early 1970s that phenobarbital and polycyclic aromatic hydrocarbons induce not only P-450, but UDPGT as well (Vainio et al. 1974). Mulder (1970) reported that phenobarbital increased p-nitrophenol UDPGT activity nearly 90%. Bock et al. (1988) have isolated and characterized the 3-MC-inducible form of (phenol) UDPGT, and found that 3-MC (40 mg/kg intraperitoneally in olive oil; single treatment) increased microsomal UDPGT activity with 1-naphthol almost four times, whereas having no effect with testosterone as the substrate. As with the MMFO, different types of inducers induce different enzymes. For a complete review of the inducible characteristics of UDPGT, the reader is referred to Burchell and Coughtrie (1990). The induction of UDPGT, however, is not a common mammalian characteristic, and might be distinctive to the rat. Astrom et al. (1987) reported that 3-MC caused a sixfold increase in microsomal UDPGT activity (with 1-naphthol) in male rats, had at best modest effects in guinea pigs and C57BL/6 mice (17% and 20% increase, respectively), and caused actual decreases in hamsters and DBA/2 mice (27% and 21%, respectively). The different isozymes also have different lobular distribution in the rat liver. As could be expected, those that prefer xenobiotic substrates (p-nitrophenol) have a distribution similar to the MMFO, with the largest amount in the centrilobular region. 3a- and 170-hydroxysteroid UDPGTs were uniformly spread across the lobule, with equal amounts in the centrilobular, midzonal, and periportal regions (Knapp et al. 1988).

As reviewed by Mulder (1986), male rats generally have higher hepatic UDPGT activity than female rats. There are exceptions, particularly with endogenous substrates; females, for example, have higher 17,6-steroid UDPGT activity than males. Mulder suggests that differences between sexes were quite variable; and dependent on assay conditions. This was confirmed by Astrom et al. (1987), who demonstrated that there was no difference in the microsomal activity toward 1-naphthol unless the preparations were treated with a detergent. This situation might also be

complicated because there are sex-related differences in age-related changes. Chengelis (1988a) reported that microsomal p-nitrophenol UDPGT in Sprague-Dawley rats peaked in both sexes at 39 weeks of age, but males had significantly higher activity at 12, 26, and 39 weeks of age. There were no sex-related differences after 52 weeks of age, and activity fell off precipitously in senescent rats. In contrast, while Galinsky et al. (1986) identified no age-related changes in UDPGT activity with variety of substrates, males (5 months old) had consistently higher activity than females (also 5 months old). Borghoff et al. (1988) also reported decreases in UDPGT activity in male Fischer 344 rats. Using 4,4'-thiobis(6-t-butyl-m-cresol) as a model, they detected a decrease in the amount of glucuronide formed, decreased microsomal activity *in vivo*, and age-related decreases in UDPGT as substrate. Thus, gender as well as age-related changes could play a role in glucuronide formation.

Developmental changes in UDPGT have been characterized in the rat, as reviewed by Burchell and Coughtrie (1989). For the isozymes most involved in xenobiotic metabolism, there are two developmental clusters. Activities toward planar phenolic structures (e.g., 4-nitrophenol) develop prior to birth and reach a perinatal maximum 2 days postpartum. Activity toward more bulky molecules (morphine, chloramphenicol) does not appear until after birth and reaches a perinatal maximum 25 to 30 days postpartum. These changes should be kept in mind when one is examining the metabolism or toxicity of xenobiotics in neonatal animals.

Considerable strain variation is demonstrated by UDPGT. The Gunn rat, for example, is an inbred substrain of the Wistar rat that has life-long hyperbilirubinemia because of low UDPGT activity with bilirubin. The Gunn rat has very low activity with a variety of planar phenolic substrates, and activity is not inducible by 3-MC (Buschell and Coughtrie 1989), but almost the expected level of activity with testosterone and similar substrates. Boutin et al. (1984) reported that the microsomal activity of Wistar rats with 1-naphthol was 34 nmol/min/mg and with testosterone was 6.4 nmol/min/mg. In Gunn rats, these activities were 113 and 4.3 nmol/min/mg. Other genetic variations have been described in Wistar rats; approximately 50% to 60% have high (whereas 40%–50% have low) 3hydroxysteroid UDPGT (Knapp et al. 1988). Although such differences obviously will alter the metabolism of some endogenous chemicals, the implications for the metabolism of xenobiotics is unclear. One should be aware, however, that different Wistar rats can produce unpredictably different metabolic profiles with some chemicals.

Glutathione and the Glutathione S-Transferase(s)

This system is probably the most important conjugation system in the rat liver with regard to "inactivation" of activated metabolites. Glutathione is a tripeptide (y-glutamyl cystinyl-glycine) that accounts for over 90% of nonprotein thiols in the liver (Levine 1983). From a xenobiotic viewpoint, the cystinyl group is the most important. Glutathione readily reacts (nucleophilic attack) with arene-oxides, epoxides, and aryl and alkyl halides to form thio-ethers. The oxidation of reduced glutathione (GSH) to the dithiol (GSSG) also plays an important role in protection against oxidative stress (Boyd 1980). As reviewed by Kaplowitz et al. (1985), the transport, synthesis, and activity of the glutathione redox cycle (glutathione reductase, glutathione peroxidase) acts to keep glutathione at a fairly constant level. The concentration of (reduced) glutathione in rat liver tends to range between 4 and 8 btmol/g liver (e.g., see Chengelis 1988c; Igarashi et al. 1983; Moron et al. 1979), and is present in much larger amounts than the oxidized disulfide form by an approximately a 20:1 ratio (Igarashi et al. 1983). No consistent sex-related differences in glutathione content have been reported. The effects of different inducing agents on hepatic glutathione levels have not been thoroughly explored, but at least one author has reported that treating rats with phenobarbital results in increases in glutathione (Utley and Mehendale 1989). Within the hepatocyte, glutathione exists in different distinct pools (Levine 1983). Reed (1990) recently reviewed the toxicological implication of mitochondrial, as opposed to cytosolic, glutathione. About 10% to 15% of hepatic glutathione in the rat is in the mitochondrial fraction. For example,

the cytotoxic effect of erthacrynic acid in isolated hepatocytes correlated with depletion of mitochondrial and not cytosolic glutathione.

Although glutathione is generally considered a cosubstrate in a synthetic reaction (for every mole of conjugate, 1 mole of xenobiotic and 1 mole of glutathione are consumed), glutathione can also play a catalytic role. In the rat (and probably other species as well), glutathione is involved in the metabolism of formaldehyde (Levine 1983) and dihalomethanes (Ahmed and Anders 1978). In both cases, the first step is the formation of a conjugate that is then the substrate for a final enzyme. For example, bromoethane is first conjugated (with release of one halide ion) to an s-halomethyl-glutathione intermediate; this is further metabolized by a cytosolic enzyme to form formaldehyde, another bromide ion and intact glutathione.

Additionally, although glutathione conjugates are generally inactive endp roducts, there are numerous examples of glutathione conjugates that are toxic or activated metabolites in and of themselves (Caldwell 1982; Picket and Lu 1989). Cysteine-s conjugates of tetrachloroethylene (TFE), chlorotrifluorethylene, and other chlorofluorocarbons are potent nephrotoxins in rats. *In vitro* glutathione conjugates of ethylene dibromide lead to the formation of an active metabolite (as measured by DNA adduct formation). The same results were obtained using both purified enzymes and isolated hepatocytes (human and rat), suggesting that the rat is a good predictive model for these types of activation reactions.

Glutathione conjugates are both actively further metabolized and excreted (Levine 1983) in the rat and most species. As reviewed by Picket and Lu (1989), GSH conjugates undergo stepwise cleavage (first by y-glutamyltranspeptidase, then by cysteinylglycine dipeptidase) of two amino acid residues to a cysteine conjugate. This is a break point; the cysteinyl conjugate is either acetylated (by N-acetyl transferase) to a mercapturic acid or cleaved to a free thiol (by the enzyme cysteine conjugate 6-lyase). This latter reaction has been shown to have important toxicological implications in the rat in that the free thiols formed, for example, are responsible for the nephrotoxicity discussed previously.

The reactions between glutathione and activated xenobiotics are catalyzed by the enzyme glutathione S-transferase (GSHT). The structure and molecular biology of this enzyme has been thoroughly explored, especially in the rat (Boyer 1989; Keen and Jacoby 1978; Picket and Lu 1989). In general, roughly eight different isozymes of the rat cytosolic enzyme have been identified. They represent different heterodimeric combinations of different monomeric structures. This organization contributes to the very broad substrate specificity displayed by the rat cytosolic GSHT (Keen and Jakoby 1978). A brief survey of the literature indicates that the most common model substrates used for examining total activity are 1-chloro-2,4-dinitrobenzene (CDNB), p-nitrobenzyl chloride, and 1,2-dichloro-4-nitrobenzene (DCNB). This has been used probably because they are a good substrate for the widest spectrum of isozymes.

The total activity of cytosolic GSHT is high in the rat; as much as 5% of the cytosolic protein in the rat can be GSHT. As reviewed elsewhere (Boyer 1989; Picket and Lu 1989), these macromolecules also have binding and transport rules distinct from the role they play in xenobiotic metabolism. They are extremely stable; rat cytosolic preparations can be left at refrigerator temperatures for several days with no change in GSHT activity. Rarely is GSHT activity the rate-limiting step in glutathione conjugation. Generally, the amount of glutathione is rate limiting and glutathione depletion has important metabolic and toxicological consequences. For a variety of metabolically activated hepatotoxins, cell damage does not occur until the metabolism results in glutathione depletion. In comparison to other species, the activity of GSHT in the rat is by no means the highest. Astrom et al. (1987) reported that the hamster, mouse, and guinea pig all had greater activity then the rat with CDNB as the substrate. Gregus et al. (1985) have reported that species-related differences in glutathione S-transferase can be highly substrate dependent. Perhaps the best way to summarize GSHT activity in the rat in comparison to other species is that the rat might not always have the highest activity, but it generally has good activity with a wider range of substrates than most species. Rodents, in general, tend to have higher activity than nonrodents.

Down and Chasseaud (1979), for example, compared GSHT activity in cytosolic preparations in the rat, baboon, and rhesus monkey with four different substrates; the rat had from 5 to 20 times the activity depending on substrate. As discussed elsewhere (see chapter 9), primates have higher amounts of EH than rats. As GSHT and EH serve the same deactivating role in the metabolism of potential toxins, this difference does not necessarily result in phylogenetic differences in toxicity, but can result in predictable differences in metabolites formed.

Hepatic GSHT in the rat is influenced by a variety of factors such as age, sex, strain, and diet. As reviewed by Mulder (1986), data on sex-related differences have been highly complex, and often dependent on the substrate examined and the age of the rat. Chengelis (1988a) examined age- and sex-related changes in hepatic GSHT in Sprague-Dawley rats with two substrates (p-nitrobenzyl chloride and CDNB). Activity was greater with both in males than females only in rats 26 through 78 weeks of age; there were no significant differences in young (4–12 weeks) or senescent rats (104 weeks of age). In both sexes, activity peaked at 52 weeks of age. This is consistent with the earlier findings of Igarashi et al. (1983), who also demonstrated that there were no sex-related differences in GSHT activity in young adult rats. The different monomeric subunits are evidently under different controlling influences that complicate generalizations about sex-related differences in the rat. For example, Hales et al. (1982) reported that orchidectomy resulted in decreased activity toward three substrates, but an increase toward a fourth. James and Pheasant (1978) reported that GSHT activity is very low in neonatal rats. Given the complex tapestry of such controls, it is sometimes difficult to predict the extent glutathione conjugation will play in the metabolism of a specific chemical in the rat, and the presence or (absence) thereof will always have to be confirmed experimentally.

In the rat, GSHT is also an inducible enzyme, although (as could be expected) the different monomers are differentially affected by different agents. Baars et al. (1978) examined the effect of phenobarbital, 3-MC, and tetrachlorodibenzo-p-dioxin (TCDD) treatment of rats on cytosolic GSHT activity with three different substrates (styrene oxide, 1,2-butylene oxide, and CDNB). In general, although all three treatments caused increases in activity with all three substrates, 3-MC appeared to be the best inducing agents. Down and Chasseaud (1979) reported that DDT was a better inducing agent than phenobarbital, and that neither agent increased GSHT activity in non-human primates. Astrom et al. (1987) compared the inducing effect of 3-MC on GSHT activity (with CDNB) in four different rodent species; increases were induced in the rat and hamster, but not the mouse or guinea pig. In general, induction of GSHT activity is a trait seen in relatively few species, including the rat, where only 3-MC and DDT appear to induce at best relatively modest increases in this type of activity.

Inhibition of GSHT can occur; for example, Aitio and Bend (1979) reported on the inhibitory effect of various common solvents (e.g., ethanol). Dierickx (1982) reported on the inhibitory effect of divalent metal ions (such as Hg2l). Jakobson et al. (1979) reported that oxidized glutathione (GSSG) and S-octylglutathione are effective inhibitors of GSHT. As a tool in the study of xenobiotic metabolism, inhibition of GSHT in the rat has not been widely used. This is probably because the enzyme has such high activity that partial inhibition has little effect on actual conjugate formation. As mentioned, glutathione availability tends to be the limiting factor in glutathione adduct formation. Most investigators have relied on glutathione depletion not GSHT inhibition to study the involvement of this system in metabolism and toxicity. Dielthylinalonate is a substrate for GSHT (Early and Schnell 1972) that rapidly depletes hepatic glutathione, and, therefore, is frequently used in studies that require glutathione depletion. For example, Chengelis (1988c) reports that diethylinalonate (640 mg/kg intraperitoneally, neat) resulted in an 86% decrease in hepatic glutathione content in 60 min.

Not all the GSHT isozymes are present in the rat liver under all circumstances. Ito and colleagues (1989) have identified a placental form that is expressed in altered hepatic or preneoplastic foci in rat liver. They have demonstrated that this is an excellent marker for these types of changes, and has the advantages of being detectable (by immunohistochemical staining) in formalin-fixed tissues.

There is also ample evidence that there is a distinct microsomal GSHT in the rat (Boyer 1989). Although Boyer has suggested that this enzyme plays an important role in xenobiotic metabolism, the full implications of this enzyme in metabolism and toxicity in the rat remain to be clarified.

Other Enzymes

As mentioned in the introduction to this section, all species have a wide variety of esterases. For example, 15 different isozymes have been described for the rat brain and five for the rat liver (Leinweber 1987). Any ester given to a rat will be rapidly hydrolyzed. Like all other species, the rat possesses alcohol dehydrogenase (ADH). Unlike the situation in other species, however, methanol is not a substrate for rat ADH, being oxidized to formate by the catalase/hydrogen peroxide system (Tephly et al. 1964).

Extrahepatic Xenobiotic Metabolism

That various tissues other than liver possess xenobiotic-metabolizing ability has been recognized for quite some time (Litterst et al. 1975). In general, given the relatively small size of these organs and the relatively low activity of the salient enzymes (compared to the liver), extrahepatic metabolism rarely plays a major role in the overall disposition of xenobiotics. For example, Litterst et al. (1975) noted that the lungs had only 7% of the cytochrome P-450 of the liver on a microsomal basis and only 35% of the microsomal protein of the liver in the rat. A possible exception to this generalization is the intestine. The presence of the gut flora gives this organ considerable hydrolytic and reductive capability, which plays a major role in enterophepatic circulation. This is especially true in the rat, as it has a very effective biliary excretory capacity. The organs most frequently examined are the lung, liver, kidney, adrenal, and GI tract. Although the metabolic capabilities of these organs might not necessarily play a major role in xenobiotic disposition, they can play major roles in target organ toxicity. This short review highlights some examples of this phenomenon in the rat.

Adrenal Cortex

The adrenal cortex is a steroidogenic organ and, therefore, actually quite rich in cytochrome P-450-dependent MMFO. (The testis and ovary are also quite rich in these enzymes, but are not discussed here.) There are two different systems: microsomal and mitochondrial. The latter system is distinctive because of the presence of an additional protein, adrenodoxin, in the MMFO system, which serves as the electron carrier between NADPH:cytochrome P-450 reductase and the hemoprotein. These systems are primarily involved in the production of glucocorticoid and mineralocorticoid by this organ. As a generality, the toxicity associated with the MMFO in the adrenal gland is not the production of toxic reactive intermediates but is secondary to the effects of xenobiotics on adrenal hormone production. For example, 7a-thiosteroids, such as spironolactone, are suicide substrates and cause the destruction of adrenal cytochrome P-450 (Menard et al. 1979; Sherry et al. 1988). Ketoconazole also blocks adrenal steroidogenesis by inhibiting cytochrome P-450-dependent activities (Loose et al. 1983). Veltman and Maines (1986a) reported that relatively small doses of mercury can cause major alterations in adrenal MMFO activity. A single dose of HgC12 (30 nmol/kg subcutaneous) in male rats caused (24-hr) increases in adrenal mitochondrial cytochrome P-450 accompanied by increases in 1 1 P-steroid hydroxylase and side-chain cleavage activities. In contrast, adrenal microsomal cytochrome P-450 was decreased, accompanied by decreases in 2la-steroid hydroxylase activity. These effects would result in decreased corticosterone production and increased progesterone production. In a continuation on their investigations on heavy metals and adrenal hormone formation, Veltman and Maines (1986b) also reported that 7 days of treatment with cupric chloride significantly decreased 1 1 0-hydroxylase activity, which resulted in a decreased

plasma level of corticosterone. The metabolic concerns associated with TCDD are normally focused on the liver, but Mebus and Piper (1986) have demonstrated that TCDD can cause decreases in adrenal 2la-hydroxylase activity. Brownie et al. (1988) reported that methylandrostenediol treatment decreased 1 1flhydroxylase activity and that, therefore, the resulting accumulation of 1 1 p-deoxycorticosterone might play a major role in androgen-induced hypertension in rats.

Hence, there are numerous examples of xenobiotics exerting potential toxic effects by disrupting adrenal steroid hormone production in the rat. It is important to note that such effects are not always accompanied by morphological evidence of adrenal damage. Unless one specifically examines for effects on adrenal steroidogenesis, these inhibitory effects on adrenal MMFO are difficult to assess in the context of a routine toxicity study.

Gastrointestinal Tract

The GI tract has two separate identifiable sources of xenobiotic metabolizing capability: that associated with the gut flora and that associated with the mucosa. As reviewed by Rowland (1988), the reactions catalyzed by the gut bacteria are largely hydrolytic and reductive in nature. In comparison to other species, rats tend to have the highest activity in 13-glucuronidase, but also have high nitrate, nitrite, and azo-reductase activities. The high 8-glucuronidase activity doubtlessly contributes to the high tendency of rats toward the enterohepatic circulation of xenobiotic metabolites. The metabolism of metronidazole by the rat provides an example of the important role that the gut flora can play in the metabolism of a synthetic chemical. N-(2-hydroxyethyl) oxamic acid is formed from metronidazole via the reductive action of intestinal bacteria. When metronidazole is given to routinely maintained laboratory rats N-(2-hydroxyethyl), oxamic acid is excreted in the urine, whereas none is detected in the urine of germ-free (gnotobiotic) rats (Koch and Goldman 1979; Yeung et al. 1983).

The GI mucosa itself has low but detectable cytochrome P-450-dependent MMFO activity in the rat. The relationship between intestinal MMFO activity and chemical carcinogenesis has attracted considerable attention. Strobel and coworkers (1980; Oshinsky and Strobel 1987) and Tamura et al. (1987) reported that isolated gut mucosal microsomes are capable of the metabolism of many of the same model substrates (e.g., ethylmorphine, p-nitroanisole) as the liver, including the carcinogen benzo(a)pyrene. The system was induced by both B-naphthaflavone and phenobarbital, and inhibited by both SKF 525-A and 7,8benzoflavone. The system produced positive results against known mutagens that required metabolic activation in the Ames assay. In fact, MMFO activity has been found along the entire intestinal tract in the rat; Bonkovsky et al. (1985) and Lindeskog et al. (1986) characterized small but measurable amounts of phenobarbital-inducible cytochrome P-450 in the small intestine. Pascoe and Correia (1985) demonstrated that intestinal cytochrome P-450 is regulated by both dietary selenium and iron concentrations; deprivation of either or both can lead to decreases in cytochrome P-450. Hence, it is clear that the GI tract of the rat possesses the ability to activate chemical carcinogens. Intestinal MMFO can be manipulated in the same manner (with regard to inducers and inhibitors) as the liver MMFO to study the activation of suspected GI carcinogens *in vivo* or *in vitro*.

Kidney

Litterst et al. (1975) was among the first to publish on the MMFO of the kidney. On a microsomal protein basis, the rat kidney has about 13% of the cytochrome P-450 of the liver, but only about 6% of the activity with aminopyrine, and scantly detectable activity with aniline. Endou (1983) examined the distribution of cytochrome P-450 along the nephron in rats, and found that it was localized only in the proximal tubule. Within the proximal tubule, the straight segment possesses higher amounts than the convoluted tubules. This was confirmed by Sugita et al. (1988); they also demonstrated that starvation induced cytochrome P-450 in the convoluted tubule, whereas 3-MC

induced preferentially in the straight portion. Cojocel et al. (1988) demonstrated that cephladoxin rather specifically causes the depletion of cytochrome P-450 in the rat kidney cortex. Babany et al. (1985) and Barry et al. (1987) explored the relationship between renal and hepatic MMFO activity, and noted that procedures (e.g., inhibition of the hepatic MMFO, partial hepatectomy, cholestasis, and biliary cirrhosis) that decrease MMFO activity resulted in increased renal MMFO. These reports suggest that there is an inverse relationship between activities of the hepatic and renal MMFO.

In an interesting similarity to the liver, the kidney also develops preneoplastic lesions that have different enzyme concentrations and staining characteristics than the surrounding normal tissue. Tsuda et al. (1987) demonstrated that the preneoplastic lesions induced by N-ethyl-N-hydroxyethylnitrosamine in the kidney and liver differed in that in the liver these foci contained decreases in all forms of cytochrome P-450 examined and increases in EH, whereas in the kidney the altered foci contained increased levels of the cytochrome P-450 isozyme PB3a and decreases in EH. This observation also demonstrates that the rat kidney also has considerable EH activity. Nephrotoxic doses of HgC12 induce large increases in renal EH activity. Greater increases were observed in Sprague-Dawley than in Fischer 344 rats. Whether this indicates a role for WH in HgC12 nephrotoxicity, or whether this is a generalized response to nephrotoxicity remains to be established.

Not only is the renal cytochrome P-450 localized in the proximal tubule, but also anatomically located in the renal cortex of the rat and probably other species as well. Thus, agents that require metabolic activation via the MMFO tend to produce renal damage that is restricted to the renal cortex. Acetaminophen, for example, causes renal cortical necrosis in rats (Newton et al. 1985). In addition, Fischer 344 rats are more susceptible, whereas Sprague-Dawley rats are resistant. Newton et al. (1985) reported that when rats were treated with radiolabeled acetaminophen, the extent of covalently bound radioactivity in the renal cortex was much higher in Fischer 344 than in Sprague-Dawley rats. These results clearly suggest that acetaminophen nephrotoxicity in the rat is due to metabolic activation, and that strain-related differences are due to strain-related differences in renal MMFO activity. Beierschmitt et al. (1986) reported that there were also age-related differences in the sensitivity of Fischer 344 rats to acetaminophen toxicity. At 600 mg/kg (intraperitoneally), acetaminophen caused severe renal lesions in old (22–25 months of age) rats, but no evidence of damage in 2- to 4-month-old rats and only intermediate damage in middle-aged (12–15 months) rats. Tarloff et al. (1988) confirmed this and further demonstrated that the aforementioned strain-related differences between Sprague-Dawley and Fischer 344 rats became less noticeable as the animals aged. At 12 months of age, rats of both strains have equivalent sensitivity to acetaminophen nephrotoxicity, a finding not necessarily due to age-related differences in renal MMFO activity.

Lungs

Relative to the rat liver, the rat lung has even less MMFO activity than the kidneys. According to Litterst et al. (1975), the lung has only about 7% of the cytochrome P-450 of the liver (on a microsomal protein basis) and also only 35% of the microsomal protein. As reviewed by Mitchell and Boyd (1983), however, there is ample evidence that the lung has sufficient MMFO activity to generate toxic reactive intermediate. In fact, for a few chemicals, such as 4-ipomeanol, the enzyme kinetic constants are such that MMFO metabolism in the lung is favored over metabolism in the liver, so that toxicity is restricted to the lung (Mitchell and Boyd 1983). Garst et al. (1985) demonstrated a positive correlation between pulmonary cytochrome P-450 content and species differences in the pulmonary toxicity of 4-ipomeanol; the rat tends to be among the most sensitive of species.

Also as reviewed by Mitchell and Boyd (1983) and Guengerich (1990), the lung is extremely heterogeneous with regard to cell type. The Clara cells tend to be relatively rich, whereas type I epithelial cells are devoid of MMFO activity. Keith et al. (1987) have demonstrated the presence of specific cytochrome P-450 isozymes in both Clara and type II epithelial cells. Hence, toxins that

required activation tend to have these two cell types as targets. The molecular biology and enzymology of pulmonary cytochrome P-450 has been most thoroughly explored in the rabbit, but some work has been completed in the rat (Guengerich 1990). Rat pulmonary cytochrome P-450 exists as several different isozymes (some of which are indistinguishable from the corresponding isozymes of the liver). Pulmonary cytochrome P-450 in the rat is induced by chemicals such as phenobarbital and 3-MC, and different isozymes are induced by different classes of inducers. For example, Robinson et al. (1986) reported that 3-MC induction increases the levels of a pulmonary cytochrome P-450 that is identical to 3-MC-induced hepatic cytochrome P-450. Rampersaud and Walz (1986) demonstrated that rat lung contains at least six different cytochrome P-450s, one of which is cytochrome P-450b, whereas cytochrome P-450e (which is coinduced in the liver with cytochrome P-450b) was not induced under any circumstances in the lung. Hence, despite the similarities, the genetic controls over pulmonary cytochrome P-450 differ somewhat from those of the liver.

In a fashion similar to the liver, however, different isozymes show different sensitivities to different inhibitors. For example, Rabovsky and Judy (1989) reported that rat pulmonary MMFO activity toward benzyloxyphenoxazone was exquisitely sensitive *in vitro* to I-octanol inhibition (IC50 ~ –3.8 pM), whereas activity toward ethoxyphenoxazone was completely unaffected by I-octanol (at the limits of solubility). Naslund and Halpert (1984) reported that chloramphenicol (100 mg/kg intraperitoneally or IV) is a selective suicide substrate for the pulmonary cytochrome P-450 isozyme involved in the 2-hydroxylation of n-hexane. Elovaara et al. (1987) reported that inhalation of m-xylene (300 ppm) resulted in the selective destruction of pulmonary cytochrome P-450 in rats with no effects on any other enzyme system and visible morphological changes. Pyykko et al. (1987) reported that various substituted benzenes also caused decreases in pulmonary cytochrome P-450 and 7-ethoxycoumarin deethylation activity, but increases in 7-ethoxyresorufin deethylation activity. Rietjens et al. (1988) reported that prolonged (7 days) exposure of rats to ozone (by inhalation: 1.6 Mg/M^3) results in increases in cytochrome P-450 owing to proliferation of Clara cells. There was, however, a shift in cytochrome P-450 isozymes such that activity toward 7-ethoxycoumarin decreases, whereas activity toward 7-pentoxyresorufin increased. Thus, the MMFO of the rat lung is localized to two main cell types, and (like that of the liver) is controlled by different cytochrome P-450 isozymes. These different isozymes can be induced or inhibited by a variety of different chemical treatments.

Most of this discussion has focused on the pulmonary MMFO. The rat lung, however, also has appreciable flavin-dependent MMFO activity (Tynes and Hodgson 1983; Ziegler 1988). This system has been shown to play an important role in the species selectivity of different rodenticides. For example, a-naphthylthiurea (ANTU) causes pulmonary edema in the rat, but not in larger species. Evidently, this difference is due to species differences in activity toward ANTU (and the formation of reactive metabolites), as reviewed by Mitchell and Boyd (1983).

Xenobiotic metabolism by the lung has important implications in toxicology and (based on the preceding paragraph) the rat is an appropriate model for the study of this system. The first concern has to do with localized (pulmonary) toxicity due to the formation of reactive metabolites. Inhalation can result in direct exposure of the lung to relatively high (enzyme-saturating) amounts of potential toxins (although inhalation is not required for a chemical to be a pulmonary toxin, e.g., 4-ipomeanol). For example, 2-nitrofluorene is a potent carcinogen that is rapidly converted *in vitro* to the potent mutagen (and presumed ultimate carcinogen) 9-hydroxy-2-nitrofluorene by rat lung microsomes (Tomquist et al. 1988). Agents that induce pulmonary MMFO activity will result in increased pulmonary sensitivity to procarcinogens. The second concern with regard to pulmonary MMFO activity has to do with pulmonary first-pass metabolism of chemicals administered by inhalation. Metabolism by the lung can influence toxicity at more distal sites. Hexane, for example, is metabolized to a neurotoxic chemical, 2,5-hexanediol. Toftgard et al. (1986) reported that the lungs can facilitate the formation of this metabolite by catalyzing the formation of 2-hexanol, which is then transported to the liver for further metabolism to 2,5-hexanediol. These implications are of concern because for the majority of the general human population, inhalation is one of the major

routes of incidental exposure to environmental and occupational chemical hazards. Thus, chemicals that influence pulmonary MMFO activity could have potentially serious human health implications. As noted, pulmonary MMFO activity can be altered under circumstances where no other noticeable effects occur, and this is the type of effect that would be easily missed in a traditional toxicology study. The rat would provide a good model for the study of the toxicological implications of pulmonary MMFO induction or inhibition in a tier 2 or single endpoint type of study.

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CHAPTER 4

The Hamster

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CONTENTS

TOXICOLOGY

The hamster is the third most frequently used laboratory animal following the rat and mouse (Renshaw, 1975), though its use in toxicology is somewhat limited. While historically the hamster saw extensive use in carcionogenesis testing, as will be overviewed, this has changed. It has many beneficial features as a laboratory animal because of its unique anatomical and physical features, reproductive ease, rapid physiological development, low incidence of spontaneous diseases, short life span, and a high susceptibility to induced pathological agents. Hamsters historically have been used in several fields, especially in carcinogenesis because of its low incidence of spontaneous tumors, but currently see most of their use in testing associated with buccal delivery of drugs. Hamsters have also been a contributing agent in blood vessel physiology because their cheek pouches with thin vascularized walls are very accessible. The hamster is also a major model in diabetes research.

Taxonomy and History

The hamster belongs in the subfamily Cricetinae of the family Cricetidae in the order Rodentia. Members of Cricetidae (also including the lemming, deer mice, and gerbils) are characterized by short legs, thick bodies, and large cheek pouches that are used to transport and store food. They have incisors that grow continuously and cuspidate molars which do not. Of the 50 species of hamsters in the Cricetinae, eight species are used in medical research.

Species

The following discussion involves the eight hamster species used in the laboratory. Table 4.1 lists these hamsters' common and scientific names and their chromosome numbers.

Table 4.1 Common and Species Names and Chromosome Number

Common Name	Species Name	Chromosome Number
Syrian (Golden)	*Mescricetus auratus*	44
Chinese (Striped, Black)	*Cricetus griseus or barabensis*	22
European (Common, Black, Field)	*Cricetus cricetus*	22
South African	*Mystromys albicaudatus*	32
Rumanian (Newtoni's)	*Mesocritceus newtoni*	38
Turkish (Kurdanti)	*Mesocricetus auratus*	42/44
Armenian (Gray, Migratory)	*Crectulus migratorius*	22
Dzungarian (Hairy-footed)	*Phodopus sungorus*	28

Syrian Hamster

Eighty percent of all hamsters used in research are of this species, making them the most common laboratory hamster. The remaining 20% are primarily Chinese, followed distantly by European, Armenian, Rumanian, Turkish, South African, and Dzungarian hamsters. The Syrian hamster was first described as a new species (Cricetus auratus) in 1839, and was originally native to the arid, temperate regions of Southeast Europe and Asia Minor. For almost 100 years, no hamsters were caught in the wild. The only evidence that the species existed was the preservation in alcohol of two hamsters, one in London and the other in Beirut. The hamsters were finally obtained from the wild starting in the 1900s, and have since been breed easily in captivity. The hamster lives in deep tunnels which ensure cool temperatures and increased humidity and is a nocturnal animal. The Syrian is virtually tailless and has smooth short hair with normal coloration of reddish gold with a grayish white ventral portion. The dorsal side may have a black stripe. The ears are pointed with dark coloration and the eyes are small and dark. The average life span is two years, but these animals can live up to three years. The hamster is 14–19 cm in length and weighs 114–140 g at adulthood. The female hamster is usually larger and longer than the male.

The Syrian hamster was first used in the laboratory in 1930 to study the Mediterranean disease kala-azar. Israel Aharoni (Hebrew University, Jerusalem, Israel) collected 11 young golden hamsters from Syria in 1930 while on a zoological expedition. The litter with their mother had been found in their burrow 2.5 meters under a wheat field. Aharoni and his wife kept the hamsters in their house until one night when they all escaped. Nine of these hamsters were recovered and given to the animal facilities supervisor of the Weizmann & Seiff Institute, Jerusalem, Israel. Five of these nine escaped the first night in the new facility, leaving only one female. After mating, the female gave birth to a litter of healthy pups. After a year's time, these hamsters produced more than 300 offspring and are today's ancestors of the laboratory-bred Syrian hamster. There is no record of any other captures of Syrian hamsters from the wild population.

The Syrian hamster has been involved in endocrinology, oncology, virology, physiology, parasitology, genetics, and pharmacology research. The cheek pouch of the Syrian hamster has provided the physiological technology for studying microcirculation and the growth of human tumors.

Chinese Hamster

The Chinese hamster is a native species to China. This hamster weighs 39–46 grams and is 9 cm long at adulthood. Its life span is approximately 2.5–3.0 years under standard laboratory conditions. Though the Chinese hamster is smaller than the Syrian, its testicles, spleen, and brain are larger.

The Chinese hamster was used for the first time in 1919. Hamsters were used to determine the best therapy for the patients with pneumonia due to the fact that mice were extremely scarce at the time. In addition, the Chinese hamster was also used to study TB, influenza, diphtheria, and rabies. In December 1948 (right before the Communist takeover of China), Robert B. Watson was given 10 female and 10 male hamsters from C. H. Hu of the Peking Union Medical College. After being moved from China to San Francisco, the hamsters ended up in New York. V. Schwenter of the

Harvard Medical School obtained the hamsters and successfully bred them in the laboratory. Of the original 20 hamsters, four of the females and three of the males produced offspring, givingrise to the present laboratory Chinese hamster population. The Harvard colony has since become extinct; however, colonies were established at the Upjohn Company (which became Pharmacia and most recently was aquired by Pfizer), Kalamazoo, Michigan, and the C. H. Best Institute in Toronto, Ontario, in the 1960s.

The Chinese hamster has been used primarily in research for cytogenetics because of its low chromosome number (Fenner, 1986). It is also used in diabetes mellitus because (a) some strains have very high incidences of the disease and (b) the course of the disease in this species is similar to that seen in humans.

European Hamster

The European hamster was originally found in a West Germany industrial area, with its natural habitat in the lowlands of Central and Eastern Europe. The European hamster is a very aggressive animal, and in the wild each adult lives in its individual burrow. This hamster has a body that is dorsally reddish brown and ventrally black with white patches laterally and has a white face and feet. They are about the size of a guinea pig, averaging 27–32 and 22–25 cm in length and weighing 450 and 350 g for males and females, respectively. Males reach sexual maturity at 60 days of age, whereas females at 80–90 days of age. In the wild, it hibernates in the winter months and is mainly a seed eater. In their natural habitat, European hamsters can live up to 8 years, whereas under laboratory conditions, the average life span is 5 years. This lifetime decrease is believed to be due to the lack of hibernation afforded a laboratory-raised European hamster (Mohr et al., 1987).

The European hamster has been used primarily in hibernation studies and in inhalation studies because its tidal volumes are the largest of any laboratory rodent species.

Armenian Hamster

The Armenian hamster is originally from the Union of the Soviet Socialist Republic (U.S.S.R.). Its body size, care weight, and maintenance are comparable to that of the Chinese hamster. The Armenian hamster was first introduced as a laboratory animal in 1963. It was first brought to the United States as a part of the U.S.A.–U.S.S.R. Cultural Exchange Program. Scientists in the United States wanted to find more species similar to the dwarf hamster (like the Chinese hamster), and the Armenian species is one of the only species found. The main applications of the Armenian hamster can be found in the areas of cytogenetics and oncology, although otherwise its use if fairly limited.

Turkish Hamster

The Turkish hamster is native to Iran and Turkey and was originally trapped in 1962. At adulthood, its average body weight is 150 grams and its typical life span is a little less than 2 years, though they have lived as long as 4 years. These hamsters interbred readily and produce offspring with a diploid number of 44. Some populations of the Turkish hamster have a diploid number of 42 chromosomes and others have 44. Hamsters with a diploid number of 42 hibernate less than those with 44. Other than hibernation research, Turkish hamsters have been used in immunology, genetics, and reproductive behavior research (Yerganian, 1972; Cantrell et al., 1987).

Rumanian Hamster

The Rumanian hamster is native to the Bucharest area and is used in the surrounding laboratories. It was initially trapped and described in 1965. Its size and management are similar to that of the

Syrian hamster, though it does not breed as well as the Syrian. The Rumanian hamster adult averages 100 grams with its face being more pointed than the Syrian hamster, but comparable in appearance to the Turkish hamster.

Dzungarian Hamster

The Dzungarian hamster used for research in photoperiodism and thermal regulation, native to the U.S.S.R., was first trapped in Siberia and provided to the United States by the U.S.S.R. The current Dzungarian hamster population is the result of mating one female to two males who were domesticated in 1965.

The Dzungarian hamster is a very timid species with the males being 11 cm long and 40–50 gm in weight and the females are 9 cm long and weigh 30 g at maturity. The Dzungarian hamster has a short tail about 1 cm in length, which is usually concealed by the body fur. The fur on the dorsal side is gray with a dark-brown or black stripe from the nape of the neck to the base of the tail and the ventral fur is white. The average life span has been reported to be 1 year by Herberg et al. (1980) and 2 years by Heldmaier and Steinlechner (1981).

South African Hamster

The South African is the only member of its genus and the only hamster native to Africa. The first colony was established in South Africa in 1941. South African hamsters are nocturnal, solitary burrowing rodents in their natural habitats. Distinct form other hamsters, it lacks cheek pouches. The hamster has gray to brown fur on its dorsal aspect with white on the ventral surface, feet, and tail. The tail is 5–8 cm long and its ears are erect with dark, bright eyes. Adult males and females weigh 145 and 95 g, respectively (Hall et al., 1967). The typical life span is 2.4 years, with a maximum life span of 6.2 years (Davis, 1963).

Husbandry

The reader is referred to Field et al. (1999) for a complete source in this area.

Housing, Caging, and Bedding

Hamsters should be housed individualy unless they have been housed together since weanlings. If raised together, hamsters will sometimes fight, though they tend not to cause each other too much physical harm (if of the same sex and if there are no offspring present). European hamsters should be housed one animal per cage and only brought together for mating purposes.

Caging requirements are illustrated in Table 4.2. Caging material is usually composed of rigid plastic (polycarbonate, polystyrene, and polypropylene), galvanized metal, glass, stainless steel, and hard alloys of aluminum, but never wood (it will only take a hamster a short time chew through wood). Cages may have solid or open wire mesh bottoms. Hamsters tend to have fewer stress-related deaths and more rapid growth in solid-bottom cages with direct bedding.

If solid-bottom cages are used, bedding materials should be provided in the cage so that the hamster is able to build a nest.

Pregnant females should be housed separately in solid-bottom cages with a caging material as described above and not be housed in suspended cages with open wire mesh bottoms. A nursing female and her young should have at least 121 in^2 of floor space. With dwarf hamsters, there should be at least 25 in^2 of floor space for the mother and her young.

Table 4.2 Space Recommendations and Regulations for Hamsters

Recommendations of the Laboratory Animal Resources National Research Council Guide for the Care and Use of Laboratory Animals (National Research Council, 1996)

Weight of animal (g)	Type of housing	Floor area/animal (cm^2)	Height (cm^2)
<60	Cage	64.5 (10 in^2)	15.24 (6.0 in^2)
60–80	Cage	83.9 (13.0 in^2)	15.24 (6.0 in^2)
81–100	Cage	103.2 (16.0 in^2)	15.24 (6.0 in^2)
>100	Cage	122.6 (19 in^2)	15.24 (6.0 in^2)

Regulations of Animal Welfare Act (PL89-544 as amended PL91-579) and the Animal Welfare Act, Code of Federal Regulations (1985).

Age	Minimum space per hamster (in^2)		Maximum population/enclosure
	Dwarf	Other	
Weanling to 5 weeks	5.0	10.3	20
5–10 weeks	7.5	12.5	16
≥10 weeks	9.0	15.0	13

The interior height of the cage should be 5.5 in for hamsters other than the dwarfs and 5 in for the dwarfs.

Temperature, Humidity, and Lighting Requirements

Hamsters are generally more adversely affected by higher temperatures than lower ones. Temperature ranges for the nonbreeding hamster are 69–75°F (20–24°C) (Fox, 1980) and for breeding 72–74°F. If temperatures drop below 4°C, the hamster will begin to hibernate (Schermer, 1967).

Humidity requirements are 40–60% with a lighting cycle of 12 hours light and 12 hours dark. This lighting also fulfills the requirement necessary for breeding. In uncontrolled light and temperature environments, failures in hamster reproduction have been observed in winter owing to decreased light and in the summer to increased temperatures.

Water and Diet

Water

Water should be available *ad libitum*. Water can be provided by an automatic watering system with a lixit accessible to the smallest hamster or by a water bottle with a sipper tube. Fluid requirements are 30 ml/day for the Syrian hamster, 11–13 ml/100 g/day for the Chinese hamster, and 5 ml/100 g/day for the European hamster.

Diet

The optimal diet for a hamster includes approximately 16–24% protein, 60–65% carbohydrates, and 5–7% fat. Most animal facilities use standard rodent chow for hamsters, athough hamsters do have a higher requirement for zinc (0.6%), copper (10 ppm), and potassium (20 ppm) than rats (Newberne et al., 1979). Copper may need to be increased during studies when the hamster may be extremely stressed, and should be considered by the principal investigator. Some studies suggest that a soybean meal may offer improved nutritional efficiency for hamsters. During lactation, nutritional requirements for female hamsters may increase, such as is seen in the rat. The female will show signs of extreme weight loss and maternal cannibalism during lactation if not enough nutrition is being provided.

Hamsters start eating solid food at days 7–10 of age. Syrian hamsters consume 5.5–7.0 g of food during their growth and development. Adult and pregnant hamsters usually consume 10–15 g/day. Unlike rats, males and females consume similar amounts of food. Hamsters, like other rodents, are coprophagic.

Feed is usually provided *ad libitum*. Hamsters do the majority of their eating at night, usually bingeing every 2 hours. Hamsters are hoarding animals and, therefore, will remove the food pellets

from the feeder and pile them in a corner of the cage which are usually opposite of the corner used for urination and defecation. Because of this trait, exact feed consumption for hamsters is difficult to determine. Feeders should have slots large enough to allow the hamster with its broad muzzle access to the food.

If hamsters are fasted for several hours or up to 4 days, they do not increase their food intake to compensate for the loss. If food is only available a certain time each day, a hamster will eat exactly as much as they would ordinarily eat if the food were *ad libitum*; however, hoarding activity is increased. This is in sharp contrast to the rat, which will compensate for periods of fasting.

Diseases and Spontaneous Tumors

Amyloidosis

Amyloidosis (a noninfectious disease that occurs in aging hamsters) is the principle cause of death of hamsters in long-term studies (Renshaw, 1975). In one report, a colony had an 88% incidence of the disease after 18 months of age. The onset of the disease may be due to a deficiency in the immune system because the first histopathological sign is deposition of immune globulins in blood vessels. Edema, proteinuria, hypercholesteremia, and ascites have also been associated with the disease.

The occurance of amyloidosis varies from colony-to-colony. It can be found in the liver, kidney, stomach, adrenal, thyroid, and spleen. The clinical signs and histopathological findings are similar to that seen in humans with the nephrotic syndrome.

Amyloidosis can be induced in adult hamsters by daily 1-ml sc injections of 50% casein Hammerstein in 0.3 M $NaHC0_3$, pH 7.5 (Gruys et al., 1979). It can also be induced in animals treated with diethylstilbestrol.

Antibiotic-Associated Enterocolitis

Following treatment with gram-positive selective antibiotics, moribundity and mortality have been reported in hamsters. The cause of the disease may be due to the transformation of the intestinal microflora, which coupled with the small size of the animals may compromise their normal homeostasis.

Calcinosis

Calcinosis is characterized by mineralization of connective tissue in nearly every organ of the hamster and is seen most particularly in the arteries. The initial stages of the disease are characterized by the precipitation of mineralized material in the elastic fibers in the arteries. In the later stage of the disease, the lesions can spread to larger areas of the vessel.

Multifocal Retinal Dysplasia

Multifocal retinal dysplasia is found to have a 2% occurrence in LAK:LVG Syrian hamsters. This disease is diagnosed through ophthalmoscopic examination in hamsters from 6 weeks of age to 9 months. Using the Ophthalmoscope or slit lamp, the dysplasia foci are seen as either retinal streaks or small, circular areas of cream-colored depigmentation. Histologically, the focal dysplasias are manifested as invaginations and rosettelike structures composed of elements of the photoreceptor layer, outer-limiting membrane, and outer nuclear layer (Schiavo, 1980).

Polycystic Disease

Hamsters have a cyst incidence of 76% at ages over 1 year (Gleiser et al., 1970). The cyst occur most commonly in the liver (Renshaw, 1975). The lesions appear to be due to congenital defects

of normal ductal structures such as the bile duct (Van Hoosier et al., 1984). No associated clinical signs have been reported.

Hamster Enteritis

Hamster enteritis, or wet tail disease, has also been called proliferative ileitis, terminal ileitis, and enzootic adenocaracinoma, among other names. It is the most common and significant disease of hamsters, especially the Syrian species. Hamster enteritis is a disease characterized by animals that are excoriated, lethargic, irritable, anorexic, emaciated, and the caudal area is wet due to the diarrhea associated by the disease. Death usually occurs from 48 hours to a week later after the onset of symptoms. Disease symptoms include ulcerations of the cecal mucosa, inflammatory lesions of the ileum, cecum, jejunum, and colon, and the rectum contains yellow semifluid material. The specific cause is unknown, but *Escherichia coli* have been associated with the etiology. It is seen especially just after weaning but can occur at all ages. The epizootiology and transmission is through direct contact and by contact with formites. To prevent the occurrence of the disease, obtain hamsters from suppliers with a minimal history of the disease. Orally administered antibiotics are the most commonly recommended treatment for this disease (Frisk, 1987).

Pneumonia

After hamster enteritis, bacterial pneumonia is the second most common disease affecting hamsters. The common causes are *Pasteurella pneumotropica, Streptococcus pneumoniae*, and *Streptococcus agalactiae*. The clinical signs include anorexia, nasal and ocular discharge, and respiratory distress. Contributing and predisposing factors of the disease include stress and significant variations in environmental conditions. Stressful situations should be avoided and affected animals should be isolated. The diagnosis is made through assessment of lesions, clinical signs, and microbiology laboratory results. Should treatment be required, antibiotics is the etiological agent that should be used.

Lymphadenitis

Lymphadenitis in the hamster is considered to be comparable to the condition seen in guinea pigs. In the hamster, the causative agent is either Staphylococcus aureus, β-hemolytic *Streptococcus* Lancefield group C, or *Streptobacillus moniliformis*. Cervical lymphadenitis is a chronic disease in which clinical signs appear several weeks after infection. After neck swelling due to cervical lymph node abscesses, anorexia and death follow.

Tyzzer's Disease

Tyzzer's disease (caused by *Bacillus piliformis*) is seen more frequently in the mouse than in the hamster. The etiological agent is. The clinical signs are lethargy, diarrhea, and dehydration. Disease observations include enterocolitis, multiple white myocardial nodules, dilated cecum, colon containing semifluid stools, lymphadenitis, and multifocal necrotizing hepatitis (Zook et al., 1977). The diagnosis of the disease is by demonstration of B. *piliformis* in affected tissues following Giemsa or silver staining. Death is most common within 48 hours.

Salmonellosis

Salmonellosis is very uncommon in the hamster. The causative organism is *Salmonella enteriti*dis. The disease has a rapid onset with death in a few days. Disease symptoms and lesions include

multifocal necrosis of the liver and septic thrombi involving the veins and venules. Infected animals, food, and bedding should be isolated to prevent further colony infections.

Lymphocytic Choriomenigitis

Lymphocytic choriomenigitis (LCM) is transmittable to humans and is caused by a ribonucleic acid (RNA) virus of the arenavirus group. The clinical signs of the virus vary with the strain, the hamster's age and the time of infection. Transmission is through aerosol exposure, direct contact, or formites. Fifty percent of those infected as newborns or congenitally develop a chronic progressive fatal disease characterized by inactivity or weight loss (wasting disease). Necropsy findings include chronic glomeruonephropathy and widespread vasculitis (Genovesi et al., 1987). Hamster colonies should be tested for antibodies and infected animals should be eliminated in order to control the disease. Hamsters implanted with tumors developed LCM and tested positive for the antibody to the virus. The causative agent is tumor formation. Students and staff working with the infected hamsters developed LCM, as did naive hamsters located near them. The spread of infection was halted by elimination of the infected hamsters and tumor cell lines (Biggar et al., 1977).

Type C Virus Infection

Type C virus appears to be similar to the retrovirus type C oncovirus. The disease was first observed in human adenovirus-induced hamster tumors. One report indicates that lymphomas in hamsters may be associated with this agent. Transmission of type C virus is unclear, but it is believed that the agent may not be expressed until activation by chemical or physical agents or by infections caused by other oncoviral agents. Untreated adult male hamster had an osteocarcinoma which contained particles resembling type C virus Yabe, et al., 1972). Transmittion of the tumor with tissue extracts, has not been successful.

Parasitic Infections

Protozoa

Fecal smears of hamsters show the presence of an infinite number of various protozoa. Although protozoa may have a role in enteric diseases, this is unclear because similar numbers and kinds of protozoa are found in both healthy and sick animals. Table 4.3 lists the protozoa found commonly in hamsters, along with the location and prevalence of these protozoa in a hamster colony (Wantland, 1955).

Nematodes

Nematode infections in hamsters are usually caused by *Syphacia obvelata* (mouse pinworm) and *Syphacia muris* (ratoxyurid). *Syphacia obvelata* is only present in less than 1% of infections; however, infection rates can be very elevated in isolated colonies. The pinworm is found in the

Table 4.3 Location and Prevalence of Protozoa in the Hamster

Organism	Location	Prevalence (%)
Trichomonas sp.	Cecum, colon	99
Endamoeba muris	Cecum	33
Giardia sp.	Small intestine	9
Chilomastix bettencoutri	Cecum	9
Hymenolepis nana	Small intestine	>1
Syphacia obvelata	Intestine	>1

intestine and causes gross lesion resulting in inflammation of the large intestine. Treatment consists of 10 mg/ml piperazine citrate (in drinking water) twice in a 7-day period (Unay et al., 1980). *Syphacia muris* is the result of direct contact with infected rats.

Mites

Acariasis in hamsters is caused by infestation by one of two species, *Demodex cricetic* or *D. aurato.* Infection rates are high; though clinical signs of skin disease are very rare. Clinical signs observed included alpoecia on the back and rump with dry, scaly skin. Ear mites in the hamster are caused by the *Notoedres* sp., a tropical rat mite *(Srnithonysaus baacote),* and the nasal mite *(Spleorodens clethrionomys).*

Cestodes

The dwarf tape worm, *Hymenolepis nana*, and *H. diminuta* are found in the small intestine of the hamster and are commonly benign infections. When in large number, they can cause impactions and obstructions which may also produce mucoid or catarrhal inflammation of the bowel (enteritis). Diagnosis of infection involves observation of eggs in the feces or mature worms in the intestine. Animals that are new in a population should be quarantined, and infected animals should be isolated and treated with niclosamide.

Spontaneous Tumors

Table 4.4 lists the most common spontaneous tumors in hamsters by their incidence. The most frequent tumors are seen in the adrenal cortex and intestinal tract, followed by the lymphoreticular system, the endometrium, endocrine system, and ovaries of aging females (Sher, 1982). The benign tumors found are usually adenomas of the adrenal cortex and polyps of the intestinal tract. In a study by Dontenwill et al. (1973), denocarcinomas were correlated with age with a rate of greater than 50% in hamsters over 100 weeks of age. The rate of small intestinal adenocarcinomas (0.8%) seen by Fabry (1985) was higher in hamsters than in rats or mice. Lymphosacromas are the most common malignant tumors of the Syrian hamster. Tumors of the pituitary, lung, urinary bladder, liver and mammary gland are virtually unknown in the hamster, but these do occur spontaneously in older rats and mice (Homburger et al., 1979). Genetic drifts seen in many colonies of hamsters may influence the rate of spontaneous tumors as in the rat and mouse.

Table 4.4 Indicence of Spontaneous Tumors in Syrian Hamsters

Neoplasm	Males	Females	Total
Adreanl adenoma	12.7	9.4	11.0
Lymphoreticular neoplasm	3.7	2.3	3.0
Uterus endometrial polyp		3.0	
Uterus endometrial carcinoma		3.0	
Adrenal carcinoma	3.0	2.0	2.5
Pancreas islet cell adenoma	3.7	1.3	2.5
Vagina papilloma		2.0	
Stomach papilloma	1.7	1.7	1.7
Thyroid carcinoma	1.0	2.0	1.5
Uterus leiomyoma		1.0	
Small intestine adenocarcinoma	0.3	1.3	0.8
Pituitary adenoma	0.0	1.3	0.7
Pancreas islet cell carcinoma	0.7	0.7	0.7
Ovary fibroma		0.7	
Ovary theca cell tumor		0.7	

Animal Identification

Hamsters are identified by punching, tagging, or coding of the ear or ear tattooing, which is done aseptically.

Dosing Procedures

Oral Administration

In dosing a hamster orally (p.o.), the animal is grasped by the skin of the neck and back. The polyethylene Gather (2–3 cm in length) or a gavage tube (metal 18- or 20-gauge) is passed into the mouth via the interdental space. The tube is passed gently into the esophagus and the fluid administered. This method is similar to the procedure done in the mouse and rat.

Subcutaneous Administration

The same restraining procedure for subcutaneous (sc) dosing is used for the hamster as described above for oral administration. The needle is inserted into the skin that is tented by pulling up a fold of skin on the back firmly between the thumb and index finger immediately behind the head. The injection is made into the skin parallel to the back because the hamster's loose skin enables large volumes to be injected sc in comparison to other rodent species of the same size (Collins, 1979).

Intradermal Administration

First the fur over the desired injection site is shaved. Restraining the animal as described above, the needle (30-gauge) is advanced just a few millimeters into the skin. If there is abruptly little resistance, then the needle has been pushed through the skin. Withdraw and advance the needle again. After administration of the material, a small welt will be visible.

Intramuscular Administration

The muscles of the posterior and anterior thighs of the hamster are the most frequently used sites for administrations of an intramuscular (im) dose. Restraining the animal as described above with an assistant where one leg is held by the doser. The quadriceps are held between the forefinger and the thumb of the doser. The agent is injected into the muscle mass.

Intraperitoneal Administration

When dosing a hamster intraperitoneally (ip), the animal is restrained as described above. The needle should be pushed parallel to the line of the leg through the abdominal wall and into the peritoneal cavity. By following the leg line, the doser can avoid administration into the urinary bladder and the liver. Administration may occur when there is no resistance to the needle passage.

Intravenous Administration

For intravenous administrations (iv) in the hamster, it is usually best that the hamster is anesthetized. The veins which can be used are the femoral, jugular, and cephalic. The areas should be shaved, a skin incision made to expose the vein, and then a needle may be placed into the vein and the agent administered.

Blood Collection Techniques

Retro-Orbital Method

This is the method of choice for collecting blood samples from the hamster. The method for collection is the same as described for the rat. A 23-gauge needle or a microhematocrit tube can be used to obtain the blood. Single 3 ml samples of blood may be collected retro-orbitally, but such is usually terminal. Repeated sampling is limited to a volume of 0.5 ml is best for the animal. The use of anesthesia is preferable in the hamster.

Cardiac Puncture

Cardiac puncture in hamsters is a delicate procedure that requires practice. This is because the heart can be hard to locate or can rotate away from the needle. A 25-gauge 3/a-in needle may be used to withdraw a safe volume from the heart of 1–2 ml with minimal damage. Repeated sampling from the heart is ill-advised because the mortality rate due to the blood withdrawn can be high (Wechster, 1983). Anesthesia is suggested in the hamster. Upon exsanguination, 5 ml can be withdrawn from a 95-g hamster (Schermer, 1967).

Tail Clipping Method

The tail clipping method is good for only a maximum of six samples because the tail is short. Place a suction bell (which is connected to a water pump) on the base of the tail to facilitate blood flow. If the hamster is placed in a narrow tube with the hind legs protruding, anesthesia is not necessary.

Femoral Vein Method

For collecting blood from the femoral vein, a tourniquet is placed above the stifle and the fur over the vein clipped. A skin incision is made to expose the vein and a 25-gauge $^{5}/_{8}$-in needle is placed into the vein.

Jugular Vein Method

When using the jugular vein method, the hamster should be anesthetized, the area shaved over the jugular, a skin incision made, and a 25-guage 5/8-in needle placed into the vein and the blood taken.

Saphenous Vein

Using a body tube to immobilize the animal, the skin over the ankle is stretched, allowing for ease in shaving the area around the vein. A 25-gauge, 5/8 inch needle is inserted into the vein and blood is withdrawn. This method is still (2005) the recommended approach to nonterminal blood collection (Hem et. Al., 1998).

Urine Collection

Urine should be collected over 17–24 hours as the animal voids. A preservative such as thymol may be added to the collection vessel prior to starting. Catherization of the ureter can be done; however, there is always the risk of blood or tissue contamination. A hamster's urine is normally a thick, milky fluid.

Analgesia and Anethesia

Analgesia

Buprenorphine (0.5 mg/kg/sc) given every 8 hours provides good analgesia for the hamster. Hamsters are easily restrained if held by an experienced handler and pre-anesthesia is not necessary.

Preanesthesia

Hypnorm (1ml/kg/ip) provides sufficient analgesic for superficial procedures, and diazepam (5ml/kg/ip) provides sedation, but not analgesia.

Anesthesia

Table 4.5 lists the concentrations of suggested anesthesias, site of administrations, the concentration and length of sedation (Flecknell, 1987).

Inhalation Anesthesia

For inhalation anesthesia, the method is the same as described for the rat. The first choice for hamsters is methoxyflurane followed by ether, halothane and enflurane.

Euthanasia

The hamster can be euthansized by inhalation and by physical and parenteral methods. Asphyxiation is the best method by using carbon dioxide from dry ice or a gas cylinder followed by ether, halothane (respiratory arrest in 30 sec), or methoxyflurane. Physical methods include cervical dislocation and decapitation with a guillotine (should only be done by an experienced research to prevent unnecessary pain and distress). Sodium pentobarbital (150 mg/kg) may be given *iv, ip*, intracardically (which may be painful), or into the thoracic cavity.

Physical Parameters

Neonatal Body Weights

Syrian hamsters weigh 2–3 g and Chinese hamsters weigh 1.5–2.5 g at birth. Neonate hamsters are hairless with closed ears and eyes. Table 4.6 covers information about the early development of the eight laboratory hamsters.

Table 4.5 Anesthesia Data for Hamsters[a]

Compounds	Concentration	Time of Sedation (min)
Fentanyl-fluanisone[b] + diazepam	1 ml/kg	60
Fentanyl-fluanisone-midazolan[b]	4 ml/kg	30–40
Ketamine-xylazine[b]	200:10 mg/kg	70
Pentobarbital sodium	35 mg/kg	30–60
Thiopental sodium	40 mg/kg	
Alphaxolone-alphadolone[c]	15 mg/kg	20–60
Pentobarbitone[c]	36 mg/kg	20–50

[a] Aministered intrapertoneally.

[b] Provides surgical anestheisa.

[c] Provides sedation/light anesthesia.

Table 4.6 Neonatal Data for Hamsters

Species	Birth weight (gm)	Day				Teeth at birth
		Eyes open	Ears open	Pups solid food	Weaned	
Syrian	2–3	15	5	7–10	21	Yes
Chinese	1.5–2.5	10–14	10–14		21–25	
Dzungarian	1.8	10	3–4	10	16–18	Yes
European						No[a]
Armenian		14	14	14	18	
Rumanian						
Turkish		12–13		12–13	20	
South African	6.5	16–25	3–5	21–25	No[a]	

[a] Incisors erupt at 3–5 days.

Body Weights and Weight Gains

The average adult body weight for each of the species is covered in the species section of this chapter.

In a study by Borer et al. (1977), Syrian hamsters gained 2 g/day from birth to 5 weeks of age. From days 30 (weight = 65 g) to 70 the hamsters gained 1 g/day. From day 70–88 the hamsters gained 0.3 g/day. Syrian hamsters at maturity weighed from 100 to 135 g.

Normal fetal and maternal weight gain is well documented by Davis (1989).

Dentition

The dentition of a Syrian hamster is bunodont, monophyodont, and brachyodont. The incisors of the Syrian species grow irregularly depending on age and sex of the hamster. The adult European hamster has one set of permanent teeth which consists of four continuously growing incisors and 12 molars.

Life Spans

The typical life spans for each of the laboratory species are discussed in the species section of this chapter.

Sexual Maturity

Female hamsters reach sexual maturity at about 4–6 weeks of age. During this time, there are 10 mature and 25 reserve follicles in each ovary. The female's first spontaneous estrus and ovulation are at 4–5 weeks and 30 days, respectively. In immature females, usually around 4 weeks of age, ovulation may be artificially induced with 30 IU pregnant horse serum (Magalhaes, 1970). The estrus cycle is 94 hours or > 4 days in length with four distinct stages: proestrus, estrus, metestrus, and diestrus. Identifying the stage may be determined from the differentiation of a vaginal smear through examination of cell types. During estrus (which occurs before and after ovulation) the female will display lordosis and can mate. The end of estrus is marked by the appearance of a copious postovulatory discharge. This discharge is creamy, white, opaque, and very viscous with a strong cheesy odor. Ovulation occurs regularly every 4 days, 9–10 hours after the peak concentrations of luteinizing hormone (LH). The breeding life of a hamster is typically about 10–12 months, or after the production of six litters.

Males reach sexual maturity at 6–7 weeks of age and have a breeding life of about 1 year, with the testicles descending at day 26 and 30, respectively.

The secondary sex characteristic of the Syrian hamster is a scent organ (flank organ) which is found on the flank. The male touches the female's organ with his paws during copulation. The intensity

Table 4.7 Reproductive Data for Hamsters

Species	Litter size (pups)	Gestation period (days)	Sexual maturity	
			Males	Females
Syrian	11	16	6–7 weeks	4–6 weeks
Chinese	5	21	8–12 weeks	12 weeks
Dzungarian	4	18	35–40	90–139
European	7–9	15–17	60 days	80–90 days
Armenian	6–7	18–19		
Rumanian		16		
Turkish	6	14–15	7–8 weeks[a]	7–8 weeks
South African	3	38	4–7 months	4–7 months

[a] Occasionally do not reach maturity until 5–6 months of age, and then they may undergo spontaneous testicular regression.

of the pigmentation is an indication of androgen activity and the males have a darker pigmentation than females. This pigmentation is first seen at 25 days of age and is more marked at 35 days of age.

Breeding

Table 4.7 presents the basic parameters of hamster reproductive function.

Environmental Effects

Seasonal changes in reproduction are rarely observed because most laboratory hamster colonies are maintained in controlled environments. In uncontrolled temperature and light environments, reproduction can be hampered due to decreased light and sometimes increased temperatures in the summer that result in small litter sizes and lack of pregnancies.

Copulation

When a mating situation is allowed, there is a short period of investigation before mating. A female will demonstrate her willingness to mate by assuming , a posture where the back is held flat and firm with the legs braced and the tail held erect and vertical. The male hamster will groom himself and the female before mating. They will repeatedly mate for 20–60 minutes with mounting and copulation taking place several times a minute. A unreceptive female may attack a male and bite him on the face or scrotum, but rarely does a male attack a female during mating. Females can also become aggressive after mating and can attack as described above.

Sperm penetration is usually 2–4 hours after ovulation. Fifty-six to 343 million sperm can be obtained from the female reproduction tract after copulation, which is approximately 40–45% of the number of spermatozoa present in the vas deferens and epidymis of a mature male (Magalhaes, 1970). The observation of a copulation plug is an sign of successful mating as well as examination for postovulatory discharge on days 5 and 9 of pregnancy will also confirm. On the other hand, if discharge is present, the female is having a normal estrus and therefore is not pregnant. The female may experience a pseudopregnancy which is marked by a discharge on day 10. In pseudopregnancy, the corpora lutea persist and function, but for a shorter time than a normal pregnancy, which is the result of copulation with a sterile male.

Pregnancy

If the breeding day is day 0 and fertilization is successful, on day 10 the female hamster shows an increase in body weight and a characteristic abdominal distension. The typical pregnancy length

is 15 days and 7–17 hours. The Syrian's gestation periods over 6 months of age and 1 year are over 16 and 17 days, respectively.

Parturition

The pregnant female becomes restless and alternates between eating, grooming and nest building along with an increased respiratory rate usually hours before delivery. Hamsters that construct small or tiny nests frequently have smaller litters or none at all. Licking of the perineal region indicates the onset of birth. Babies are either born breech or head first and once birthed, they are licked clean and separated from the membranes and umbilical cord by the mother. The placenta is eaten immediately or stored as food. Litter sizes vary with the age of the female, types of diet, genetic factors, temperature, caging material and nesting material. In breeders older than 14 months, the litter size usually decreases. The male-to-female ratio at birth is close to equal.

During the lactation period, the mother will show excessive weight loss, which can be avoided if humidity is kept over 40% and animals have adequate water and food. Maternal cannibalism is not uncommon, particularly during the young's first week of life. The neonates appear to be biting the mother and the mother retaliates by killing and eating the young. Additional reasons for cannibalism include stress to the mother, especially if there are unusual loud noises, or if the litter is too large and the mother reduces it to a manageable size. In the case of Dzungarian hamsters, the mother and young may be housed with the father, since both parents participate in the care of the offspring. The young of a South African hamster remain attached to the mother's nipples and travel with her until they are 15 to 20 days old. However, the maxium litter size is only four because the female has four nipples.

Respiratory Rate and Oxygen Consumption

The respiratory rate for hamsters (breaths/min) is 33 minimally and 127 maximally. The average respiratory rate is 74 and the average resting respiratory rate is 30–33 breaths/min (Robinson, 1968). These animals are nose breathers and have a resting oxygen consumption of 2.3 ml/g/hr.

Body Temperature

Core (rectal) temperature for Syrian hamsters is 99.5°F (37.5°C).

Blood Pressure

The blood pressure measured by cannulation of the carotid artery of the hamster is 111 mmHg (Storia et al., 1954). The blood pressure measured on the cheek pouch by Berman et al. was 90 ± 11.3 mmHg. Another measurement of blood pressure by photoelectric tensiometry was 108 mmHg. Though there can not be a direct comparison of these measurements because of the techniques used, the values obtained are similar and can be used as reference values for each method described.

Heart Rate

The average mean heart rate (beats/min) of the Syrian hamster is 450 with a range of 300–600.

ECG Patterns

The normal P-Q interval is 48 msec with a range of 40–60, QRS interval of 15 msec with a range of 13 to 20, T- and P-wave amplitude of 0.33 t 0.07 mV and 0.19 t 0.03 mV, respectively. For

conducting measurement of ECGs, the hamster needs to be anesthetized because of its aggressive behavior. The ECG tracings of hamsters are similar to human ECG tracings (Lossnitzer et al., 1977).

Clinical Laboratory

Typical clinical chemistry values for Syrian hamsters are listed in Table 4.8. This next section will cover several interesting aspects of the hamster and its clinical chemistry. In contrast to humans, the hamster has a lower bilirubin, cholesterol, creative phosphokinase, alkaline phosphatase, lactic dehydrogenase, and A/G ratio values and higher blood urea nitrogen amylase, bicarbonate, phosphorus, asparatate aminotransferase, and a2-globulin values. Blood collection times should be noted because hamsters are day sleepers and light photoperiods chemistry values can be variable. Along with the anesthesia used that could affect chemistry values, these should be taken into account when analyzing clinical chemistry data.

Glucose

Thiobarbiturate anesthesia can produce glucose levels as high as 300 mg/dl in adult male Syrian hamsters. The hyperglycemia can exist for 5 hours after anesthesia exposure and there is not

Table 4.8 Clinical Chemistry Values for Syrian Hamsters

Test	Units	Mean values		Range both sexes
		Male	Female	
Bilirubin	(mg/dl)	0.42	0.36	0.20–0.74
Cholesterol	(mg/dl)	54.8	51.5	10.0–80.0
Creatinine	(mg/dl)	1.05	0.98	0.35–1.65
Glucose	(mg/dl)	73.4	65.0	32.6–118.0
Urea Nitrogen	(mg/dl)	23.4	20.8	12.5–26.0
Uric Acid	(mg/dl)	4.58	4.36	1.80–5.30
Sodium	(mEq/L)	128	134	106–146
Potassium	(mEq/L)	4.66	5.30	4.0–5.9
Chloride	(mEq/L)	96.7	93.8	85.7–112.0
Bicarbonate	(mEq/L)	37.3	39.1	32.7–44.1
Phosphorus	(mg/dl)	5.29	6.04	3.4–8.24
Calcium	(mg/dl)	9.52	10.4	7.4–12.0
Magnesium	(mg/dl)	2.54	2.20	1.9–3.5
Amylase	(Somogyi units/dl)	175	196	120–250
Enzymes				
Alkaline phosphatase	(IU/L)	17.5	15.4	3.2–30.5
Acid phosphatase	(IU/L)	7.45	6.90	3.9–10.4
Alanine transaminase	(IU/L)	26.9	20.6	11.6–35.9
Aspartate transaminase	(IU/L)	124	77.6	37.6–168
Creatinine phosphokinase	(IU/L)	101	85.0	50–190
Creatinine kinase	(IU/L)	23.1		
Lactic dehydorgenase	(IU/L)	115	110	56.0–170.0
Serum proteins				
Total protein	(g/dl)	6.94	7.25	4.3–7.7
Albumin	(g/dl)	3.23	3.50	2.63–4.10
α_1-globulin	(g/dl)	0.64	0.55	0.30–0.95
α_2-globulin	(g/dl)	1.85	1.70	0.9–2.70
β-globulin	(g/dl)	0.56	0.83	0.1–1.35
γ-globulin	(g/dl)	0.71	0.67	0.15–1.28
A/G ration		0.87	0.93	0.58–1.24

Sources: Mitruka, B.M. and Rawnsley, H.M. (1981) *Clinical Biochemical and Hematological Reference Values in Normal Experimental Animals and Humans*. 2nd ed. Masson, New York, and Loeb, W.F., and Quimby, F.W. (1999) *The Clinical Chemistry of Laboratory Animals*, 2nd Ed., Taylor & Francis, Philadelphia, PA.

correlation to the length of hyperglycemia compared to the duration of the anesthesia. Hyperglycemia has also been reported in hibernating hamsters (Newcomer et al., 1987).

Lipids

Lipids found in the hamster include cholesterol, triglycerides, phospholipids, and fatty acids. Cholesterol concentrations in hamsters are high compared to other laboratory animals, but they are lower than human levels. Photoperiods of 10 hours or less can cause a decrease in cholesterol, but other lipids such as plasma triglycerides are not affected. In hibernation, serum lipids increase. A strain of spontaneous hypercholesterolemic Syrian hamsters show increase in cholesterol when exposed to low temperatures.

Urea Nitrogen

Hamsters that develop kidney disease during aging have increased urea nitrogen levels as seen in other laboratory animals.

Enzymes

Aspartate aminotransferase (AST), lactic dehydrogenase (LDH), alanine aminotransferase (ALT), and creative phosphokinase can contaminate serum obtained by cardiac puncture owing to the high concentrations of these enzymes already in the heart.

Alkaline Phosphatase

The alkaline phosphatase in the hamster is made of isoenzymes from liver, bone, and intestine. Alkaline phosphatase is a more sensitive indicator of liver damage than bilirubin or alanine aminotransferase. Dramatic increases are indicative of bile duct obstruction. Immature hamsters have 2- to 3-fold higher elevation of values compared to adults.

Aspartate Aminotransferase

AST activity is low, but increases after muscle injury. Increased AST levels have been found in hamsters with liver neoplasms.

Creative Kinase and Lactic Dehydrogenase

Cardiomyopathic hamsters display elevated creative kinase and lactic dehydrogenase activities. This level is usually around 730 IU/L, whereas normal creative kinase levels are 23.1 IU/L.

Thyroid Hormones

Thyroid hormones are of interest because of the relation to the hamster's hibernation. Basal T3 and T4 decrease with age with T_4 levels in 3-month-old hamsters around 6.75 ± 0.75 mu-g/dl and 3.59 t 0.16 mu-g/dl in 20-month-old hamsters. T3 levels in 3-month-old hamsters are 62 ± 2 ng/dl and 42 ± 3 ng/dl in 20 month-old-hamsters. These fluxuations are also seen in humans and in other rodent species.

After administration of thyroid-stimulating hormone (TSH), older hamsters show less of an increase in T3 and T4 levels. During short photoperiods there is a decrease in TSH, T3, and T4. Pregnant hamsters may metabolize thyroid hormones differently because there is a decrease in protein-bound iodine during pregnancy.

Reproductive Hormones

In estrus there is a single luteinizing hormone (LH) surge while the follicle-stimulating hormone (FSH) is biphasic. The first FSH surge occurs concurrently with LH. The following burst is thought to be responsible for the initiation and/or maintenance of follicular growth for the next estrus cycle. Maintenance of functional corpora lutea is believed to be performed by a combination of prolactin, FSH, and a small amount of LH.

Progesterone is the dominant hormone during the first 2 days of estrus, decreasing on day 3 but increasing again on day 4. Initial levels of progesterone are dependent on LH. Estradiol levels are low for the first 2 days and increase and decrease on day 4.

Adrenal Hormones

Corticosterone levels are 3 to 4 times higher than cortisol during the day hours. Adrenocorticotropic hormone (ACTH) stimulation increases both hormones; however, cortisol levels are stimulated at a higher rate. Basal cortisol levels are 0.45 ± 0.04 μg/dl and 0.38 t 0.09 μg/dl in males and females, respectively, and corticosteriod levels are 7.4 t 1.9 mg/dl (Tomson et al., 1987). Pregnant hamsters may produce large amounts of cortisol (30 μg/dl) whereas non-pregnant females have relatively low levels (0.3 μg/dl) in contrast to other species. Chronic stress can increase plasma cortisol levels.

Glucocorticord level patterns are circadian pattern as in other rodents.

Proteins

Chinese hamsters that have spontaneous diabetes have α_2 as 10–30% of their total proteins, whereas control hamsters have only 3–8%. Asymptomatic hamsters with significantly elevated α_2 proteins do develop chemical or clinical diabetes later on.

Hematology Values

Normal Syrian hamster hematology parameters are listed in Table 4.9. Hematological values for European and Chinese hamsters are listed in Table 4.10. A hamster has a blood volume of 6–9%

Table 4.9 Hematological Values for Syrian Hamsters

		Male		Female	
Test	**Units**	**Mean**	**Range**	**Mean**	**Range**
RBC	×10E6/mm³	7.5	4.7–10.3	6.96	3.96–9.96
HgB	g/dl	16.8	14.4–19.2	16.0	13.1–18.9
MCV	μ^3	70.0	64.0–77.6	70.0	64.0–76.0
MCH	μμg	22.4	19.9–24.9	23.0	20.2–25.8
MCHC	%	32.0	27.5–36.5	32.6	27.8–37.4
Hct	%	52.5	47.9–57.1	49.0	39.2–58.8
Sedimentation rate	mm/hr	0.64	0.32–0.96	0.50	0.30–0.70
Platelets	×10E6/mm³	410	367–573	360	300–490
WBC	×10E6/mm³	7.62	5.02–10.2	8.56	6.48–10.6
Neutrophils	×10E6/mm³	1.68	1.11–2.25	2.48	1.88–3.08
Eosinophils	×10E6/mm³	0.07	0.04–0.12	0.06	0.04–0.08
Basophils	×10E6/mm³	0.08	0.05–0.10	0.04	0.03–0.05
Lymphocytes	×10E6/mm³	5.6	3.69–7.51	5.81	4.41–7.20
Monocytes	×10E6/mm³	0.19	0.12–0.26	0.20	0.16–0.25

Source: Mitruka, B.M. and Rawnsley, H.M. (1981) *Clinical Biochemical and Hematological Reference Values in Normal Experimental Animals and Humans*. 2nd ed. Masson, New York.

Table 4.10 Hematological Values for European and Chinese Hamsters

Parameter	Units	European Range	Chinese Range
RBC	×10E6/mm³	6.04–9.10	4.4–9.1
HgB	g/dl	13.4–15.5	10.7–14.1
MCV	μ³	58.7–71.4	53.6–65.2
MCH	μμg	18.6–22.5	15.5–19.1
MCHC	%	26.4–32.5	27.0–32.0
Hct	%	44.0–49.0	36.5–47.7
WBC	×10E6/mm³	3.4–7.6	2.7–9.6
Neutrophils	×10E6/mm³	3.5–41.6	14.8–23.6
Eosinophils	×10E6/mm³	0–2.1	0.3–3.1
Basophils	×10E6/mm³	0–0.2	0.0–0.5
Lymphocytes	×10E6/mm³	50.0–95.0	68.1–84.8
Monocytes	×10E6/mm³	0–1.0	0–2.4

Source: Mitruka, B.M. and Rawnsley, H.M. (1981) *Clinical Biochemical and Hematological Reference Values in Normal Experimental Animals and Humans.* 2nd ed. Masson, New York.

of their body weight. The highest safe volume for one bleeding is 5.5 ml/kg. The typical volume from adult hamsters for diagnostic use is 1 ml. Hematological values for a hamster vary extensively because they are deep day sleepers, though values between males and females are close. The variations seen are changes in blood volume and quantity of blood components.

Erythrocytes

Hamster erythrocytes have a diameter of 5–7 um. South African hamsters have larger erythrocytes than other hamsters and laboratory rodents. A small portion of erythrocytes show polychromasia (Schermer, 1967). Erythrocytes have a life span 50–78 days and Desai (1968) saw an increase in erythrocyte longevity during hibernation. Nucleated or basophilic cells are rare, but reticulocytes are found from 3–4.9%.

Leukocytes

Hamster leukograms are comparable to those of other laboratory rodents. During photoperiods, the total leukocyte numbers range from 5000 to 10,000/ul, whereas during hibernation counts decsend to 2500/ul. During sleep, the lymphocyte:neutrophil ratio is 45%:45%, whereas in awake animals the neutrophil percentage lies between 17 and 35%.

Coagulation

Hamster blood begins to coagulate at 15–20 sec (Schermer, 1967) with a mean coagulation time of 142 sec (Desai, 1968).

Trypanosomes

Hamsters often have trypanosomes present in the blood. These microorganisms are parasitic but not pathogenic and have been observed in other laboratory animals, including sheep and monkeys, but not in the large numbers as seen in the hamster. The counts of trypanosomes can be significant, sometimes equaling the leukocyte counts.

Blood Gases and pH

Blood gas values are Pa_{02} 71.8 ± 4.9 mmHg, PaC_{02} 41.1 ± 2.4 mmHg, $HC0_3$ 29.9 ± 2.9 mEq/L, and the blood pH is 7.48 ± 0.03. Analysis of exercising hamsters show a increase of 12.9 ± 7.9 in Pa_{02}, a decrease of 6.6 ± 2.6 Pa_{C02}, and a decrease of 3.5 ± 2.3 in $HC0_3$ concentrations.

Urine Values

Urine volume can range from 5.1–8.5 ml/24 hours in normal hamsters. The urine volume can be as high as 25 ml/day in diabetic Chinese hamsters. Sodium and potassium concentrations are 70 and 120 mmol/L, respectively. Proteins are excreted about 10 times the rate of humans, the pH is basic, and cholesterol is the main lipid excreted.

Species Peculiarities

The hamster cheek pouch is distinctive because it accepts heterologously neoplastic tissue, but rejects normal human tissue. This led to the discovery that a biological difference is present between malignant and nonmalignant tissues. The cheek pouch has been utilized for the transplanting of neoplastic tissue for evaluation of growth. Once this method was standardized, it became a screening tool for chemotherapeutic agents (Newcomer et al., 1987).

The cheek pouch is transparent, very accessible and it is ideally suited for *in vivo* studies of microcirculation and the behavior of formed blood elements. These characteristics have also made the hamster the model of choice for buccal administration and evaluation of oral and mucosal irritation. The hamster cheek pouch is the standard model for evaluating acute or cumulative (28 day) irritation/tissue tolerance, as well as providing a popular model for the induction and study of squamous cell neoplasia (Heller et. al., 1996).

Strain-Related Considerations

In a study by Althoff and Mohr (1973) that compared the chronic respiratory response of the Chinese, Syrian, and European hamsters to diethylnitrosamine (DEN) and dibutylnitros amine (DBN), strain-related differences were found. In the Chinese hamster, DEN did not yield neoplasms in the respiratory tract, however in the Syrian species, DEN caused tumors in the trachea and lungs followed by the nasal cavity. In European hamsters, DEN produced benign and malignant tumors in the respiratory tract and caused death after 15 weeks of daily treatment.

DBN caused papillary tumors and malignant neoplasms in the nasal and paranasal cavities in the Chinese hamster. In the Syrian, DBN produced tumors, firstly in the trachea, then the nasal cavities and lungs. At the high dosages in European hamsters, DBN caused carcinogenic effects in the trachea, lungs, and nasal cavities. In the lose dose groups, lung carcinomas prevailed.

Hamsters are good models to study respiratory carcinogenesis; however, different species can have different responses to a chemical as shown by this study. The spontaneous rate of respiratory tumors, metabolism, and nature of the chemical needs to be known before cross-species extrapolation can be performed.

Typical Study Protocols

Carcinogenicity Toxicity Testing

Hamsters are very suitable animals for carcinogenicity testing because of a low occurrence of spontaneous tumor development, but they are highly susceptible to experimentally induced carcinogenesis. The incidence of spontaneous tumors in Syrians is reported to be lower than the incidence seen in mice or rats (Homburger et al., 1979; Mohr, 1979). Although the hamster has a short lifespan, substance-related effects and neoplasms occur rapidly. Hamsters are not typically used in testing, and a 2004 inquiry of (the more than 90) known contract laboratories succeeded in only identifying one with up-to-date experience in handling such studies.

Carcinogenicity protocols used for rat studies are satisfactory enough to be used for hamster studies. However, changes that need to be addressed include blood collections that should be kept

to a minimum and the length of the study usually needs to be shortened (to 96 weeks) owing to the hamster's shorter life span.

Hamsters are recommended for long-term testing with aromatic amins, polycyclic hydrocarbons, and other agents suspected of being pulmonary carcinogens (Aufderheide et al., 1989). Urinary bladder carcinomas induced by aromatic amines can take up to 7 years to induce in dogs, but can cause neoplasms in less than 1 year in hamsters (Witschi, et al., 1993).

Nitrosamines produced tumors in the hamster in the nasal cavity, stomach, liver, pancreas, lung, trachea, and occasionally the esophagus. The frequent site for nitrosamine tumor induction in the rat is the esophagus, demonstrating species specificity for a target organ site. Some nitrosamines are more toxic in the rat than the hamster and vice versa (Newcomer et al., 1987). The hamster does show a nitrosamine-induced pancreatic tumor related to pancreatic tumors in humans.

Inhalation and Intratracheal Studies

Hamsters are used widely in inhalation studies for toxicological research. The hamster is useful because it has a lower occurrence of spontaneous respiratory tumors and of respiratory diseases (Wemer, 1979). Its respiratory epithelium is similar to that of the human than other laboratory rodents. The hamster has similar lung absorption characteristics to those of the rat and mouse for aldehydes, ozones and other irritant gases (Morris, 1997; Steinberg et al., 1990; and Kannbe et. al., 1991). Owing to its more mixed breathing pattern, it compares favorably to the rat (an obligatory nose-breather) for studying fiber and particulate inhalation (Hesterburg et. al., 1997; Geiser et. al., 1990; Gelzleichter et. al., 1999; Warheit and Hartsky, 1993 and Warheit et. al., 1997). In cigarette smoke studies, specific in-bred species of the hamster are the only laboratory rodents where carcinogenesis can be induced by inhalation. Laryngeal cancer in the hamster is caused tar fractions or cigarette smoke and has been found to be histologically identical to the cancer found in humans (Homburger et al., 1979). For these reasons, the hamster continues to be popular in studying the toxicity of cigarette smoke and its mechanisms (Tafassian et al., 1993 and DiCarlantonin et al., 1999).

Acute and subacute inhalation toxicity studies using the hamster have examined nickel monoxide (Ni0), cobaltous oxide (Co0), and chrysotile asbestos. Hamsters exposed to asbestos for 11 months developed asbestoses and those exposed to Ni0 developed pneumoconiosis, occupational disease states seen in humans.

For whole body exposure inhalation study designs, the exposure chamber should be large enough to allow an adequate number of animals to be exposed at once. It should also be equipped with the means to regulate temperature and humidity and have identical chambers for all treatment groups. The hamsters should be housed individually, but if there are space limitations, animals could be housed in groups. The orientation of the cages should be rotated after each exposure and the animals should be housed in an area other than the inhalation chamber when not being treated. This in turn will reduce the contamination of the exposure chamber by bodily fluids.

Nose-only exposure with the animals being restrained in tables is currently more popular, but hamsters do show significant stress related physiology responses to such restraint, including marked weight loss (King-Herbert et al., 1997).

Animals are randomly distributed to test groups based on body weights as done in other types of animal studies. The animals should have free access to water at all times and feed should be available when animals are not being exposed unless exposure times are very lengthy. If feed is provided during exposure, then the feed is also being exposed to the test materials and may be an important variable of the study. The number of exposures, whether once or several times a day, and the length of exposure time can be decided by the investigator; however, once decided, exposure time should remain consistent during the study. The concentration and particle size of the aerosol should be addressed periodically and the aerosols should be evenly distributed in the chamber (Raabe et al., 1973) Data may be collected concerning clinical signs, mortality, body weights,

pharmacokinetics, hematological and clinical chemistry functions, organ weights, and gross and microscopic observations.

The same procedure as described for inhalation studies are used for intratracheal instillation studies except animals are exposed to the control and test articles via intratracheal administration. The usual dose volume is 0.2 ml per animal and the animal is usually anesthetized during dose administration. The length of the study and the number of treatments per day may be decided by the principal investigator.

Teratology Studies

The hamster provides a popular alternative species for teratology and reproductive toxicity studies due to its short pregnancy period, predictable estrus, rapid embryonic development, and a low incidence of spontaneous malformations (Wlodorezyk, B et al., 1995; Williams et al., 1991; Wolf et al., 1999; Gomez et al., 1999 and DeSesso et al., 1998).

Retinoic acid (vitamin A) has been shown to be a teratogen in hamsters (Frierson et al., 1990; Eckholf and Willhite, 1997; Willhite et al., 1996 and 2000). Thaliomide has been found to be a teratogen in specific in-bred strains of the Syrian hamster making it a viable alterative to the use of rabbits. Other hamster teratogens are cyclophosphamide (Shah et al., 1996), dinocap (Rogers et al., 1989), hydrocortisone, colchicine, vincristine, vinblastine, heavy metals such as cadmium compounds, organic and inorganic mercury, 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), and 2,4-D (2,4-dichlorophenoxyacetic acid) alone or contaminated with dioxin (Newcomer et al., 1987). In teratology studies, a number of aspects should be taken into account such as the embryonic development of the hamster, the strain-specific fertility seen in hamsters, and the age of the mother.

Toxicology Studies

The protocols used for rats in acute and long-term toxicity studies can be used for the hamster; however, blood collection should be kept to a minimum and the length of the test may need to be adjusted due to the shorter life span of the hamster.

The greater part of toxicology work involving hamsters has been buccal inhalation and respiratory studies, although intratracheal has also been a popular route due to the precise control of doses (Biswas et al., 1993). The hamster has been a useful model for studies in toxicity, but it has some biochemical and physiological characteristics not seen in other rodent species. The hamster has a significant resistance to certain pharmacological agents, such as barbiturates, morphine, and colchine, but is very sensitive to halothane and sevoflurane induced changes in diaphragmatic contractility (Kagawa et al., 1998). The hamster oral LD_{50} of colchicine is 600 times the lethal dose known to humans, whereas morphine when given to hamsters at the LD_{50} does not produce a narcotic reaction. These characteristics should not he considered a hindrance to the use of hamsters in toxicology studies.

Of 304 compounds analyzed by the IARC, 130 were carcinogenic in at least one rodent species. Of the 130, only 38 compounds were tested in both hamsters and rats, 35 were tested in hamsters and mice, and 78 tested in both rats and mice. Of those tested in hamsters and rats, 84% of the compounds had similar results in both species, 86% in both mice and hamsters, and in mice and rats 90% had similar results. Based on this information, hamsters are not more or less sensitive to toxicity than other rodent species used in long-term testing (Arnold et al., 1979).

Chinese Hamster Ovary (CHO) Cell Chromosome Aberrations

This assay is used to evaluate the ability of a compound to induce chromosome aberrations clastogenic responses in the Chinese hamster ovary (CHO) cells. The idea that 60% of the drugs listed in the Physicians Desk Reference have positive CHC chromosome aberration findings associated with

them have raised some concerns about this assay. The CHO cells used in this assay may be obtained from the American Tissue Culture Collection, Rockville, Maryland (Brusick, 1982), with the original cells being obtained from a Chinese hamster.

The assay is divided into two parts, nonactivation and activation. The S9 rat liver is the activating agent and EMS (0.5 µl/ml) is the positive agent for the nonactivation, whereas for the activating studies it is dimethylnitrosamine (DMN) at 0.5 µl/ml). The dosages selected for the test article are one toxic (loss of growth potential) and four lower (usually in a half-log series) concentrations. These dosages are found in a range-finder with the cells exposed to the test article for 4 hours and incubated for 24 hours. The solvent used to dissolve the test article is used as the solvent vehicle for the control and positive control articles.

The CHO cells are grown in 10% fetal calf serum (FCS)/Ham's F12 media, with the cell density kept around 1.5×10^6 per 75 cm^2 plastic flask. In the assay, about 0.25×106 cells per well per test article concentration are tested. The cells are exposed to the test agent for 2 hours at 37° C and the cells used in the activation section receive the S9 rat liver along with the test article before the 2 hour activation. Then the cells are washed with sterile saline and given new media. For each dose, half of the plates per treatment group will receive 5-bromo-2'-deoxyuridine (BrdU) at a concentration of 10µM. After hour 17, the cells are then incubated for 24 hours with colcemid (2×10^{-7} M). After incubation, the metaphase cells are collected by mitotic shake-off and then swollen with 0.0075M KCl solution, washed with methanol: acetic acid (3:1) fixative, dropped onto glass slides, and air dried. The slides are then stained with 10% Giemsa (pH 6.8) and the slides are scored for chromosomal aberrations like chromatid and chromosome gaps, breaks and deletions.

Syrian Hamster Embryo (SHE) Cell Transformation Assay

The SHE assay is genotoxicity assay currently being promoted for use as an alternative for the CHO and also as a screening assay for clastogens (LeBoeuf et al., 1996). The Syrian hamster embryo (SHE) cell transformation assay looks at the potential of chemicals to induce morphological transformation in karyotypically normal primary cells. This induction has been shown to correlate well with the carcinogenicity of many compounds in the rodent bioassay. Typically, the assay has not received use due to the technical difficulty (Leonard and Lawwerys, 1990). This was the topic of an entire issue of *Mutation Research* (1996) and is currently being further evaluated by regulatory agencies, using a low pH technique which is technically easier to use and reproduce (LaBoerf et al., 1996; Kerckaert, et al. 1996; Aadema et al., 1996; Isfort et al., 1996; Custer et al., 1996; Isbort et al., 1996).

Models of Diseases

Cardiomyopathy

The genetic origin for cardiomyopathy comes from a recessive autosomal gene defect, which induces a metabolic degenerative lesion in striated muscles and limits the life span to 146 days. The main activity is consistently aimed at the heart (Bajusz, 1969; Gertz, 1973). Nonvascular myocardial disease, within hamsters, mirrors the same pathologic and clinical afflictions found in humans. This can be seen, as the animal appears normal, but the cardiac muscle is invariably diminished. Histologically, lesions appear within 350 days of birth in males and 25 to 30 days in females. Acute myolysis occurs within the first lesion, where the myofilaments are dissolved. This is healed, but is replaced with connective tissue within 100 days (Gertz, 1973). Subcutaneous edema signifies the clinical appearance of the disease, but there is no method to show that the edema is present or that the lesion is on the heart with electrocardiograms. After the lesion formation, the alteration of cardiac excitation and high-frequency QRS component of the ECG report can be viewed. Ascites, hydrothorax, and hydopericardium become prevalent throughout the latter stages

of the disease. Finally, the laboratory animals become hyperneic and cyanotic within the terminal stage. Congestive changes in the enlargement and increased volume can be seen in the liver, spleen, kidneys, and heart. These hamsters show considerable change as they are treated with digitalis, diuretics, and salt restrictions. With the appearance of similar disease effects and side effects, the model can consistently represent the effects of heart failure due to cardiac muscle degeneration along with the associated therapy involved within humans.

Dental Caries

Dental caries signifies the poor development and the poor calcification of hamster teeth. The disease can be transmitted between hamsters, and it should be considered infectious. The introduction to the laboratory animals can be through the introduction of cariogenic microflora. The microflora are added to the drinking water or can be found in infected feces. This leads to carious lesions in the molars, which increases the translucent nature of the enamel and increase cavities due to depressions or fissures. The degeneration then reaches the dentine and the pulp of each tooth, where bacterial infections, inflammation, and complete necrosis of the molar results in the complete removal of the tooth. For this disease, the laboratory tests showed that fluoride worked best to limit the effects of dental disease, and this information was later used in clinical trails for humans (Keyes et al., 1960).

Diabetes Mellitus

The influence of diabetes mellitus was seen in 1959 and 1969 for the Chinese hamster and South African hamster populations, respectively (Stuhlman, 1979). However, only small strains of the Chinese hamster populations receive the disease through a recessive gene. Generally, the Chinese hamster is affected rapidly within one to three months of birth, and the indications for the disease include human characteristics. Generally, the following indications show the presence of diabetes mellitus: polyuria; polydipsia; glycosuria; ketonuria. Normal Chinese hamsters have lab values for glucose ranging 110 ± 6 mg/100 ml, but diabetic hamsters show levels ranging 200 to 800 mg/100ml. Variation is applicable to the diagnosis of the disease from chronic diabetes to insulin dependant, and the related side effects such as cataracts. This variability can also be seen in parameter discrepancies for native insulin values, as well as the influence of hereditary consequences of the disease.

The South African hamster holds the disease as an inherited non-sex linked polygenic trait. This specific genus has a 22% inclination of diabetes mellitus, where hyperglycemia is variable, much like humans, with age of onset, degree of severity, incidence, and rate of progression. However, it is consistently not influenced in the aspects of age, sex, or obesity values. The obesity value influence is not prevalent in diabetic South African hamsters, but it is considered applicable to Chinese hamsters.

Disease pathogenesis, secondary complications, exact genetic mechanisms, and possible therapeutic designs can be studied with the models created from effect of diabetes mellitus in hamsters.

Leprosy

The leprosy bacilli was first introduced to the hamster in 1937, where it was found to be the first animal susceptible to the organism within laboratory conditions. Leprosy research in hamsters has reduced as the leprosy bacilli have been grown in cell cultures and in the tail and foot pads of the mouse (Frenkel, 1987).

Muscular Dystrophy

By restricting the vitamin E in the diet of a hamster, from weaning till death, conditions for muscular dystrophy (MD) syndrome will peak. However, the cumulative effect will not be seen

until the eleventh or twelfth month. Genetic tendencies for MD can be found in the Syrian hamster strains afflicted with cardiomypathy (BIO 14.6 and BIO 53.38). Within 60–200 days, after birth, sign for the disease appear within all of the skeletal muscles and the heart. Plemorphic lesions, which include: focal degeneration of the myofibrils; coagulation necrosis; contraction clot formation; alignment of muscle nuclei within the fiber (Homburger and Bajusz, 1970), were found. The syndrome permits the myofibrils, within the muscles, to become a granular substance where the nuclei have burst and are no longer viable. Generally, the alterations are evident at the hamsters have morphological manifestations within 33 days after birth, with a death at 220 days from cardiac failure.

This model is excellent to study the mechanism of MD from an *in vivo* system to intact animals.

Osetoarthritis and Degenerative Joint Disease

After 2 years of age, hamsters generally develop joint afflictions in the form of osteoarthritis and degenerative joint disease. The overall effects include the separation of the cartilage and zone of calcification layers, and this creates conditions perfect for sclerosis and dislocation of the joint. Also the ligaments are affected through fibrillation and the synovial membrane becomes fibrotic. Agents associated with the disease include: *Mycoplasmas; Streptobacillus moniliformis, Corynebacterium kutscheri;* hormonal imbalances; chemical and physical maladies. With the application of such agents, the disease is a considerably better model as it mirrors the reaction seen in humans.

Pancreatic Cancer

The most difficult neoplasm to induce into laboratory animals is pancreatic cancer. However, it is best induced in Syrian hamsters, as long as the population is treated with nitrosamines. These hamsters produce some of the same situations as in human pathogenesis when considering neoplasms, histogenesis, and enzymatic patterns. During pancreatic carcinogenesis, hamsters occasionally develop diabetes, which is also a pathogenetic response in humans.

PATHOLOGY

Background: Milestones in the Hamster Life Cycle

A newborn Syrian hamster *Mesocricetus auratus* weighs approximately 2 to 3 g; an adult of 6 months weighs approximately 150 g. For males, spermatogenesis commences at approximately day 25 of age; sexual maturity is reached at day 40 for females, earliest ovulation occurs from weeks 4 to 8. The average estrus cycle is 4 days, whereas gestation occurs in 16 days.

Litters vary in size from 4 to 12 pups. Females of some strains are able to produce six litters or more (Van Hoosier and Ladiges 1984). Normal weaning time is 21 to 28 days. Breeding capability for males and females is approximately 12 months.

The average life span for these naturally nocturnal animals is approximately 2 years, with a 3-year maximum life expevtation (Van Hoosier and Ladiges 1984). However, specific closed colonies have had mean survival times of 63 to 79 weeks for males and 50 to 63 weeks for females (Pour et al. 1979; Redman et al. 1979; Slauson et al. 1978), whereas another colony had a mean survival time of 106 weeks for males and 97 weeks for females (Kamino, Tillmann, Boschmann, et al. 2001; Kamino, Tillmann, and Mohr 2001).

Special Anatomical Features

Anatomically, a unique morphological feature of the Syrian hamster is its well-developed cheek pouches, which are highly distendible evaginations of the lateral buccal walls. The hamster utilizes

them to transport and store food. For experimental purposes, these readily accessible structures are eversible and can be utilized as sites for normal or abnormal tissue transplants (including tumor implantations). Immunological rejection does not occur owing to the absence of an intact lymphatic drainage pathway (Van Hoosier and Ladiges 1984).

The upper GI tract is unusual in that the esophagus enters between the forestomach and glandular stomach. Also at this junction, the limiting ridge of the hamster stomach represents a distinct constriction, forming the two separate compartments.

Nonneoplastic Lesions

The published incidence rates of the most frequent nonneoplastic lesions of untreated outbred Syrian hamsters are listed in table 4.11. Because most of these lesions were tabulated using only one or two references and proper historical control data have not been published, the actual incidences are probably lower than those given in table 4.11. Various factors that are known to increase frequency rates include age; sex; diet; breeding methods; genetic drift; hormonal imbalance; the presence of transmissible bacterial, viral, or parasitic agents; and the extent of gross necropsy and histopathological evaluations.

Amyloidosis

Systemic amyloidosis is a principal cause of death in aging hamsters (Van Hoosier and Ladiges 1984). In one colony, a high frequency of 88% occurred in hamsters over 18 months of age and a lower frequency of 42% was found in hamsters 13 to 18 months of age (Gleiser et al. 1971). In another colony, systematic amyloidosis was noted as the cause of death of approximately 25% of males that were sacrificed at week 53 and 33% of females that were sacrificed at week 40 (Slauson et al. 1978).

By analysis of serum electrophoretic patterns, there was a decrease in albumin and a concomitant increase in total globulin, primarily a sharp transient rise in $\alpha 2$ serumglobulin, as detected in 12-month-old hamsters (Van Hoosier and Ladiges 1984). By transmission electron microscopy, amyloid deposits were identified as finely granular, amorphous material that was located in the mesangial matrix and between endothelial cells and the basement membranes of renal glomeruli.

By light microscopy, the initial lesion appears to be located in the walls of glomerlar capillaries, forming so-called "wire-loop" lesions. In addition, amyloid accumulation in epithelium and connective tissue has been seen primarily in hepatic periportal areas, adrenal cortex, pancreas, and adjacent to splenic lymphoid follicles. Less frequent sites are the lungs, ovaries, testes, and epididymides.

For positive diagnosis by light microscopy, Congo Red stain and the birefringence of these areas in polarized light are utilized (Gleiser et al. 1971). Biochemically, two proteins, namely amyloid A (AA; major component) and amyloid P (AP; minor component), have been identified in isolated fibrils from aged hamsters (Brandwein et al. 1981; Coe and Ross 1990). Only the AP component is under sex hormone control in the Syrian hamster, which distinctly predisposes females to acquire amyloidosis normally with aging. Testosterone inhibits the hepatic synthesis of the AP component homologue (called female protein), which is normally expressed from 100- to 200-fold times greater in the female Syrian hamster, as compared to the male (Coe and Ross 1990).

Arteriolar Nephrosclerosis

Arteriolar nephrosclerosis was observed at approximately 75% incidence of control animals (Slauson et al. 1978). Progressive renal arteriolar sclerosis with subsequent glomerulosclerosis (fibrinoid necrosis) and tubular degeneration or atrophy with fibrosis were described. Degenerative vascular changes (fibrinoid necrosis) were seen in the testes, ovaries, and uterus. Although limited serological analyses for anti-LCM antibody were negative, chronic viral infection was considered as the potential cause of the disease.

Table 4.11 Incidence of Various Nonneoplastic Lesions in Untreated Outbred Syrian (Golden) Hamsters

Organ	Lesion	% Incidence in Males	% Incidence in Females	Reference
Adrenal glands	Cortical cyst	2	8	Pour, Mohr, Althoff, et al. (1976a)
	Cortical hyperplasia	10–72a	5–61a	Pour, Mohr, Althoff, et al. (1976a) Deamond et al. (1990) Kamino, Tillman, Boschmann, et al. (2001)
Arteries	Vascular calcinosis	5–13	10	Pour, Kmoch, et al. (1976)
Colon/cecum/ ileum	Enteritis (colitis/typhlitis/ proliferative ileitis)	10–73	20–73	Pour, Kmoch, et al. (1976) Pour, Mohr, Cardesa, et al. (1976) Frisk and Wagner (1977a,b)
Heart	Thrombosis	10–73	20–73	Pour, Kmoch, et al. (1976) McMartin (1977) McMartin and Dodds (1982) Deamond et al. (1990)
Kidneys	Arteriolar nephrosclerosis	72	80	Slauson et al. (1978)
	Nephrocalciosis	15–31	16–20	Slauson et al. (1978) Deamond et al. (1990)
	Calcification	63a	47a	Kamino, Tillmann, Boschmann, et al. (2001)
	Infarct	42a	47a	Kamino, Tillmann, Boschmann, et al. (2001)
	Pyelonphritis, acute/chronic	6–13	8–13	Pour, Kmoch, et al. (1976)
Liver	Cholangiectasis/cystic change	21–60a	12–70a	Pour, Mohr, Cardesa, et al. (1976) Deamond et al. (1990) Kamino, Tillmann, Boschmann, et al. (2001)
	Cholangitis	30–49	29–31	Pour, Kmoch, et al. (1976) Pour, Mohr, Cardesa, et al. (1976)
	Clear cell foci fatty change	— 51a	35a 59a	Kamino, Tillmann, Boschmann, et al. (2001)
Pancreas	Islet cell hyperplasia	37–64	30–81	Pour, Mohr, Althoff, et al. (1976a)
Parathyroid glands	Hyperplasia (principal cells)	83	22	Pour, Mohr, Althoff, et al. (1976a)
Ovaries	Cyst	—	6	Pour, Mohr, Althoff, et al. (1976a)
Sternum	Cartilage degeneration	97a	97a	Kamino, Tillmann, Boschmann, et al. (2001)
Stomach/ forestomach/ glandular-pylorus region	Erosions/ulcerations	4–8	5–20	Pour, Kmoch, et al. (1976) Pour, Mohr, Cardesa, et al. (1976)
Testes	Tubular atrophy	40a	—	Kamino, Tillmann, Boschmann, et al. (2001)
Uterus	Granular cell foci	—	54a	Kamino, Tillmann, Boschmann, et al. (2001)
Vertebra	Chondrosis	30–83	10–81	Pour, Kmoch, et al. (1976)
Multiple organs	Amyloidosis	16–88	16–88	Gleiser et al. (1971) Pour, Kmoch, et al. (1976) Deamond et al. (1990) Kamino, Tillmann, Boschmann, et al. (2001)

Note: Maximal incidence rate includes animals surviving up to 33 weeks of age.

Atrial Thombosis

Certain colonies of Syrian hamsters have been maintained and utilized as prospective animal models. For instance, a high incidence (73%) of aging male and female acromelanic Syrian hamsters had atrial thrombosis, accompanied by a consumption coagulopathy (McMartin 1977; McMartin and Dodds 1982).

The thrombi were located primarily in the left atrium, resulting from localized hemostasis secondary to cardiac failure. Atrioventricular valvular thickening and bilateral ventricular hypertrophy were often present. Pulmonary edema and pleural effusion were commonly seen at gross necropsy, which correlated clinically to hyperpnea, tachycardia, and cyanosis. Microscopically, myocardial degeneration and necrosis were associated with calcification and fibrosis. Similar cardiac lesions were noted at a frequency of 40% per sex in a closed colony of APA strain, random-bred hamsters (Doi et al. 1987). Glomerulonephorosis was also noted with no apparent correlation to the cardiac thrombosis as well as chronic renal disease characterized by tubular dilation or atrophy and proteinaceous casts.

A cardiomyopathic hamster model with progression to myocardial failure has been described (Bajusz et al. 1969; Gertz 1973). Within 65 days of age, hamsters of both sexes of the inbred strain BIO 14.6 developed myocardial degeneration or necrosis with calcification as well as generalized myodystrophy. At 9 to 12 months of age, marked ventricular hypertrophy, thrombosis of the left atrium, and hepatic chronic passive congestion were seen, indicative of an animal model for congestive heart failure.

Generalized Vascular Calcinosis

Generalized vascular calcinosis was noted in the aorta and coronary and renal arteries (Pour, Kmoch, et al. 1976). Later, this abnormality was ameliorated by modifications in the diet (Birt and Pour 1983; Pour and Birt 1979).

Hemorrhagic Necrosis

In the late 1970s, spontaneous hemorrhagic necrosis (SHN) was described in late-term fetal hamsters at days 14 and 15 of gestation (Keeler and Young 1978;Young and Keeler 1978). It was characterized by multifocal but coalescent zones of parenchymal hemorrhage, edema, and necrosis affecting the subependymal capillary vasculature of the rhage, edema, and necrosis affecting the subependymal capillary vasculature of the forebrain, thalamus, medulla, and spinal cord. Litter viability was decreased.

Fetal brain development and differentiation were influenced by intrauterine environmental factors, especially a diet deficient in vitamin E. Supplementation of diet with vitamin E throughout gestation and lactation completely prevented SHN and fully restored litter viability to normal levels (Keeler and Young 1979). Cross-breeding of susceptible and nonsusceptible strains eliminated the disease (Keeler and Young 1978).

Bacterial Infections

Hamster Enteritis

Several important bacterial infectious agents have been known to affect the health status of the Syrian hamster as well as to produce complications in histopathological evaluations. Foremost,

hamster enteritis (HE) remains a disease of great concern. It is usually manifested early as an epizootic disease with a high mortality of approximately 90% of weanling hamsters from 3 to 8 weeks of age (Frisk and Wagner 1977b).

In this chapter, HE is the term used to describe wet tail, or wet tail disease, which also has been referred to as proliferate ileitis, regional enteritis, terminal ileitis, atypical (transmissible) ileal hyperplasia, and enzootiv intestinal adenocarcinoma (with localized invasion to adjacent muscularis). HE appears to include a spectrum of various stages of these lesions.

Clinical symptoms and signs include diarrhea, dehydration, lethargy, anorexia, and irritability. The disease progresses to staining of the perineum, tail, and ventral abdomen. Death occurs from 48 hr to 1 week after the onset of symptoms.

Gross necropsy lesions range from mild acute changes such as ileal hyperemia orhemorrhage to marked distension of segments of the ileum. Mucosal areas might become ulcerative and necrotic, eventually penetrating into the muscular layers, resulting in multiple abscesses of the subserosa. Enlargement of the mesenteric lymph nodes and peritonitis with adhesions also develop. Intussusceptions of the colon and prolepses of the rectum have been described for hamsters that recovered from HE (Jacoby et al. 1975).

Microscopic lesions range from mild acute enteritis with epithelial hyperplasia of the columnar cells to marked epithelial hyperplasia associated with chronic inflammation and fibrosis. Other regions of the mucosa have epithelial degeneration or necrosis and hemorrhages, ulcerations, or abscesses (Frisk and Wagner 1977b) Associated lesions include lymphadenitis of the mesenteric lymph nodes (Amend et al. 1976).

Etiologically, intracytoplasmic bacteria have been demonstrated by transmission electron microscopy (Frisk and Wagner 1977b; Frisk et al. 1981; Johnson and Jacoby 1978; Wagner et al. 1973). Intracytoplasmic particles resembling rod-shaped bacteria have been detected with indirect fluorescent-antibody technique using serum from hamsters with HE (Frisk et al. 1981; Jacoby 1978; Jacoby et al. 1975).

Specifically, the bacterium *Escherichia coli* has been isolated from the intestinal tract of hamsters with HE (Amend et al. 1976; Frisk et al. 1978, 1981; McNeil et al. 1986). In one facility, *E. coli* was isolated early in the disease process, whereas *Campylobacter* (Dillehay et al. 1994) *sp.* was observed in later stages within hyperplastic epithelial cells (Frisk and Wagner 1977b). In addition, Jacoby (1978) demonstrated by immunofluorescence an intracellular antigen morphologically compatible with gram-negative rods like *Campylobacter fetus,* whereas Dillehay et al. (1994) demonstrated campylobacter-like organisms by transmissions electron microscopy (TEM). Thus, two or more organisms might act synergistically to produce the disease, as neither *E. coli* nor *C. fetus* cultured alone could reproduce the natural disease (Van Hoosier and Ladiges 1984).

Similar gross and microscopic changes as just described for HE were noted predominately in the ceca of Syrian hamsters (Barthold and Smith 1984; Rehg and Lu 1982).

The most effective treatment for HE has been oxytetracycline in the drinking water, as compared to neomycin, dimetrdazole, and tetracycline hydrochloride (McNeil et al. 1986). Other antimicrobials (i.e., clindamycin, lincomycin, ampicillin, vancomycin, erythromycin, cephalosporins, and gentamycin) caused fatal gastrointestinal toxicity to hamsters with clinical symptoms, including diarrhea, dehydration, anorexia, and hypothermia, (Bartlett et al. 1978; Bartlett et al. 1977; Hawkins et al. 1984; Lusk et al. 1978). In another report, effective treatment was demonstrated by vancomycin hydrochloride, which did not cause major adverse effects (Boss et al. 1994).

The anaerobe *Clostridium difficile* was found to be the direct cause of the enterocolitis producing overgrowth of the ileal/colonic/cecal flora. Gross pathology consisted of distension, hyperemia, and hemorrhage of the ileum, cecum, and ascending colon. Histological sections revealed mucosal acute and chronic inflammation and congestion and hemorrhages as well as luminal collections of inflammatory cells, mucin, and sloughed epithelial cells.

Tyzzer's Disease

As compared to HE, Tyzzer's disease has been noted much less frequently. The only outbreak of Tyzzer's disease in a colony bred in the United States was reported in 1977 (Zook et al. 1977). Two other epizootics were reported in Japan (Nakayama et al. 1975; Takasaki et al. 1974).

Clinical symptoms include the sudden onset of diarrhea, dehydration, and lethargy; deaths occur within 48 hr. At gross necropsy, the most common lesion is yellow-white foci (1–2 mm diameter) in the liver, corresponding microscopically to areas of necrosis with inflammatory infiltrates. Secondary changes are thickened and discolored portions of the terminal ileum, cecum, and colon. These regions correspond microscopically to coagulative necrosis associated with epithelial cell sloughing, submucosal edema, polymorphonuclear and mononuclear cellular infiltrates, multinucleated giant cells, and fibrosis.

The causative agent is a gram-negative, pleomorphic bacterium, *Bacillus piliformis*. Positive diagnosis has been demonstrated with PAS and Gewmsa stain within the cytoplasm of intestinal epithelial cells as well as hepocytes and myocardial fibers.

Other Bacterial Infections

The possible roles of bacteria (e.g., *Pasteurella pneumotropica* and *Sriptococcs pnemomoniae*) as well as *Mycoplasma pulmonis* in the etiology of pneumonia in Syrian hamsters have not been clearly defined (Van Hoosier and Ladiges 1984).

Viral Infections

Lymphocytic Choriomeningitis

Syrian hamsters and contaminated tumor cell lines were likely sources of the infectious RNA LCM virus, which infected various laboratory personnel between 1965 and 1974, as well as other naive hamsters (Biggar et al. 1977; Skinner and Knight 1979). Commercial distribution of the cell lines ceased in 1974.

Clinical signs and symptoms of LCM occur rarely in hamsters, even in those with chronic infections for months. However, ataxia, conjunctivitis, dehydration, and tremors have been noted in a few hamsters. Death occurs following chronic progressive infection characterized by viremia, viruria, and high titers of virus in tissues (Parker et al. 1976).

Experimentally, complement-fixing antibodies have been detected in young adult hamsters by 10 days after inoculation.

Microscopic lesions include conjunctivitis, chronic glomerulonephritis, generalized vasculitis, and lymphocytic cellular infiltrates in the liver and kidneys.

Sendai Virus

Parainfluenza 1 (Sendai) is an RNA agent of the paramyxovirus group that has been implicated as a cause of hamster pneumonia. An enzootic form of the disease was reported at a research facility (Profeta et al. 1969). At gross necropsy complete consolidation of the lungs has been observed. Occasional deaths have been reported in suckling hamsters (Van Hoosier and Ladiges 1984).

Adenovirus

Syrian hamsters from 10 production colonies, including major commercial suppliers in the United States, had subclinical adenoviral infections (Gibson et al. 1990). Serum antibodies reacted

with mouse adenovirus strains K87 and FL by indirect fluorescence, which correlated to the presence of intranuclear inclusion bodies in ileal enterocytes by light microscopy (LM) and TEM.

Fungal Infections

Hamsters are susceptible to various fungal infections. Histoplasmosis, for example, has been studied extensively; hamsters are highly sensitive and therefore useful for diagnostic purposes. Most fungi grow in the spleen, lymph nodes, and liver (Van Hoosier and Ladiges 1984).

Parasitic Infections

Cestodes

Hymenolepis nana, the dwarf tapeworm, is the most prevalent internal parasite found in hamsters (Van Hoosier and Ladiges 1984). Their size varies from 25 to 40 mm in length, and they are usually found in the small intestine.

Although the consequences of infection are usually benign, impactions followed by deaths have been observed, dependent on the number of parasites and degree of intestinal occlusion.

Preventive measures rely on routine sanitation of cages and effective insect and vermin control. Yomesan (niclosamide) has been reported as effective and safe for treatment of cestode infections in hamsters (Ronald and Wagner 1975). Laboratory personnel should be aware of the possible transmissible potential and receive proper hygienic procedures.

Mites

Two species of mites (*Demodex aurati* and *D. cricetti)* have been identified in hamster epidermis (Flatt and Kerber 1968; Owen and Young 1973). *D. aurati* resides in hair follicles, whereas *D. criceti* inhabits folds in the epidermis.

Both species appear to be asymptomatic, low-grade pathogens. However, clinical skin disease in which *D. aurati* was identified resulted in demodectic mange consisting of marked alopecia and scab formation in 50% of male hamsters (Estes et al. 1971). Predisposing factors included sex, age, and stress.

Neoplastic Lesions

The incidence rates of naturally occurring tumors in untreated outbred Syrian hamsters are generally low. The most frequent tumor types are listed in table 4.12, as reported in references from 1970 to 2001. Significant low spontaneous tumor rates (< 2%) are noted for the respiratory tract, pituitary gland, and mammary gland of both sexes and the genital tract of male hamsters. Such low incidences establish the Syrian hamster as a good candidate for chemical carcinogenicity studies, including morphological studies utilizing electron microscopy, cytology, and histochemistry (Becci et al. 1978; Hess, McDowell, Resau, et al. 1981; Hess, McDowell, and Trump 1981; Schreiber et al. 1974).

Because most of the tumor types in table 4.12 are listed with only one or two references and factual historical data have not been published, the actual incidence rates are probably lower than those given. Various factors that cause these differences include diet, water quality, survival age, sex, breeding methods, genetic drift, hormonal imbalances, extent of gross necropsy and microscopic examinations, and the presence of transmissible agents.

One or more of these predisposing factors might become relatively predominant and complicate the frequency of historical tumor data. For example, in one report the majority (75%) of lymph

Table 4.12 Incidence of Various Neoplasms in Untreated Outbred Syrian (Golden) Hamsters

Organ	Tumor Type	% Incidence in Males	% Incidence in Females	Reference
Adrenal glands	Cortical adenoma	10–49a	2–23b	Pour, Mohr, Althoff, et al. (1976a)
	Cortical earcinema, Phenochromocytoma	3	2	Pour, Mohr, Althoff, et al. (1976a) Kamino, Tillmann, and Mohr (2001)
Colon/cecum	Adenocarcinoma (a)	7	20	Fabry (1985)
Harderian gland	Adenoma	3	—	Pour, Mohr, Cardesa, et al. (1976)
Liver	Cholangioma	4	7	Pour, Mohr, Cardesa, et al. (1976)
	Hepatocellular adenoma	4	0	Pour, Mohr, Cardesa, et al. (1976)
Lymphreticular system	Malignant lymphoma (lymph sarcoma)	2–53	2–53	Ambrose and Coggin (1975) Pour, Mohr, Althoff, et al. (1976a) Coggin et al. (1983) Kamino, Tillmann, and Mohr (2001)
Ovaries	Granular cell tumor/thecoma	—	3–4	Kamino, Tillmann, and Mohr (2001) Pour, Mohr, Althoff, et al. (1976a)
Pancreas	Islet cell adenoma	4–14	2–3	Kamino, Tillmann, and Mohr (2001) Pour, Mohr, Althoff, et al. (1976a)
Parathyroid glands	Adenoma	2–5	4–7	Kamino, Tillmann, and Mohr (2001) Pour, Mohr, Althoff, et al. (1976a)
Pituitary gland	Adename	—	6	Kamino, Tillmann, and Mohr (2001)
	Adenocarcinoma	—	3	Kamino, Tallmann, and Mohr (2001)
Skin	Melanoma	2	2	Turusov (1982)
Spleen	Hemangioendothelioma	1–3	—	Pour, Mohr, Althoff et al. (1976b)
Stomach (forestomach)	Papilloma	5–8	3–5	Pour, Mohr, Althoff, et al. (1976b) Kamino, Tillmann, and Mohr (2001)
Uterus	Endometrial carcinoma	—	5–7	Kamino, Tillmann, and Mohr (2001) Pour, Mohr, Althoff, et al. (1976a)
	Endometrial polyp	—	12	Pour, Mohr, Althoff, et al. (1976a)
	Leiomyoma	—	3–10	Kamino, Tillmann, and Mohr (2001) Pour, Mohr, Althoff, et al. (1976a)
Vagina	Papilloma	—	4	Pour, Mohr, Althoff, et al. (1976a)

sarcomas was horizontally transmitted, suggestive that infectious agent(s) were involved (Van Hoosier and Trentin 1979). Similarly, five epizootics of poorly differentiated lymphocytic lymphomas (53% incidence) were horizontally transmitted in two facilities by a vitriolic agent (Coggin et al. 1983). The predominant sites were the large and small intestines with involvement of mesenteric and cervical lymph nodes.

METABOLISM

Although comparative studies of hamster and human metabolism are not extensive, some exist (e.g., Young et al. 2001, for methyl mercury metabolism and deposition and Rockwood et al. 2003, for methemoglybin reductase) and there is, as we shall see, sufficient data to evaluate comparative metabolism.

Hepatic Microsomal Mixed Function Oxidase

Most of the work pertaining to xenobiotic metabolism has concentrated on the Syrian or Syrian golden strain, with some on the Chinese. Therefore, most of the following discussion is based on this particular strain. As in all other species studied, the liver is the major site and the MMFO system is the major system involved in xenobiotic metabolism in the hamster. Identified P-450 isoform activities are listed in table 4.13, and some of the salient enzyme activities are summarized in table 4.14. The liver-to-body weight ratio on the nonfasted hamster is slightly higher than that of the rat, and can range from 3.5% to 5.4% depending on sex, age, and protein content of the diet (Birt et al. 1983). CYP-450 concentrations are higher than those of rats, ranging between 0.95 and 1.35 nmol/mg microsomal protein in most published reports. A concentration of 1.85 nmol/mg (for naive hamsters) has been reported (Chiang and Steggles 1983). As in all species, cytochrome P-450 in hamsters exists as a family of isozymes, but the number of different isozymes has not been as well characterized in the hamster as in other rodent species. Nelson et al. (1996) and Sagami et al. (1991) have identified 13 different cytochrome P-450 isozymes. Concentrations of cytochrome P-450 also tend to be somewhat higher than those of the rat, ranging from 0.43 to 0.58 nmol/mg. The activity of NADPH: cytochrome C reductase is in the same range as that of the rat (i.e., 200–320 nmol/min/mg). It has not been established if this enzyme in the hamster plays the rate-limiting role in MMFO activity that it does in the rat.

Metabolism of the cytochrome P-450-dependent monooxygenase, when evaluated in hamster liver microsomes by measuring aniline hydroxylation, benzylphelamine demethylation, and benzo(a)pyrene hydroxylation, defluaranation of enflurane was evaluated by measuring free fluoride metabolites. All were inducbile with *in vivo* treatment of animals suggesting that pure *in vitro* effects are of limited value in predicting *in vivo* metabolism (Chen et al. 1995). Furthermore many of the activities are not limited to the liver but are also present in the lungs and other tissues (Sagami

Table 4.13 Specific CYP Activities Identified in the Hamster

Isoform		
CYP	1A1	
CYP	1A2	
CYP	2A8	
CYP	2A9	
CYP	2C25	
CYP	2C26	
CYP	2D20	
CYP	2D27	
CYP	2E1	
CYP	3A10	
CYP	7A1	
CYP	11A1	(11B-hydroxylase and 19-hydroxylase but not alelosterone synthane; Veronneau et al. 1996)
CYP	17A1	

Source: Nelson et al. (1996), Sagami et al. (1991).

et al. 1991). Ohhira et al. (1999) have demonstrated phenobarbital induction of CYP isoenzyme metabolism of triphenyltin.

The saturating activities of the hamster MMFO with various common model substrates are also summarized in table 4.14. In general, activity tends to be higher than that of the rat. Few differences in qualitative species MMFO substrate selectivity have been identified between the rat and hamster. There are interesting and important quantitative differences. For example, the fact that carbon tetrachloride has been reported to be a hepatocarcinogen in hamsters but not in rats is apparently due to differences in the rates of metabolism. Castro et al. (1989) have demonstrated that hamsters have a much higher rate (both *in vivo* and *in vitro*) of production of reactive metabolites that bind to nuclear protein than rats. Weyland and Bevan (1987) examined the *in vivo* disposition of benzo(a)pyrene in different rodent species. The major difference between the hamster and the other species was that increased amounts of radioactivity were retained in the lungs of hamsters at lower doses with a proportional decrease in the amount of radioactivity excreted into the bile. Bergman et al. (1984) studied species differences in a-naphthaflavone microsomal metabolism. Total metabolism was essentially the same in rats and hamsters, and the same three chemicals made up the majority of the metabolites in both species (ANF-5,6-oxide, ANF-6-phenol, and ANF-7,8-dihydrodiol). The oxide, however, was the major metabolite in rats and the dihydrodiol was the major metabolite in hamsters, indicating a site-selective species difference.

Birt et al. (1983) studied the effects of age, gender, and dietary protein on the hepatic MMFO in hamsters. In general, a high protein diet (10% vs. 40% lactalbumin) tended to cause slight

Table 4.14 Summary of Hepatic Xenobiotic Metabolizing Enzymes in Hamsters

Enzyme	Concentration or Activity	Comments and References
Cytochrome P-450	0.95–1.35 nmol/mg	Burke and Prough (1976) Smith et al. (1986) Ardies et al. (1987) McCoy and Koop (1988) Blaich et al. (1988)
Cytochrome b_5	0.43–0.58 nmol/mg	Blaich et al. (1988) DeMarco and McCoy(1985) Smith et al. (1986)
NADPH: cytochrome P-450 reductase	200–320 nmol/min/mg	Blaich et al. (1988) Burke and Prough (1976) Smith et al. (1986)
MMFO activities		
Aniline	0.50–1.2 nmol/min/mg	Burke and Prough (1976)
Benzphetamine	6.0–12.5 nmol/min/mg	Fuji et al. (1985)
7-ethoxycoumarin	5.0–14 nmol/min/mg	Smith et al. (1986)
Benzo(a)pyrene	0.3–2.5 nmol/min/mg	Ardies et al. (1987) McCoy and Koop (1988) Blaich et al. (1988)
Epoxide hydrolase (with styrene oxide) microsomal	12.6–25.9 nmol/min/mg	Pacifici et al. (1981) Oesch and Wolff (1989)
UDP-glucuronosyl transferase (with 4-nitrophenol)	40–50 nmol/min/mg	Hietanen and Vainio (1976) (untreated microsomes; no trypsin or detergents)
Glutathione S-transferase (with chlorodinitrobenzene)		Igarashi et al. (1983) Lam (1988)
Cytosolic	7–8 μmol/min/mg	Morgenstern et al. (1984)
Microsomal	0.9–1.0 μmol/min/mg	
Protein estimates		Birt et al. (1983)
Microsomal	25–35 mg/gram liver	Lechner and Gomes (1975)
Cytosolic	?	

Note: mg = mg protein.

increases in the levels of microsomal protein and tended to cause the most pronounced sex- and age-related differences. When the results of only the low-protein diet (which would be most representative of standard hamster chow) were evaluated, some interesting observations become apparent. In general, microsomal protein tended to increase as a function of age, whereas MMFO activity either increased or remained about the same in comparing activity in young versus old hamsters. Although a few isolated differences were identified, consistent and convincing differences between sexes were not demonstrated. Cytochrome P-450 also tended to increase as a function of both dietary protein content and age. Changes in cytochrome P-450, however, were not reflected in equivalent increases in MMFO activity. For example, in 18-week-old female rats (10% lactoglobulin) the cytochrome P-450 concentration was 0.58 nmol/mg protein and AHH activity was 132 ng/min/mg, whereas in 60-week-old rats (40% lactoglobulin) cytochrome P-450 content was 1.62 nmol/mg and AHH activity was 126 ng/mg/min. This suggests the possibility that there are age- or dietary-related variations in the isozymic character of cytochrome P-450 in hamsters.

Microsomal Induction

As amply reviewed elsewhere, *in vivo* treatment of mammals with a wide variety of organic chemicals can result in increases in MMFO activity by a process called enzyme induction. The prototypical inducing agent is phenobarbital, and its effects in hamsters have been well documented. Lechner and Gomes (1975) reported treatment of hamsters with phenobarbital (80 mg/kg/day for 2 days, po) induced about a 20% increase in microsomal protein, a 75% increase in cytochrome P-450, and significant increase in MMFO activity with both aminopyrene and aniline. McCoy et al. (1981) compared the effect of both phenobarbital and 3-methylcholanthrene (3-MC) in both rats and hamsters. Both treatments caused increases in cytochrome P-450 in both species, although there were slight quantitative differences in amount induced. When benzo(a)pyrene metabolism was examined, however, there was a marked difference in species response: Phenobarbital increased metabolism in hamsters, but 3-MC did not, whereas phenobarbital did not increase this activity in rats, but 3-MC caused a two orders of magnitude increase. Steggles and Chiang (1983) used an inducing regimen of a single intraperitoneal dose of phenobarbital (50 mg/kg) followed by 4 days of treatment with 0.1% phenobarbital in the drinking water. This resulted in a 40% increase in cytochrome P-450. The MMFO activity on a milligram protein basis was significantly increased with all substrates; with benzphetamine and 7-ethoxycoumarin, activity on a nanomole P-450 basis was significantly increased as well. Smith et al. (1986) treated hamsters with phenobarbital (70 mg/kg intraperitoneally for 4 days) and observed a 65% increase in cytochrome P-450 as well as a significant increase in cytochrome b5 that were accompanied by increases in MMFO activity toward both benzphetamine and ethoxycoumarin. This increase in cytochrome b5, however, has not been confirmed by other investigators (Blaich et al. 1988). Blaich et al. (1988) directly compared the inducing effects of phenobarbital on MMFO activity of both rats and hamsters. As expected, significant induction of activity toward ethoxycoumarin (the prototypical substrate for the isozyme of cytochrome P-450x induced in the rat by phenobarbital) was obtained in both species. In contrast, phenobarbital induced slight increases in rats toward both benzo(a)pyrene and ethoxyresorufin (substrates of P-448 induced by 3-MC in rats), whereas in hamsters phenobarbital increased activity only toward benzo(a)pyrene. Hence, phenobarbital is an effective inducing agent in hamsters, but the response of hamsters is somewhat different than that of rats. In general, phenobarbital effectively induces the activity in both species toward substrates, such as benzphetamine and ethoxycoumarin, of rat cytochrome isozyme P-450e. In contrast, phenobarbital and 3-MC are equally efficacious in hamsters in inducing increases in the metabolism of substrates, such as benzo(a)pyrene, of rat cytochrome isozyme P-450d (P-448). As detailed later, hamsters and rats differ considerably in their induction with 3-MC-type agents.

The effect of traditional cytochrome P-450- or P-448-inducing agents has been studied in the hamster and the response of the hamster to some of these agents has been shown to be different

from that of the rat. The treatment of hamsters with 20 mg/kg intraperitoneally once daily for 3 days of 3-methylcholanthrene (a classic or prototype inducing agent of cytochrome P-450 in rats) did not increase MMFO activity toward benzo(a)pyrene, ethylmorphine, or benzphetamine, but did increase activity toward biphenyl 2.4- to 3.4-fold. The activity of NADPH: cytochrome P-450 reductase was not induced, and this observation has been generally confirmed by most subsequent investigations on MMFO induction in the hamster.

Hietanen and Vainio (1976) examined the inducing effect of DDT (1,1,1-trichloro-2,2bis([p-chlorophenylj)ethane) on *in vitro* benzo(a)pyrene metabolism in four different species, including the hamster. In terms of baseline activity, the animals had the following rank: guinea pig > hamster > mouse > rat. Treatment with DDT (single dose, 160 mg/kg po), however, increased the activity twofold in hamsters, whereas actually causing slight decreases in the other species. These early papers suggest that hamsters are relatively resistant to 3-MC induction, while still responding to halogenated aromatics. This was confirmed by Chiang and Steggles (1983), who noted that 3-MC induced increases in microsomal cytochrome P-450 (and thereby did induce some increases in MMFO activity on a milligram protein basis), but did not increase MMFO activity per nanomole of cytochrome P-450 toward benzphetamine, 7-ethoxycoumarin, benzo(a)pyrene, or p-nitrophenetole.

Blaich et al. (1988) also reported data that suggest that although 3-MC induces increases in microsomal benzo(a)pyrene metabolism, these increases seem to be due mostly to increases in total microsomal protein rather than a specific isozyme. Comparable treatment of rats with 3-MC induces much larger increases in microsomal benz(a)pyrene metabolism.

On the other hand, Chiang and Steggles (1983) reported that polychlorinated-biphenyls (single dose, 300 mg/kg intraperitoneally) induced large increases in cytochrome P-450 and had about the same effect on benzo(a)pyrene metabolism as 3-MC, but, unlike 3-MC, increased MMFO activity per nanomole cytochrome P-450 toward benzphetamine and p-nitrophenetole. Interestingly, 6-naphthalflavone (BNF), which in the rat is considered to be a 3-MC-type inducer, induced a different pattern in hamsters than 3-MC did, in that it induced increases in microsomal 7-ethoxycoumarin metabolism, both on a milligram microsomal protein and nanomole cytochrome P-450 basis, but actually decreased benzphetamine metabolism (Ardies et al. 1987; Chiang and Steggles 1983; Smith et al. 1986). Across the board, 7-ethoxyresorufin deethylation is induced by 3-MC, BNF, and polyhalogenated hydrocarbons in hamsters as it is in rats (Blaich et al. 1988; Chiang and Steggles 1983; Smith et al. 1986). The response is much greater in rats; 3-MC induces a thirtyfold increase in rats as opposed to a threefold increase in hamsters (Iwasaki et al. 1986). Thus, with regard to cytochrome P-450 or P-448 induction in the hamster, three points should be kept in mind. First, 3-MC-type agents are less effective in inducing aromatic hydrocarbon hydroxylase (AHH)-type activity in hamsters than in rats. Second, different agents of this class have more selective effects on MMFO activity than in rats (e.g., BNF induces activity different than 3-MC). Third, halogenated hydrocarbons are the most effective members of this class in inducing generalized increases in MMFO activities and, hamsters might, in fact, be the most sensitive rodent to these agents (e.g., DDT).

The prototypical type 3, or steroidal inducing agent, pregnenolone-16(a)-carbonitrile has no effect on MMFO activity in hamsters (Chiang and Steggles 1983).

Sex-Related Differences

Unlike the situation in the rat, there are no consistently reported marked sex-related differences in cytochrome P-450 concentrations or in MMFO activity. There are some suggestive hints in the literature, however. Blaich et al. (1988) examined MMFO induction in male and female hamsters, and found that 3-MC increased the cytochrome P-450 content in both sexes: 1.34 to 2.27 nmol/mg for males and 1.24 to 2.58 nmol/mg for females. Cytochrome b5, in contrast, was increased only in females: from 0.43 to 0.48 in nmol/mg males and from 0.53 to 0.71 nmol/mg in females. As in the rat, MMFO induction was accompanied by large increases in ethoxyresorufin deethylation: from

0.14 to 1.16 nmol/min/mg for males and from 0.08 to 1.39 nmol/min/mg for females. With regard to ethoxycoumarin deethylation, however, activity was significantly increased in males (5.37–7.47 nmol/min/mg) but decreased in females (5.27–3.23 nmol/min/mg). Phenobarbital had the same inducing effect regardless of the sex in hamsters.

What are the toxicological consequences of MMFO induction in hamsters? There are a few examples in the literature that can be discussed here. Blaich and Metzler (1988b) studied the effects of microsomal enzyme induction on diethylstilbestrol metabolism and toxicity in hamsters. They observed that pretreatment of male hamsters with 7,8 benzoflavone decreases the incidence of kidney tumors but increases the incidence of liver tumors.

Ioannides et al. (1981) compared baseline and induced (with phenobarbital and 3-MC) mutagen (benzo(a)pyrene and 2-acetylaminofluorene) activation of the hamster, guinea pig, mouse, and rat in the Ames assay, which uses the S9 liver fraction (a combination of cytosol and microsomes). In all three species, 3-MC induction increased the mutagenicity of benzo(a)pyrene, whereas phenobarbital decreases it. Given the previous discussion that phenobarbital and 3-MC have approximately the same effect on microsomal benzo(a)pyrene metabolism (Chiang and Steggles 1983), these results would suggest that there are other factors involved in mutagenicity microsomal metabolism.

Santhanam and Lotlikar (1989) reported that pretreatment of hamsters with BNF increased the extent of aflatoxin B, binding to hepatic deoxyribonucleic acid (DNA) *in vivo*. They further reported that this response differentiates the hamster from the rat (in which BNF decreases aflatoxin "activation") and might make the hamster an attractive model to study aflatoxin-induced carcinogenesis.

Ethanol Induction and Metabolism

The role of the MMFO in ethanol metabolism is the subject of some debate. There is ample evidence to demonstrate, however, that ethanol induces a specific isozyme of cytochrome P-450. As it has been reported that the hamster more avidly drinks ethanol than the rat (Ardies et al. 1987), the hamster might be a more attractive model for the study of ethanol metabolism and toxicity. Thus, several investigators have studied the inducing effect of ethanol in the hamster.

DeMarco and McCoy (1985) compared the effect of two different 28-day ethanol treatment regimens (drinking water vs. liquid diet) in hamsters. They observed that only the liquid diet regimen was effective in increasing cytochrome P-450 content, but this increase was accompanied by a decrease in cytochrome b5 content and benzo(a)pyrene hydroxylase activity.

Fuji et al. (1985) used the drinking water regimen (10% ethanol v/v for 3 weeks) and also noted that ethanol causes modest increases in cytochrome P-450 in hamsters accompanied by decreases in MMFO activity on a nanomole cytochrome P-450 basis with benzphetamine, 7-ethoxycoumarin, and benzo(a)pyrene. In contrast, there were increases in aniline hydroxylation, dimethylnitrosamine demethylation, and ethanol oxidation activity. As discussed by Fuji et al. (1985), these data are sufficient to conclude that ethanol induction in the hamster is different from that observed in the rat, in which a large specific increase in 7-ethoxycoumarin deethylation occurs. These data also suggest that ethanol induces a specific isozyme of cytochrome P-450 at the expense of other isozymes in the hamster.

Ardies et al. (1987) compared the effect of ethanol with other inducing agents (phenobarbital, BNF, and isoniazid) in hamsters. The resultant MMFO of each inducing agent was distinctive with regard to the composite picture produced by the ethyl isocyanide binding spectrum, induction of cytochrome b5 effects on activity with specific substrates, and pattern produced by SDS-PAGE on microsomal protein. Consistent with previous publications, the MMFO of ethanol-treated (10% v/v ethanol in drinking water) hamsters had increased activity with ethanol and aniline but not with benzphetamine or 7-ethoxycoumarin (Ardies et al. 1987).

Using both SDS-PAGE and immunoprotein methods, McCoy and Koop (1988) further confirmed the existence of a distinctive cytochrome P-450 induced by ethanol in hamsters. Hence,

ethanol does induce a specific cytochrome P-450 in hamsters that, depending on treatment regimen, might or might not be reflected by an increased total cytochrome P-450 that has a rather narrow substrate specificity.

TCDD Metabolism and Induction

Rats and hamsters display a hundredfold difference in sensitivity to 2,3,7,8-tetrachlorodibenzo-dioxin (TCDD). Hamsters are, in fact, among the least sensitive species studied, having an LD50 between 1 and 5 mg/kg to this chemical. This observation has stimulated work comparing and contrasting the metabolism and the inducing effect of TCDD in hamsters and rats. Wroblewski and Olson (1988) studied the metabolism and inducing effects of TCDD in isolated rat and hamster hepatocytes. Interestingly, hepatocytes isolated from uninduced animals of both species had approximately the same rates of TCDD metabolism, and pretreatment with both 3-MC (50 mg/kg intraperitoneally for 3 days) or TDCC (single doses; 5 mg/kg in rats and 500 mg/kg in hamsters) caused approximately the same five- to sixfold increase in TCDD metabolism in both species. TCDD pretreatment, however, induced increases in benzo(a)pyrene metabolism in rats and not hamsters. The cytochrome P-450-specific inhibitors metyrapone and a-naphthaflavone (ANF) both had inhibitory effects on TCDD metabolism in rats, with ANF being far more effective. In hamsters, metyrapone had no effect and ANF had only a slight and transitory effect on TCDD metabolism. In an earlier work, Wroblewski et al. (1988) noted that neither 3-MC or TCDD induced increases in benzo(a)pyrene metabolism (when expressed on nanornole cytochrome P-450 basis), but both caused large increases in ethoxyresorufin metabolism in the hamster. These data suggest that TCDD has different inductive effect in rats and hamsters, but do not readily support the hypothesis that the species differences in TCDD toxicity are due to differences in metabolism.

Peroxisomal Proliferation

Hepatomegaly is a commonly used indicator of enzyme induction. However, hepatomegaly can also accompany induced increases in hepatic peroxisomes. The basic biology of these agents has been reviewed elsewhere. Whereas rats and mice are very sensitive to these agents, Lake et al. (1984) reported that hamsters were far less sensitive to such agents. For example, equivalent doses of clofibrate (500 mg/kg) induced an 18-fold increase in camitine acetyl transferase activity (a standard peroxisomal marker) in rats, but only a 2.5-fold increase in hamsters. Other data presented in this paper were consistent with this observation. This group (Lake, Evans, Foster, et al. 1989; Lake, Evans, Gray, et al. 1989) expanded on these observations with studies on nafenopin. They observed, for example, that when rats, hamsters, and guinea pigs were treated under the same dosage regimen (50 mg/kg/day by gavage for 3 weeks), the resulting increases in palmitoyl-CoA transferase were tenfold in rats, 1.5-fold in hamsters, and none in the guinea pig. Watanabe et al. (1989) obtained similar results in their studies on the species differences in peroxisomal proliferation associated with bezafibrate. Thus, the hamster is far less sensitive to peroxisomal proliferating agents than the rat and, therefore, might be a more appropriate model than the rat in assessing the toxicity of peroxisomal proliferating agents.

Epoxide Hydrolase

Aromatic groups frequently are oxidized to arene-oxides or epoxides by the MMFO, which are in turn inactivated by hydrolysis to dihydrodiols by epoxide hydrolase. This is an important enzyme that has been intensely studied in a wide variety of species, except for hamsters. Initial work by Pacifici et al. (1981) suggests that the hamster might have relatively high epoxide hydrolase activity (12.6 nmol/min/mg microsomal protein) in comparison to other rodent species (hamster = guinea

pig > rat > mouse). In comparison to nonrodent species, the rankings were baboon > hamster = human > dog. Interestingly, the hamster had the highest activities of renal and pulmonary epoxide hydrolase. Oesch and Wolff (1989), in their studies on hexachloro-I, 3-butadiene metabolism, confirmed that hamsters had higher epoxide hydrolase activity than other rodent species but obtained a higher saturating activity than Pacifici et al. (1981); that is, 25.9 nmol/min/mg. Rigorous exploration of the substrate specificities, inhibitors, and other enzymological aspects of this enzyme in hamsters remains to be done.

Aromatic Amine Oxidation

Hamsters have proven to be convenient species for studying the oxidation of aromatic (primary or secondary) amines and amides. Lotlikar et al. (1967) noted that of the five most studied rodents (rat, hamster, mouse, rabbit, and guinea pig), hamsters had the highest baseline activity in the *in vitro* N-hydroxylation of 2-acetylaminofluorene, and that such activity was highly inducible by pretreatment with 3-MC. Razzouk and Roberfroid (1982) confirmed that the hamster had higher activity than the rat with 2-AAF (and 2-aminofluorene), but observed that the mouse had higher activity than the hamster. The difference between the two papers might be due to the difference in mouse strains examined. The Razzouk and Roberfroid paper did not specifically examine the relative contributions of the MMFO versus the flavine mixed function oxidase (FMFO) system N-hydroxylation in the hamster, but did present data from experiments using inhibitors that the FMFO was at least partially involved.

Beckett and Gibson (1975) examined the species-related differences in N-hydroxylation of dibenyzlamine and noted (consistent with Lotlikar et al. 1967, and Razzouk and Roberfroid 1982) that the hamster had higher activity than either the rat or the mouse (but not as high as the rabbit or the guinea pig).

McMahon et al. (1980) examined N-hydroxylation of 4-aminobiphenyl in various species. N-hydroxybiphenyl is the major metabolite of this chemical in the rat, guinea pig, C3H mouse, and hamster. The highest initial rates (*in vitro*) were seen in the hamster, whereas the lowest were seen in the rat. In contrast to the previous discussion on induction, these authors also reported that PCB treatment induced large increases in N-hydroxylation in the rat but had no effect in the hamster.

Gorrod and coworkers (Gorrod and Gooderham 1987; Gorrod and Patterson 1983) studied the metabolism of N-benzyl-4-substituted anilines, and observed that hamsters had the highest N-hydroxylation activity, although it should be mentioned that the hamster also had the highest N-debenzylation activity. As discussed by Gemborys and Mudge (1981), the hamster has a high rate of N-hydroxylation with acetaminophen, which contributes to the sensitivity of this species to acetaminophen hepatotoxicity. Little work has been reported on the FMFO in hamsters, but the data reported by Gorrod and Patterson (1983) is consistent with the hypothesis that the FMFO is largely responsible for N-hydroxylation in the hamster.

Ioannides et al. (1981), in their studies on the effects of different inducing agents on the activation of 2-acetylaminofluorene, concluded that a system other than the MMFO is involved in this process in hamsters as well as in other species. McCoy et al. (1986) studied the microsomal metabolism of nicotine in hamsters. Nicotine N'-oxide was one of the major metabolites in their studies, and using a combination of inhibitor and protease treatments, they were able to conclude that this reaction was mediated via the FMFO. In contrast, Nwosu and Crooks (1988) were unable to detect any nicotine N'-oxide as a urinary metabolite when nicotine was administered *in vivo* to hamsters. The reasons for the differences between these two papers are not evident, but the N'-oxide could either be further metabolized or simply not excreted via the urine. In general, when the combined literature is considered, the hamster has a relatively high capacity, especially compared to the rat, for the oxidation of aromatic amines and amides, and this activity is mediated, at least in part, by the FMFO.

N-Acetylation

The acetylation of aromatic amines to aromatic amides is generally considered to be the first step in the activation of aromatic amines to hepatocarcinogens (e.g., the conversion of 2-aminofluorene to 2-acetylaminofluorene). Lower and Bryan (1973) reported that the hamster has higher *in vitro* rates of aromatic amine acetylation than the guinea pig, mouse, and rat. For example, with 4-aminobiphenyl, the activity in hamster cytosolic preparations was 2.4-fold higher than that of the rat. Ioannides et al. (1981) compared the metabolic activation of 2-acetylaminofluorene to mutagens by the microsomes from different species. Hamster microsomes were extremely active in this regard, whereas rat microsomes displayed very weak activity. Given that the hamster has greater activity in the acetylation of aromatic amines and the subsequent oxidative activation of these to mutagenic intermediates, the hamster would probably be a better model than the rat in the study of the metabolism and toxicity of aromatic amines.

Interestingly, homozygous "rapid" and "slow" acetylator inbred strains of hamster have been identified and characterized (Hein et al. 1986; Trinidad et al. 1989). The slow acetylator strain has only 3% of the N-acetyl transferase activity with aminofluorene as the rapid strain, whereas the activities of other enzymes involved in aromatic amine activation are similar (Hein et al. 1986). Additional work (Trinidad et al. 1989) further established that the difference was consistent for a variety of aromatic amines (e.g., p-aminobenzoic acid) but not for nonaromatic amines (e.g., isoniazid).

Glutathione S-Transferase

Glutathione and the glutathione S-transferase (GST) enzymes comprise one of the most important systems in the phase II metabolism or deactivation of active metabolites. The capacity of this system is controlled by the amounts of glutathione available and the activity of the GSTs. As reported by James and Harbison (1982), there is little difference among rats, guinea pigs, and hamsters with regard to the concentration of reduced glutathione, ranging from 6.5 to 9.3 μmol/g liver. They also reported that SKF 525-A as well as other chemicals that form metabolic intermediate complexes with cytochrome P-450 cause decreases in glutathione in the hamster. Stein et al. (1988) reported a broader range; 4.4 (rat) to 10.6 μmole/g (mouse), with a mean for the hamster of 5.4 μmol/g. Igarashi et al. (1983) reported a similar range; 3.5 (guinea pig) to 7.8 μmol/g (mouse), with the mean for hamsters of 5.4 μmol/g. Fasting had no effect on glutathione concentrations in hamsters, but provoked decreases of 47% to 65% in the other species examined. Igarashi and coworkers (1986) have also examined GST for species-specific differences. When glutathione S-transferase activity was examined using crude cytosol as the enzyme source and 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate, hamsters had the highest activity compared to the rat, mouse, guinea pig, and rabbit. The GST of the hamster is made up of three different subunits ranging in weight from 27 to 28.5 Da, as identified by SDS-PAGE (Igarashi et al. 1986). On S-sepharose column chromatography, hamster GST activity eluted as four apparent isozymes (presumably different combinations of subunits). Rigorous exploration of the substrate specificities of the different hamster isozymes has not been reported. Lam (1988) reported a value for GST activity (7.73 ± 0.46) measured under saturating conditions that was in good agreement with that reported by Igarashi et al. (1986). They also reported that the hamster differs from other species in that subchronic treatment with BHT does not cause an increase in GST. In summary, the hamster possesses an active multizymic cytosolic GST system and adequate amounts of glutathione for this system to play a role in xenobiotic metabolism and toxicity, but it has not been thoroughly explored.

Glutathione S-transferase is generally considered to be a cytosolic enzyme. As reviewed by Morgenstern et al. (1984), however, the existence of a distinct microsomal GST has been known since the early 1980s. There is sufficient evidence that this is a distinct enzyme and not a contaminant

of microsomal preparations by cytosolic remnants. For example, in *in vitro* assay systems, it is allosterically activated by N-ethylmaleimide, and only with such activation does the specific activity approach that of the cytosolic enzymes with the more traditional substrates, such as CDNB. As reported by Morgenstern et al. (1984), the hamster has the highest specific activity for microsomal GST of any of the common laboratory species. For example, male hamsters have an activity of 957 ± 58 nmol/min/mg (with CDNB in N-ethylmaleimide-stimulated hepatic microsomes), whereas male rats have an activity of 540 ± 25 nmol/min/mg under the same conditions. Although on the basis of specific activity and total amounts, the microsomal GST comprises only a small portion of the total hepatic GST, it can still play an important role in the metabolism and toxicity of specific chemicals. For example, in hamsters hexachloro-1,3-butadiene is preferential metabolized by the microsomal GST by a ratio of 22.7 to 1.0 over the cytosolic enzyme (Oesch and Wolff 1989). Similar ratios were 37.7 and 3.3 for human and rat preparations, respectively. The hamster might, therefore, be a better species than the rat in the study of this enzyme.

Conjugation Reactions: Glucuronide, Sulfate, and Amino Acids

Enzyme systems other than GST are involved in Phase II reactions. As a loose rule of thumb, these other systems tend to act on more stable chemicals, such as aromatic acetates, phenols, or primary amines, than GST. These other systems catalyze the formation of glucuronic acid, sulfate, and amino acid conjugates. This would also include acetylation, which has been previously discussed. The activity of these systems has been at least partially examined in the hamster. Huckle, Hutson, et al. (1981) studied the species differences in the *in vivo* metabolism of 3-phenoxybenzoic acid. Glucuronide conjugates predominated in the hamster, whereas the sulfates predominated in the rat. They noted that the hamster, like other rodents and unlike the cat or ferret, form a very low percentage of amino acid (glycine and taurine) conjugates. In fact, the hamster has the lowest activity of any rodent of glycine N-acyl transferase activity measured *in vitro* under optimum conditions (Huckle, Tait, et al. 1981). Emudianughe et al. (1987a) examined the *in vivo* metabolism of 1-naphthylacetic acid, and observed that the predominant urinary metabolite in hamsters was the glucuronide (64%), whereas the glycine conjugate comprised a smaller percentage (10%). One could generalize that if a functional group could be conjugated with either glucuronic acid or an amino acid, the former will always predominate in the hamster. This, however, is not the case. Emudianughe et al. (1987a, 1987b) compared the metabolism of 1- versus 2-naphthylacetate, and found that this chemical was such a poor substrate for uridine-diphospho-glucoronosyl (UDP-glucuronosyl) transferase that even in the hamster amino acid conjugates (glycine and glutamine) were the major metabolites. Hence, depending on the substrate or metabolite available, the hamster will tend to form glucuronide metabolites at the expense of amino acid conjugates, but there are exceptions.

Relatively little work has been done on the UDP-glucuronosyl transferase (a microsomal enzyme) in hamsters since the 1970s. Hietanen and Vainio (1976) compared the *in vitro* activity of UDP-glucuronosyl transferase in the rat and hamster using p-nitrophenol as the substrate. They observed that on a microsomal protein basis, the hamster had higher activity, but when corrected for the n-ficrosomal protein and expressed on a gram liver basis, the activity of the two species was about the same (8–12 nmol/min/g). Rats have somewhat higher UDP-glucuronosyl activity with thyroid hormone (T4), a natural substrate, than hamster (Henry and Gasiewicz 1987), and TCDD treatment induced increases in both species. Interestingly, TCDD provokes completely different changes in circulating thyroid hormone concentrations in rats (decreases) and hamsters (increases), a difference that cannot be accommodated by the difference in UDP-glucur-onosyl transferase. Based on the available data, there appear to be quantitative differences in the activity of this enzyme between the rat and the hamster that will require additional work to delineate.

Acetaminophen Metabolism and Toxicity

UDP-glucuronosyl transferase and 31 phosphoadenosine-51 phosphosulfate (PAPS) sulfotransferase (a cytosolic enzyme) frequently act on the same substrates, and the differences in substrate specificity and the availability of cosubstrate (PAPS availability is often limiting) determine which conjugate is preferentially formed. This has been studied in the hamster in the metabolism of acetaminophen. As mentioned, the hamster is among the most sensitive of species to the hepatotoxic effect of acetaminophen, a phenomenon believed to be due, at least partly, to the higher rates of N-hydroxylation by hamsters. This was convincingly demonstrated by Green et al. (1984), who examined the metabolism and toxicity of acetaminophen in hepatocytes isolated from various species. As expected, the hamster produced a higher ratio of toxic to nontoxic (i.e., sulfates and glucuronides) metabolites than other species. This was confirmed by Tee et al. (1987), who demonstrated (a) that hepatocytes isolated from several different species, including the hamster, were equally sensitive to cell injury when exposed to the putative toxic metabolite of acetaminophen, N-acetyl-p-benzoquinoneimine; and (b) hamsters have higher rates of production of this metabolite than other species. Even in hamsters, however, the majority of acetaminophen is disposed of via conjugation to nontoxic metabolites.

As demonstrated by Roberts et al. (1986), sulfate formation is the preferred conjugative pathway of acetaminophen metabolism in isolated hamster hepatocytes. Miller and Jollow (1987) also demonstrated that the sulfate was the preferred conjugate in hamster hepatocytes, especially in incubations fortified with inorganic sulfate. Further, pretreatment of intact hamsters with an inhibitor of PAPS sulfotransferase (2,6dichlorodinitrophenol; 10 mg/kg intraperitoneally given 30 min before acetaminophen) greatly enhanced acetaminophen-induced liver injury. *In vivo*, however, the glucuronide is the preferred conjugate. Miller and Jollow (1987) concluded that this was due to the capacity (as evidenced by the K_{max}) of the sulfotransferase, and not the availability of PAPS. Brzeznicka et al. (1987) measured the concentrations of PAPS and observed that the concentration in hamsters was about half that in rats (approximately 68 vs. 33 nmol/g liver). These observations are not mutually exclusive, and it is likely that both the capacity of sulfotransferase and the availability of PAPS are the reasons that the sulfotransferase does not play a greater role in the metabolism (and, therefore, protection against hepatotoxicity) of acetaminophen in hamsters.

Diethylstilbestrol Metabolism and Toxicity

The sensitivity of hamsters to the heptotoxicity of acetaminophen is an example of a quantitative species difference in toxicity that has a metabolic explanation. The response of hamsters to diethylstilbestrol (DES) meanwhile is an example of a qualitative species difference; that is, DES causes kidney tumors in male hamsters, but not in any other species studied. Gottschlich and Metzler (1980) extensively studied the metabolism of DES in hamsters and could not identify any species- or sex-related differences in the metabolism of DES to explain the difference in toxicity. This work was continued (Blaich and Metzler 1988a, 1988b) and has produced some interesting findings. For example, cotreatment of hamsters with 7,8-benzoflavone and DES led to a reduction in the kidney tumor incidence and a rise in liver tumor incidence. There is still, however, no evidence that the metabolism of DES plays a role in the induction of renal tumors by this chemical in hamsters. The more rapid hepatic metabolism of DES induced by 7,8-benzoflavone probably plays a role in the kidney protection afforded by this treatment regimen.

Extrahepatic Metabolism

Up to this point, this section has focused on hepatic xenobiotic metabolism, as the liver is quantitatively the most important organ in xenobiotic transformations. Other organs can also possess

metabolic capacity that can play a role in xenobiotic metabolism and toxicity. Those of the hamster are briefly mentioned here. Burke and Prough (1976) examined the MMFO activity of hamster liver and lung with a variety of different substrates. In general, the lung had much lower activity (e.g., 0.05% for AHH), but for a few substrates activity in the lung can approach that of the liver (e.g., 77% with biphenyl), and MMFO activity in the hamster lung is essentially unresponsive to induction by 3-MC. Smith et al. (1986) demonstrated that the kidney contains cytochrome P-450 (and the other components of the MMFO) at about 10% (on a microsomal protein basis), and that increases in the activity of the renal MMFO were not induced by common hepatic MMFO inducing agents. Li et al. (1983) demonstrated that AHH activity of the male hamster kidney is about 20% of that of the kidney, and that various antiandrogenic and/or estrogenic treatments will depress the activity of the kidney but not the liver. Wiebkin et al. (1984) reported that isolated hamster pancreatic acinar cells have low (16- to 210-fold less than the rat) but measurable rates of MMFO activity with a variety of different substrates. Pretreatment of hamsters with common inducers had no effect on acinar MMFO activity. Hietanen and Vainio (1976) reported that unlike other species examined, the hamster has no measurable intestinal (duodenum) AHH activity, but does have UDP-glucuronosyl transferase activity (about 25% on a microsomal basis when compared to hepatic activity). Hein et al. (1986) demonstrated that the hamster kidney has higher levels of sulfotransferase than the liver. Kawakubo et al. (1988) reported that hamster skin has a surprisingly high N-acetyl transferase activity that could play a role in carcinogen activation in this species. Hadley and Dahl (1983) reported the MMFO activity of hamster nasal tissue to be higher than any other species examined.

Gut Flora Metabolism

As reviewed by Rowland et al. (1986), the gut flora can also play a role in xenobiotic metabolism and toxicity. The gut flora metabolic activity in rodents tends to be lytic (3-glucosidase and 6-glucuronidase) or reductive (azo, nitro, and nitrite reductases) in nature. The rat and hamster have essentially equivalent activities of 6-glucosidase (30–35 Amol/hr/g feces), azo reductase (2–3 Amol/hr/g feces), and nitroreductase (3.8–4.2 Amol/hr/g feces). The rat has much higher activities with P-glucuronidase (156 vs. 60.8 Amol/hr/g feces) and nitrate reductase (3.9 vs. 1.7 Amol/hr/g feces).

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CHAPTER 5

The Guinea Pig

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Pathology: **John C. Peckham**
Experimental Pathology Laboratories, Inc.

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CONTENTS

TOXICOLOGY

Journalists often refer to human research subjects as human guinea pigs, and the public mind has long regarded the guinea pig as the classic laboratory animal for all biomedical research and safety assessment. Actually, their use is now proportionately constant at 2% of the annual total of laboratory animals. This makes them only the third or fourth most popular species in toxicology and safety assessment.

Although not used in the numbers perceived by the general public, guinea pigs are important research animals that are used primarily in studies of immunology, audiology, and infectious diseases. They are relatively expensive, usually costing three to five times as much as rats and often more than rabbits; however, perhaps their greatest disadvantage for research use is the lack of

readily accessible peripheral veins for intravenous injections and collection of blood and serum samples. As will be seen, however, they are more widely used in safety assessment than most believe.

Species and Characteristics

The scientific name of the guinea pig is *Cavia porcellus*. Guinea pigs are hystricomorph rodents (suborder *Hystricomorpha*, order *Rodentia*) originating from South America. They are more closely related to porcupines and chinchillas than to mice or rats.

Guinea pigs have long been used as experimental animals in biomedical research because they are small, tame, and easy to handle. Of the three natural varieties (English, Abyssinian, and Peruvian), the albino form of the short-haired English variety is the most commonly utilized in the laboratory. The Hartley strain of this variety is by far the most commonly used in toxicology. Recently, the Charles River Laboratories has also developed a fertile, euthymic hairless strain that offers advantages for use in dermal studies.

The popularity of the guinea pig as a pet and research animal owes much to their docile nature. They seldom bite or scratch and will respond to attention with frequent and gentle handling. At the same time, they tend to be messy in their habits, and the development of human allergies to guinea pigs is not uncommon (in the toxicology laboratory, probably only allergies to the rabbit and rat are more common).

In many ways, this roly-poly rodent is the most attractive laboratory animal. Exceptionally curious, the guinea pig investigates any activities outside of its cage by attempting to watch, and expressing itself by a wide range of sounds from a deep chortle to a shrilling whistle. They are unable to climb, but they can jump short distances.

Guinea pigs are alert, full bodied, and have smooth, shiny skin. Their coat is dense, with the hair being clean and not marked by discharge from the nose, eyes, or ears. If allowed to feed freely, guinea pigs can become quite large over time. Males will commonly reach 1 kg at 1 year, and can weigh several kilograms at a later age.

History

The guinea pig was first scientifically described by Gesner (1516–1605) and Aldrovandus (1522–1607) in about 1580 (Wagner 1979). The use of the guinea pig as a test animal for biomedical research goes at least back to Lavoisier in 1780 (Lane-Petter 1963), who used it to measure heat production by animals.

Collins (1979) reported that (for 1965) about 2.5 million guinea pigs were used annually for research in the United States. Over the more than 25 years since, this figure has undoubtedly decreased. By 1983, total use of guinea pigs in research was 521,237 (of which 28,753 were used in toxicology).

Utilization as a Test Animal

In the broad range of biomedical research, the guinea pig has been employed as the test animal in a wide range of investigations: nutrition, pharmacology, allergy, radiology, and immunology. Complement, a substance originally isolated from the blood of guinea pigs, is a key component in serological work.

The guinea pig can be easily infected by human tuberculosis germs, and is indeed the animal of choice for diagnosis of tuberculosis in humans. Another disease that the guinea pig shares with its fellow mammal, the human, is scurvy. This disease is noninfectious, caused by a deficiency of vitamin C in the diet. An idle but interesting fact is that the only other animal subject to scurvy, the monkey, is also very susceptible to human type tuberculosis.

Table 5.1 Toxicological Endpoints Evaluated Using the Guinea Pig as a Model

Endpoint	References
Delayed contact dermal sensitization (Coombs type IV) Buehler, Draize, Landsteiner, Guinea pig maximization (GPMT), and so on, test designs	Landsteiner and Chase (1937, 1940, 1941, 1942) Draize et al. (1944) Buehler (1964) Magnusson and Kligman (1969)
Immediate hypersensitization	Ayala et al. (1988)
Photosensitization	Ichikawa et al. (1981)
Armstrong and Harber and Shalita tests	Harber and Shalita (1975)
Ototoxicity (because of early findings with antibiotics and nonsteroidal anti-inflammatory drugs)	Brummett (1983) Cazals and Guilhaume (1985) Parravicini et al. (1983)
Cataractogenesis (induction of cataracts by dermal or oral application of test substances)	Melnikova and Radionov (1979)
Pulmonary irritation and sensitization	Emerson and Cole (1983) Karol et al. (1980)
Systemic toxicity (in place of traditional species such as rat, mouse, and dog); usually due to efficacy data being developed in carrageenan colitis model in this species	Adrian et al. (1976) Lee and Thomsen (1982)
Host resistance assay (for generalized screen of effect on immune competence, in place of traditional mouse model in same assay)	
Teratology	Hoar (1969)
Inhalation	Firpo et al. (1988)

In safety assessment and toxicology studies, however, the use of the guinea pig is a bit narrower. Table 5.1 summarizes these uses. Specific protocols for some of these tests are discussed later in this chapter.

Basic Biological Characteristics

During the course of all the different kinds of toxicology studies that need to be designed and conducted, a complete knowledge of the fundamental biological characteristics is essential. Also, most studies are conducted with the measurement of various parameters to determine if a test material has had any (particularly any adverse) effect on the animals. Such measurements must start from a position of knowledge of what normal values are and what kind of variability is commonly seen.

Tables 5.2 through 5.9 summarize a range of basic biological characteristics of the guinea pig. Most of these were gained from a review of general sources (e.g., Collins 1979), although a number of special references are cited in the tables. The tables summarize physiological (table 5.2), reproductive (table 5.3), hematology (table 5.4), clinical chemistry (table 5.5), serum enzyme (table 5.6), serum protein component (table 5.7), organ weight (table 5.8), and deoxyribonucleic acid (DNA) repair (table 5.9) characteristics. Figure 5.1 shows growth curves for male and female animals.

Husbandry

Guinea pigs represent an intermediate species in terms of ease and expense of care. Although they are considerably easier to house and maintain than primates, dogs, or rabbits, they are clearly more expensive and difficult than rats or mice.The objective of all that is done for the husbandry of laboratory animals can be distilled to maintaining healthy animals in as stable an environment as possible, and as humanely as possible.

Table 5.2 Physiological Parameters

Life span	2–6 years	
Rectal temperature	38.6ºC–40.0ºC	
Respiration rate	69–160/min	
Heart rate	240–277/min (Fara and Catlett 1971) or 130–190/min (Collins 1979)	
Daily food consumption	8 g/100 g body weight	
Daily water consumption	10 ml/100 g body weight	
Percentage of total life span	Embryonic	1.8%
	Gestational	4.4%
	Puberty	4.2%
Oxygen consumption	0.76 ml O_2/g/hr	
Tidal volume	1.8 ml	
Minute volume	0.16 L	
Mean blood pressure (mmHg)	Systolic	76.7
	Diastolic	46.8
Metabolic rates (resting)	3–5 weeks of age	34.0 cal/m^2/hr
	7–9 weeks of age	33.25
	11–13 weeks of age	32.95
	5–6 months	30.8
	11–12 months	29.54

Table 5.3 Reproductive Parameters

Puberty	45–70 days
Breeding age	12–14 weeks
Gestation	59–70 days; 63 average
Weaning age	21–28 days
Diploid chromosome number	64
Litter size	1–8; 3–4 average
Estrus cycle	16–18 days

Table 5.4 Hematology Parameters

Plasma volume		30.6–38.2 ml/kg body weight
Whole blood volume		75 ml/kg body weight
Red blood cell diameter		7.1 μ
Red blood cell sedimentation rate		0.5–1.5 mm/hr
Blood pH		7.35
Red blood cells		4.5–7.0 x 10^6/mm^3
Micronuclei		0.2 ± 0.4 (1–5 days old) 0.3 ± 2.8 (adults; Zuniga-Gonzalez et al. 2001)
Hematocrit		42.0–55.2 ml/100 ml
Platelets		3.4–10.0 x 10^5/mm^3
Hemoglobin		11.7–14.5/100 ml
White blood cells		9.9–10 x 10^3/mm^3
Differential:	Neutrophils	4.2 x 10^3/mm^3
	Eosinophils	0.4
	Basophils	0.07
	Lymphocytes	4.9
	Monophils	0.43
PVC (%)		43
MCV (cuμ)		81
MCH ($\mu\mu$g)		25
MCHC (%)		30
Reticulocytes (%)		0.9–1.0
Red blood cell	Diameter	7.1 μ (Ponder 1948; Scarborough 1931)
	Life span	60–80 days (Edmundson and Wyburn 1963)

Table 5.5 Clinical Chemistry Parameters

Plasma	Calcium	5.3 mEq/L
	Sodium	145-152 mEq/L
	Chloride	105 mEq/L
	Phosphorus	5.3 mg/100 ml
	Potassium	6.8–8.9 mEq/L
	Magnesium	2.3 mg/100 ml
	Cholesterol	21-43 mg/100 ml
	Serum protein	5.4 g/100 ml
	Albumin	2.8–3.9 g/100 ml
	Globulin	1.7–2.6 g/100 ml
	Protein-bound iodine	1.8–2.2 mμg/100 ml
Whole blood	Glucose	82–107 mg/100 ml
	Bilirubin	22–39 mg/100 ml
	Non-protein-bound nitrogen	30–51 mg/100 ml
	Creatinine	0.99–1.77 mg/100 ml
	Blood urea nitrogen	30–51 mg/100 ml
	Glucose	79–107 mg/100 ml
	Uric acid	1.3–5.6 mg/100 ml
	Total lipids	94–245 mg/100 ml
	Phospholipids	25–77 mg/100 ml
	Triglycerides	0–145 mg/100 ml
	Progesterone	0–2.75 ng/100 ml
	Estrogen	0–54 pg/100 ml

Table 5.6 Serum Enzyme Activities

	Values			
	Male		Female	
Enzyme	***M***	**SD**	***M***	**SD**
Amylase (Somogyi units/dl)	295.0	31.0	269.0	28.0
Alkaline phosphatase (IU/L)	74.2	6.92	65.8	5.46
Acid phosphate (IU/L)	32.2	2.59	28.7	3.20
Alanine transaminase (SGPT) (IU/L)	44.6	6.75	38.8	7.15
Aspartate transaminase (SGOT) (IU/L)	48.2	9.5	45.5	7.00
Creatine phosphokinase (CPK) (IU/L)	0.95	0.15	1.10	0.20
Lactic dehydrogenase (LDH) (IU/L)	46.9	9.50	52.1	11.2

Source: Data from Albritton (1952), Altman and Dittmer (1964).

Table 5.7 Serum Protein Components of the Normal Guinea Pig

	Values			
	Male		Female	
Fractions	M	SD	M	SD
Total protein (g/dl)	5.60	0.28	4.80	0.34
Albumin (g/dl)	2.73	0.30	2.42	0.27
(%)	48.8	5.50	50.5	5.40
A_1-Globulin (g/dl)	0.11	0.04	0.10	0.02
(%)	1.90	0.38	2.20	0.19
A_2-Globulin (g/dl)	0.33	0.08	0.23	0.06
(%)	5.90	1.25	4.80	1.42
β-Globulin (g/dl)	1.14	0.20	0.82	0.17
(%)	20.40	4.10	17.10	3.60
γ-Globulin	1.29	0.26	1.22	0.15
(%)	23.10	4.60	25.40	3.25
Albumin/globulin	0.95	0.16	1.02	0.18

Source: Data from Burns and De Lannoy (1966) and Altman and Dittmer (1974).

Table 5.8 Organ Weights in Two Strains of Guinea Pigs[a]

	Weight (g) ± *SD*			
	Strain 2		Strain 13	
Organ	Male	Female	Male	Female
Body	802 ± 65	780 ± 69	1044 ± 69	940 ± 99
Liver	25.37 ± 2.9	29.21 ± 4.83	33.7 ± 3.89	35.5 ± 8.50
Lungs[b]	5.21 ± 0.97	5.18 ± 1.22	7.23 ± 0.94	7.44 ± 0.88
Heart	2.12 ± 0.28	2.07 ± 0.27	2.42 ± 0.33	2.26 ± 0.23
Thyroid[c]	0.061 ± 0.013	0.058 ± 0.007	0.078 ± 0.017	0.074 ± 0.924
Kidney[c]	2.94 ± 0.56	2.79 ± 0.38	2.57 ± 0.24	2.33 ± 0.15
Adrenal[c]	0.402 ± 0.13	0.394 ± 0.110	0.310 ± 0.050	0.284 ± 0.041
Spleen	0.78 ± 0.12	1.03 ± 0.27	0.73 ± 0.07	0.93 ± 0.16

[a] N = 20 in all cases.

[b] Weight of both lungs.

[c] Weight of left organ only.

Source: From Breazile and Brown (1976).

Table 5.9 DNA Repair Activity (erg/mm² UV light equiv.)

IQ	9.4
MCIQ	1.7
2-AF	14.2
IQ/2-AF	5.5
MeIQ/2-AF	0.7

Source: Loury and Byard (1985).

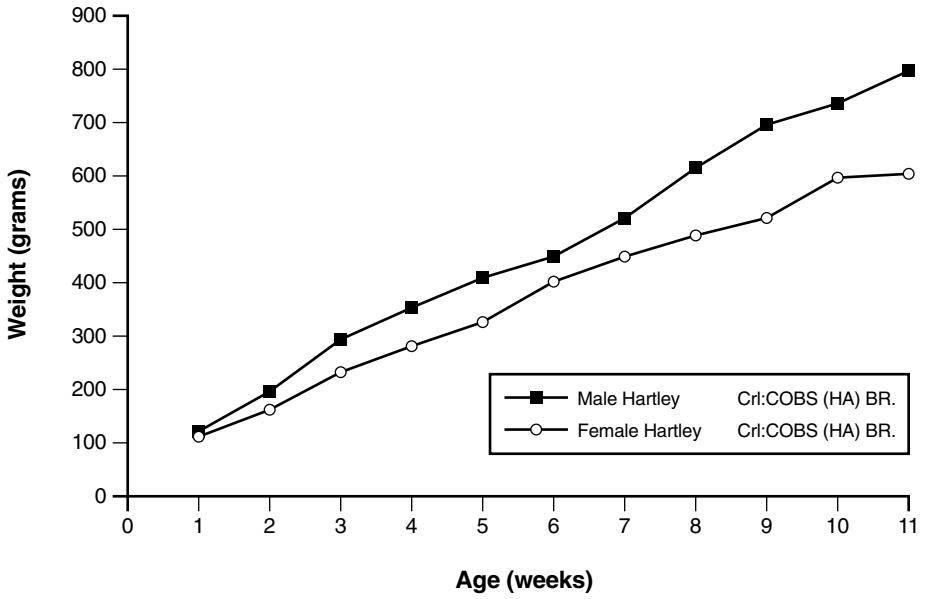

Figure 5.1 Guinea pig weight curves.

One set of broad principles that should be taken into account when assessing whether animals are being humanely maintained and utilized are the so-called five freedoms (Webster 1986). These are:

1. Freedom from thirst, hunger and malnutrition, achieved by readily accessible fresh water and diet to maintain full health and vigor.
2. Appropriate comfort and shelter.
3. Freedom from injury and disease, achieved by prevention or rapid diagnosis and treatment.
4. Freedom of movement and the opportunity to express most normal patterns of behavior.
5. Freedom from fear.

Although in toxicological research some of these five principles are not strictly possible, to the extent possible they should guide the researcher's actions.

Housing and Caging

Eveleigh (1988) recently reviewed the history of the development of caging for several laboratory species, including the guinea pig, and the interested reader is directed to that source.

Because guinea pigs do not climb, they can be kept in open-sided boxes or pens, provided that the sides are at least 10 in. high.

Guinea pigs can be housed indoors in pens on the floor, in fixed or portable tiered compartments, or in cages. Floor pens, despite their disadvantages (e.g., waste of space and spread of infection), are commonly used because of the simplicity and ease of cleaning and inspection. The general size of a pen is 40×96 in., and it can be placed on each side of the room, leaving a passage about 24 in. wide. Another arrangement uses only half of the available space each day.

Tiered compartments can also be used. These are permanent compartments made of concrete or wooden shelves or portable metal units. The permanent type are not much more economical in space than the floor pens, and they are generally more expensive and difficult to clean and disinfect. The portable type, up to four tiers, are space saving and easy to clean.

The initial cost of a caging system is greater than boxes, pens, or tiered compartments, but does save space. Generally, $30 \times 30 \times 14$ in. cages made out of a single aluminum alloy sheet are ideal. These cages can be divided by panels if needed. Each cage can hold a harem of four sows and one

Table 5.10 Minimum Floor Space per Guinea Pig

Weight (g)	Floor Area per Animal (cm^2)	Height (cm)
Up to 250	277 (43 in.)	17.8 (7 in.)
250–350	374 (58 in.)	17.8 (7 in.)
Over 350	652 (101 in.)	17.8 (7 in.)

boar. The cages will hold 12 young animals up to 350 g in weight, and the number can be reduced to 10, 8, and 6 as the weight increases. The cages for experimental animals are $10 \times 10 \times 10$ in. and are made up of 3/4-in. wire mesh to hold individual animals. A 24×16 in. floor area could be enough for 10 guinea pigs. The most common bedding material is seasoned softwood shavings. Peat moss, although more expensive, is highly recommended. Treated flax and corncobs are also good, as they have highly absorbent and deodorant properties. Cereal straw should not be used because it might be irritating to the animals and could act as a source of contamination.

The Institute of Laboratory Animal Resources (ILAR) guidelines for floor space to be provided per animal did not change with the 1985 revision of the *Guide for the Care and Use of Laboratory Animals*. Caging or housing of whatever form should provide (as a minimum) the space for each animal shown in table 5.10 (National Institutes of Health 1985).

Attention to the details of thorough cleaning is an essential component of the husbandry of guinea pigs. Obnoxious odors, the accumulation of mineral scale from the high concentration of mineral salts in guinea pig urine, and the spread of infectious diseases between animals and cages are minimized with proper sanitation of cages and racks.

Cages in which bedding is used should be cleaned and sanitized often enough to prevent an accumulation of excreta or debris. This generally means that cages should be sanitized at least once each week, and all new bedding should be added each time the cage is sanitized. Although wire floor cages do not have to be cleaned as frequently, they should be sanitized at least once every 2 weeks. The pan under the cage, however, must be cleaned frequently to prevent the accumulation of excreta. If large numbers of animals are maintained in each cage, daily cleaning of the pans might be necessary to maintain a high level of sanitation. Soiled bedding should be disposed of promptly either by incineration or in a way that will not contaminate other animal areas or areas of human habitation.

Temperature, Ventilation, and Humidity Control

Strict attention should be given to air-handling systems to control temperature, humidity, air velocity, and air pressure within the animal room. Guinea pigs are extremely susceptible to respiratory disease, and a well-designed, efficiently operating system provides the essential environmental stability for respiratory disease control.

There should be 10 to 15 changes of air per hour and no air should be recirculated unless it has been filtered to remove airborne contaminants. Care must be taken to control the velocity and direction of air flow to produce a draftless and even distribution of air to all areas of a room. An ideal state of mass air displacement is, in some instances, difficult to achieve in a room filled with animals and cages, and in these circumstances cages can be designed or arranged to minimize drafts.

Ideally, room temperatures should be maintained between 65°F and 75°F (20°C–25°C) and relative humidity at approximately 45% to 55%.

Breeding

It is general practice now to purchase guinea pigs for toxicological research from specialized vendors, and generally it is not cost effective to try to raise stocks for use in tests. However, there are specialized cases (e.g., teratology and reproductive toxicity studies) in which successful breeding

is an essential component of the test. In such cases, guinea pigs are best mated when they are approximately 3 months old or weight from 450 to 600 g. Specific considerations of experimental design generally dictate the details of arrangements for mating, but it should be kept in mind that the reproductive potential of the guinea pig is high and that most simple pair cohabitations result in pregnancy.

Mating in the guinea pig is normally detected by either the presence of sperm in the vagina or by the presence of a vaginal plug. The plug consists of a central core formed by a mixture of secretions from the male's vesicular and coagulating glands and is enclosed by a mass of flat epithelial cells apparently derived from the vaginal wall (Stockard and Papanicolaou 1919). The plug usually fills the vagina from cervix to vulva. A few hours after its formation, the plug falls out of the vagina and can often be observed as a waxy mass on the cage floor. The efficiency of utilizing plugs to predict pregnancy is usually high. Live sperm, on the other hand, can be found routinely in vaginal smears for only a few hours after copulation.

Watering

Most facilities supply water to guinea pigs either from suspended water bottles or from an automatic system through sipper tubes. If bottles are used, it is frequently the practice to supply ascorbic acid in the water. Whichever of these supply methods is utilized, however, the use of either distilled or deionized water rather than chlorinated tap water is recommended as it will reduce the endogenous rate of vitamin C deactivation. Guinea pigs do have a tendency to play with water tubes, which can result in the flooding of cages.

Nutrition

Among the commonly used laboratory species, only guinea pigs and primates require a dietary source of vitamin C (ascorbic acid). Collins and Elvehjem (1958) reported the ascorbic acid requirement for growth of immature guinea pigs is 0.5 mg/100 g body weight per day. Nungester and Ames (1948) indicated that a 300-g guinea pig requires a daily intake of approximately 6 mg of vitamin C to provide adequate protection against infection. Under intensive breeding conditions, the daily requirement of the adult female that is pregnant or lactating during most of her breeding life is at least 20 mg (Bruce and Parker 1947).

Commercial pelleted diets are manufactured to meet the requirements of the guinea pig for ascorbic acid. Additionally, they are usually fortified to compensate for ascorbic acid losses in storage, but careful attention must be paid to manufacturing dates on such food. Laboratory animal feeds must not be maintained too long in storage and must be fresh when used. Ideally, guinea pig feeds should be stored in rooms where the temperature is 50°F or less, and should not be stored in the animal room. The date of manufacture is commonly given or coded on each feed bag, and a strong effort should be made to store feed for no longer than 4 to 6 weeks from the date of manufacture. This is especially important where no green vegetable supplement is offered. Feeders should be designed to prevent guinea pigs from climbing into them. For this purpose the standard J-type feeder works very well.

Feeding guinea pigs green vegetables in addition to the pelleted diet has been a subject of continuing controversy. A green supplement on a regular basis might provide insurance against vitamin C deficiency; however, such supplements offer benefits beyond this one advantage. These benefits are most apparent in a production colony under an intensive breeding system where there is heavy stress on the pregnant or lactating female. Such supplements enhance the ability of breeding animals to maintain body weight based on the results of feeding experiments in which four different commercially prepared diets were compared using large numbers of animals held for breeding periods of 18 to 24 months. It has been observed that the feeding of a green supplement resulted

in an average increase of 10% to 44% in weaned offspring over the same pelleted feeds without a green supplement.

Guinea pigs do have some rather specific requirements for amino acids and vitamins in their diets (Navia and Hunt 1976). Besides vitamin C, they are also very susceptible to vitamin E deficiencies and diets with 34% to 33% protein are recommended.

Diseases

Scurvy

Signs of scurvy usually appear within 2 weeks after guinea pigs are deprived of vitamin C. The guinea pig diet should provide 15 to 20 mg vitamin C/kg body weight for maintenance (Clarke et al. 1980); vitamin C deficiencies frequently occur when guinea pigs are fed rabbit chow. Although rabbit pellets resemble guinea pig pellets in appearance, rabbit pellets lack vitamin C, are lower in protein, and therefore do not meet the dietary requirements of guinea pigs.

Clinical signs of a vitamin C deficiency are reluctance to move, an unkempt appearance, swellings around the joints, diarrhea, and cutaneous sores. Animals often succumb to secondary bacterial infections before the classic signs of scurvy are evident. The most prominent gross lesions seen at necropsy are hemorrhages in the muscle and periosteum, particularly around the stifle joint and rib cage, and epiphyseal enlargement, best noted at the costochondral junction.

Vitamin E Deficiency

Guinea pigs are very susceptible to vitamin E deficiencies, usually resulting in the development of skeletal muscle necrosis, but a deficiency is unlikely in animals fed commercially produced diets (Wagner 1979).

Alopecia

A diffuse loss of hair over the flanks and back develops to a degree in all sows in late pregnancy. It is more marked with an intensive breeding program. Around the time of weaning, a thinning of the hair occurs during the transition from baby fur to more mature hair (Wagner 1976). Alopecia also seems to be associated with other stress conditions, although the exact cause is not known. Nutritional and genetic factors are probably involved to some degree.

Loss of hair with a distinctive pattern or patchy distribution can result from a hair-chewing vice termed barbering. Animals might chew their own hair or that of a cagemate. The location of hair loss can usually provide a clue as to whether it is self-inflicted or has resulted from barbering by a cagemate.

Circumanal Sebaceous Accumulations

Excessive accumulations of sebaceous secretions occur in the folds of the circumanal and genital region in adult male guinea pigs. These folds must be cleansed periodically to preclude infection and unpleasant odors. At times, the sebaceous secretions form a plug that accumulates in the folds between the two halves of the scrotum. It can be removed after softening with soap and water.

Preputial Infection and Vaginitis

Male guinea pigs occasionally develop preputial infections caused by lodging of foreign material in the preputial folds. Breeding males on bedding can be affected when pieces of bedding adhere to the moist prepuce following copulation and are drawn into the preputial fornix. Treatment primarily involves removing the particle and cleansing the area.

Vaginitis in female guinea pigs is usually caused by entrapment of wood chips or their bedding in the vagina, causing a foreign body reaction. The problem is corrected by washing the area carefully and wiping away the chips. It might be desirable to place the animal on a different type of bedding until the area has healed.

Water Deprivation

Sometimes animals die of water deprivation even when ample water seems to be available. This can occur when (a) water is provided by a device with which the animal is not familiar or does not know how to operate; (b) water devices are placed too high or are otherwise inaccessible, particularly for small weanlings; (c) water is unpotable because of impurities or odors in the container or the water itself; or (d) automatic watering systems become plugged or jammed.

Table 5.11 provides an overview of the major infective and parasitic diseases seen in laboratory guinea pigs. Guinea pigs are highly susceptible to factors in the environment that lower their resistance to infection. Among these are poor sanitation, overcrowding, mixing of species, improper temperature and humidity control, inadequate diet (especially insufficient vitamin C), transportation, and experimental procedures.

Dosing Techniques

Before any dose can be administered, an animal must first be picked up and manipulated. Guinea pigs should be lifted by grasping the trunk with one hand while supporting the rear quarters with the other. Support is particularly important with adults and pregnant animals. Injured lungs could result if an animal is grasped too firmly over its back.

Oral Dosing (Gavage)

Guinea pigs can be stomach tubed using a technique similar to that for the rat. An assistant restrains the animal by grasping it around the shoulders and supporting the hindquarters to prevent undue struggling. A blunted 15- to 16-gauge hypodermic needle, polyethylene catheter (3-4 figure) or commerically manufactured dosing needle (Popper tube) as used for rats is introduced into the mouth through the interdental space and advanced gently into the esophagus. A small gag made from a solid plastic rod with a hole drilled centrally can be used to prevent the animal from biting a plastic catheter.

Subcutaneous Injection

An assistant should restrain the animal as previously described, and a small area of skin on the flank should be tented by the operator. The needle is introduced into the raised skin, parallel to the body wall. The skin of the guinea pig is thicker than the skin of smaller rodents and provides more resistance to needle passage, hence injection is easier if a short (0.5–1.0 in.) 21- to 23-gauge needle is used.

Intradermal Injection

Intradermal injection is carried out as described for rats. The thicker skin of the guinea pig makes the technique relatively easy in this species. As described previously, the presence of a small bleb of material indicates successful intradermal rather than subcutaneous injection.

Table 5.11 Some Common Infectious and Parasitic Diseases of the Guinea Pig

Name	Pathogenic Agents	Symptoms Appearing Singly or in Various Combinations	Gross Lesions
Virus pneumonia	Virus	Acute—Unthrifty, emaciated Latent—No change	Lungs
Pseudo tuberculosis	Bacterial—*Yersinia* or *Pasteurella pseudotuberculosis*	Acute—Rapid breathing, rales, diarrhea, enlarged lymph nodes, emaciation, unthrifty Latent—No change (organisms might be recovered from throat)	Lungs, liver, and spleen show whitish, caseous abcesses
Bacterial pneumonia	*Bordetella bronchiseptica* and *Streptococcus pneumonia*	Acute—Rales, discharge from nose, enlarged lymph nodes Latent—No change (organisms might be recovered from throat)	Lungs
Lymphadenitis	Bacterial—*Streptococcus pyogenes* Group C	Acute—Gross swelling of lymph nodes in neck region; unthrifty, emaciation Latent—No change (organisms might be recovered from throat)	Lymph nodes, pericardium
Salmonellosis	Bacterial—*Salmonella typhimurium, Salmonella enteritidis*	Acute—Slight diarrhea, unthrifty, emaciation Latent—No change (organisms might be recovered from feces)	Enlargement of gallbladder and liver
Wasting disease	Unknown (probably a virus)	Acute—Inappetence, rapid loss of weight, unthrifty Latent—No change	None
Salivary gland virus	Cytomegalovirus	Acute—Large eosinophillic intranuclear inclusion of the salivary gland ductal epithelium, loss of appetite Latent—None	Swollen glands

Scientific Name	Common Name	No. of Legs (Might be Rudimentary)	Food	Remarks
Gyropus ovalis	Louse	6	Blood	Adults visible to naked eye
Gliricola porcelli	Louse	6	Blood	Adults visible to naked eye

Scientific Name	Common Name	Shape	Host Tissue Invaded	Remarks
Paraspidodera unicata	Round worms	Long, narrow, smooth body	Intestines	Eggs can be seen (micro-scopically) in feces; adults can be seen by naked eye in feces
Two species: Emieria caviae Balantidium caviae	Coccidia	Spherical	Intestines	Eggs (oocysts) can be seen (microscopically) in feces

Footpad Injection

The footpads are occasionally used as injection sites, particularly for material intended to act as an antigen for antisera preparation. An assistant should restrain the animal as previously described and inject the material into the large central pad of the foot. In view of the considerable swelling that often ensues, only one pad should be inoculated, so that the animal can avoid placing full weight on that limb. Because there is little evidence to suggest that footpad inoculation of antigen results in any better antibody production than does inoculation at other sites, it is preferable to avoid using this technique.

Intraperitoneal Injection

A similar technique to that described in the rat and the mouse is used for intraperitoneal injection of guinea pigs. A second individual should restrain the animal. The operator extends one of the animal's legs and introduces the needle along the line of the thigh, into the center of the posterior quadrant of the abdomen.

Intramuscular Injection

As with other rodents, the anterior and posterior thigh are the sites most frequently used for intramuscular injection, although it is also possible to inject into the triceps muscles on the anterior aspect of the shoulder. To inject into the thigh, and assistant should restrain the animal as for intraperitoneal injection, and the operator should hold one leg firmly. If the quadriceps muscles are to be used, they should be held between the thumb and forefinger and the needle introduced at right angles to the skin into the center of the muscle mass.

Intravenous Injection

Guinea pigs have few superficial veins; those that are reasonably accessible are the ear veins and the penile vein (in males). These veins are small and fragile, and hence IV injection is difficult in this species. In large (> 500 g) guinea pigs, the ear veins should be used. The ear should first be swabbed with a small quantity of xylene to dilate the vessels, and an assistant should restrain the animal on a firm surface.

The ear should be held firmly at one edge, and a suitable vein selected. A very fine needle (29–30 gauge) should be used, and once positioned in the vein, the syringe can be steadied using the remaining fingers of the hand restraining the ear. Any movement of the animal during this procedure is likely to result in damage to the vein, and to avoid this it might be preferable to anesthetize the animal. Following successful venipuncture, the xylene should be removed from the ear using a wet swab.

Penile vein injections should always be carried out in anesthetized animals, because the procedure can cause considerable discomfort. The penis is extruded from beneath the inguinal skin by pressure at either side of the genital opening. The tip should then be grasped between the thumb and forefinger and the organ extended and rotated so that the dorsal penile vein lies uppermost. When introducing the needle, care should be taken to avoid damaging the fragile vein.

A second approach is to use the saphenous vein or the dorsolateral vein of the penis. A cutdown might be required for exposure of the saphenous vein. Guinea pig skin is tough, especially over the back, and subcutaneous injections require a degree of force.

Common Techniques

Blood Collection and Measuring Blood Flow and Blood Pressure

Obtaining blood samples from guinea pigs is almost as difficult as IV injections. Methods commonly employed include cutting the nail bed; puncture of the dorsal metatarsal vein of the marginal ear vein; puncture of the orbital sinus; vacuum-assisted bleeding of either the lateral marginal vein of the hind limb, or the lateral metatarsal vein; cardiac puncture; or via an indwelling cannula technique. Carbon dioxide anesthesia increases the yields of blood and serum particularly when exsanguinating the animal. A method of obtaining 3 to 6 ml of blood from the femoral artery has been described by Simpson et al. (1967), Burnett et al. (1968) and Hem et al. (1998) have developed a method of collections from the saphenous vein that yields samples just as good.

Blood flow through the spleen has been determined with xenon-133, based on the measurement of the rate of the disappearance of xenon-133 from the spleen. Similarly, uterine blood flow was measured near term and a technique for recording the blood pressure of fetal guinea pigs was described briefly by Davitaya and Nadirashvili (1971).

Guinea pigs lack readily accessible peripheral veins, having deeply placed vessels often covered by layers of fat. A small amount of blood can be collected from the orbital sinus or by clipping a toenail. Larger amounts of blood can be obtained from the femoral artery or vein or directly from the heart. Cardiac puncture is best carried out with the animal lightly anesthetized. The guinea pig can either be placed on its right side and the needle inserted at the site of the apex beat around the fourth intercostal space, or on its back and the needle inserted in the zyphoid area, aiming for the left ventricle. Cardiac punctures in the guinea pig, however, involve a significant element of risk.

Shaving and Tape Stripping

Two main techniques are common to most dermal toxicity studies performed in the guinea pig: shaving (for the removal of hair from an area that it is desired to apply test material to) and tape stripping (for the removal of the stratum corneum, or barrier layer, of the skin).

Shaving

The Oster Model AS electric clippers with detachable blades are most commonly used to shave guinea pigs, rabbits, and rodents, following these steps:

1. The guinea pig is restrained manually with sufficient downward pressure on the anterior (neck and shoulder) area and posterior (haunches and rump) area of the animal.
2. Initially, the Oster Model AS electric clipper with blade size 10 is used. Size 10 cuts the hair to a length of approximately 1/16 in.
3. The clipper blade is held parallel to the animal's skin. Proceeding slowly, the hair is shaved against the grain of hair growth, giving the blade a chance to feed and cut. The skin of the animal is stretched to remove wrinkling, which both facilitates shaving and reduces the risk of cutting or tearing the skin.
4. An assistant, helping to restrain the animal with one hand, uses the other hand to guide the nozzle of a vacuum cleaner in front of the electric clipper to remove hair clippings during the shaving process. The hair that is collected in the vacuum is emptied into a plastic bag and disposed of as waste.
5. After the hair of the back and both flanks of the guinea pig have been clipped to a length of 1/16 in., the size 10 blade is removed and a size 40 blade is attached to cut the remaining hair to a length of approximately 1/130 in. following steps 3 and 4.
6. The blades should be changed periodically to reduce overheating and resultant skin irritation.

Each guinea pig is examined for any signs of abrasion or irritation that might have occurred during shaving. If abrasion is present, this should be recorded.

Blade Maintenance

Proper maintenance of cutting blades is essential. Cutting blades used in one toxicity study should never be used in another toxicity study unless they are first cleaned thoroughly with an appropriate solvent, such as acetone or ethanol. Once the blades are cleaned with the solvent and oiled with clipper oil, they can be used in a different toxicity study. If several toxicity studies are performed simultaneously, it is important to use a different blade set for each individual study to prevent cross-contamination. One blade set can be identified for shaving all control animal groups of different studies.

Cleaning Blades. Should cutting blades fail to cut, examine the cutting surfaces of the upper and lower cutting blade units. If hair or foreign matter is present, remove it; blades will not cut when this material is present. Brush the blades with the small brush supplied with the clippers to remove the hair and foreign matter build-up.

If more extensive cleaning is needed, the cutting blades should be detached from the clipper. Without separating, slide the upper blade approximately halfway to either side. (Do not remove the tension spring fastened to the lower blade by two screws; doing so will disturb the cutting tension.) New blades or very dirty blades should be cleaned prior to use. This removes congealed preservatives from new blades and hard-to-remove foreign matter from used blades. Repeat the cleaning procedure by sliding the upper blade in the opposite direction. Wipe blades completely dry. If blades should separate, lift the spring with a fingertip just enough to slide the upper blade into position. Run the motor when reattaching the blade assembly. Apply a thin film of Oster oil and wipe excess oil from the blade surfaces prior to use.

Oiling Blades. Apply oil several times during use to maintain a light film on the mating faces of the blades and the tension spring guide. Always wipe excess oil from blade surfaces prior to use.

Sharpening Blades. Dull blades, improper sharpening, and excessive tensioning (to force dull blades to cut) reduce cutting efficiency and can cause the clipper to heat, slow down, and reduce the motor and blade life. When cutting blades no longer cut smoothly and cleanly, sharpening is necessary.

Tape Stripping

Tape stripping (or denuding of the skin) is performed using a tacky transparent tape product such as 1-in. wide Dermiclear (Johnson & Johnson Products, Inc.). After shaving, an assistant holds the animal while successive 10-in. lengths of tape are employed. Successive portions of the length of tape are pressed firmly over the region of skin from which it is desired to remove the outer layer of the epidermis. This is repeated until the skin becomes glossy in appearance.

The permeability of guinea pig skin is generally closer to that of humans than either the rabbit or rat (Tregear 1966).

Anesthesia

Guinea pigs are among the most difficult rodents in which to achieve safe and effective anesthesia, and they are generally considered poor risks for it. Their response to many injectable anesthetics is very variable and postanesthetic complications such as respiratory infections, digestive disturbances, and generalized depression and inappetance are frequently seen. Many of these

problems can be avoided by careful selection of anesthetic agents and a high standard of intra- and postoperative nursing care. At times, guinea pigs might exhibit a peculiar squirming movement during administration of volatile anesthetics. This movement does not signify return to consciousness, and caution should be exercised in administering additional anesthetic (Flecknell 1987).

Preanesthetic Medication

Guinea pigs are nonaggressive animals that are generally easy to handle and restrain. When frightened they run around their cage at high speed, making safe handling difficult. It is important to approach guinea pigs quietly and handle them gently but firmly. They should be picked up around the shoulders and thorax and the hindquarters supported as they are lifted clear of their cage. Intramuscular or intraperitoneal injection of anesthetic agents can then be carried out. Preanesthetic medication is therefore not usually required, but if an anesthetic is to be administered by IV injection into an ear vein, initial sedation is advantageous.

The following drugs can be used to produce sedation and restraint:

1. Fentanyl/fluanisone (1 ml/kg intramuscular) will produce restraint, sedation, and sufficient analgesia for minor procedures such as skin biopsy.
2. Diazepam (5 mg/kg intraperitoneal) produces heavy sedation and immobility, but no analgesia. The animal is easily roused by painful stimuli or other disturbances such as noise. This agent can be useful in providing sufficient sedation to allow local anesthetic techniques to be used humanely.
3. Ketamine (100 mg/kg intramuscular) immobilizes guinea pigs but does not produce good analgesia.
4. Alphaxalone/alphadolone (40 mg/kg intramuscular) produces deep sedation but requires a large volume of drug to be injected (2–3 ml for an adult guinea pig).
5. Atropine (0.05 mg/kg subcutaneous) should be administered prior to any general anesthetic to minimize the volume of bronchial and salivary secretions. It is particularly useful in guinea pigs because of their relatively narrow airways, which are prone to obstruction.

General Anesthesia

IV administration of anesthetics is difficult to achieve in guinea pigs and drugs are usually administered by the intraperitoneal, subcutaneous, or intramuscular routes. The animals should be carefully weighed and dose rates calculated accurately.

The anesthetic combination of choice is fentanyl/fluanisone together with diazepam or midazolam. This combination provides surgical anesthesia with good muscle relaxation lasting about 45 min. If a longer period of anesthesia is required, further doses can be given (approximately 0.5 ml/kg intramuscular every 20 to 30 min). Following the completion of surgery, the anesthesia can be reversed using naloxone (0.1 mg/kg IV) or buprenorphine (0.1 mg/kg IV).

An effective alternative is to administer ketamine (40 mg/kg intraperitoneal) and xylazine (5 mg/kg intraperitoneal). This combination provides about 30 min of surgical anesthesia, although the level of analgesia might be insufficient for major surgery in some animals.

Alphaxalone/alphadolone produces only light surgical anesthesia even when administered by the IV route. If additional anesthetic is administered, severe respiratory depression frequently ensues.

If sodium pentabarbitol is to be used, this is best administered at a dose of 25 mg/kg intraperitoneally to sedate and immobilize the animal. Anesthesia should then be deepened using a volatile agent such as methoxyflurane. Use of the higher dose rates of pentobarbitone (37 mg/kg intraperitoneal) which are needed to produce surgical anesthesia are frequently associated with an unacceptably high mortality.

Induction of anesthesia by an inhalational agent can be either by use of an anesthetic chamber or administration via a small facemask. Following induction, it is usually most convenient to

maintain anesthesia using a facemask, as endotracheal intubation is an extremely difficult technique to carry out in guinea pigs.

Methoxyflurane is the volatile anesthetic of choice in guinea pigs as it has a wide margin of safety and is nonirritant. Holothane can be used successfully, but it can produce profound hypotension even at normal maintenance concentrations. Ether is unsuitable for use in guinea pigs, because it is highly irritant to their respiratory tract, producing increased bronchial secretions that tend to occlude the narrow airways. In addition, bronchospasm could be produced during induction of anesthesia with ether.

Anesthetic Management

Care must be taken to prevent the development of hypothermia. Postoperative recovery is aided by administering 10 to 15 ml of warmed dextrose-saline (0–18% saline, 4% dextrose) subcuteaneously to correct any fluid deficiency. A warm (25°C–30°C) recovery area should be provided and the animal given additional subcutaneous fluid for the next few days if its appetite is depressed.

Breeding

Generally, guinea pigs should be bred in the laboratory only if such breeding represents an integral part of the study. This usually means only for reproduction and developmental toxicology studies, for which the guinea pig is an infrequent subject.

Any breeding stock should be obtained from reliable sources. Soon after weaning, any offspring for follow-on generations should be quarantined and placed in the breeding quarters early to avoid any breeding setback due to environmental changes.

Puberty in the females can occur in 4 to 5 weeks; they weigh about 250 g to 400 g at puberty. The males mature at about 8 to 10 weeks, weighing about 400 g to 600 g. The first mating, however, should be done around 12 weeks of age, when the female and male weigh about 450 g and 500 g, respectively. The guinea pig experiences postpartum estrus, and mating at this time will considerably reduce the interval between litters. The gestation period varies between 59 and 72 days, with an average of 63 days. The weight of the guinea pig at birth depends on the nutritional status of the sow and the number of pups in the litter. For single births, the weight can be 150 g; however, for three to four youngsters, the weight is generally between 85 g and 90 g. If the live birth weight is below average, the chances of survival are generally very poor. There are usually three to four litters per gestation. Development of young guinea pigs is rapid; they gain 3 to 5 g of weight per day for the first 2 months. Mature adults may weight 700 g to 750 g at 5 months of age.

Nonintensive Method

In this procedure, the individual sows (5 to 10 per boar) can be isolated throughout their breeding span (2 years). The method is wasteful of space, and generally the annual number of offspring is lower (12 per sow per year). However, it has the advantage of allowing the keeping of correct breeding records, and it is excellent for inbreeding and disease control.

Communal Farrowing

In this system of breeding, the heavily pregnant sows are removed from mating pens and allowed to litter and to rear the young communally. When the youngsters are 180 g in weight, the mothers are removed and returned to mating pens. The preferred system is to have one boar and up to 20 sows; the boar can be rotated every week if desired. Such a system yields an average of seven litters per 2-year life span.

Intensive System

Monogamous or polygamous systems can be followed. Monogamous systems are expensive, as a large number of boars have to be maintained and the yields are lower. In a polygamous system, the yields can be as high as 14 to 16 young per sow per year.

Developmental Toxicity

Guinea pigs have characteristics that make them unlike any of the other species commonly used for developmental toxicity studies (rabbits, rats, and mice). Their endocrine control of reproduction is similar to that of the human, even to its trimester characteristics, and yet pregnancy is preceded by estrus. The placenta is capable of the full range of endocrine activity, but it is labyrinthine and its functions are supplemented by a yolk sac exposed to uterine secretions. Estrus, ovulation, and fertilization can be accurately timed, as with most rodents, and data are available defining or describing most of the aspects of gestation and embryonic development. For example, normal resorption activity involves the loss of approximately 5.8% to 6.3% of implanted embryos as determined by differences between the number of implantation sites and functioning corpora lutea (Hoar 1969; Hoar and King 1967). The process of ossification of the entire skeleton of the guinea pig was detailed by Petri (1935). Structural malformations have been induced in guinea pigs in a variety of ways. Thalidomide given intraperitoneally as a saline suspension, by gavage, or in dry feed for three consecutive generations produced a "conspicuous number of cleft palates and deformities of the outer ear and shortened limbs," as well as reducing the litter size, increasing prenatal deaths, and producing smaller individual offspring, particularly from those mothers fed thalidomide in the diet (Arbab-Zadeh 1965).

Trypan blue (an azo dye) produces deleterious effects in the fetus. Pregnant guinea pigs received a single subcutaneous injection of 2 ml of 1% trypan on a single day during the period of days 6 to 13 of gestation and their offspring were recovered on day 30 of gestation or allowed to deliver. The response seen at 30 days included an increased resorption rate, growth retardation, and gross abnormalities with the maximum incidence of abnormalities (57%) resulting from injection on day 11. Every embryo from treated females was affected by the dye, with the response varying from shorter crown-rump length to gross abnormalities. The malformations found included cyst of the anterior thoracic wall (49.3%), spina bifida (33.8%), microphthalmia (5.6%), hydrocephaly (4.2%), edema (2.8%), meningocele (1.4%), and assorted other defects (2.8%). Fifty percent of the retarded or malformed embryos displayed a posterior cleft palate. Treated females going to term had a reduced litter size, their offspring displaying only those abnormalities (5.3%) that were compatible with life (Hoar and Salem 1961).

Hypervitaminosis A produces malformations in the guinea pig. Giroud and Martinet (1959a, 1959b) gave pregnant guinea pigs 50,000 IU of vitamin A on days 12 to 14 of gestation and reported an increased number of spontaneous abortions, resorptions, and a case of mandibular fissure combined with a bifid tongue. Robens (1970) gave guinea pigs 200,000 USP units/kg of vitamin A palmitate as a single dose on selected days (14–20) during organogenesis. Most of the females were allowed to deliver, although some were terminated at 50 days of gestation. Multiple structural defects, involving primarily the head region, were seen in 60.8% of offspring born following maternal treatment on days 14 to 16. Missing coccygeal vertebrae and agnathia (38.5% of offspring) were seen following treatment on day 17 of gestation. Limb defects (37.2%) were the most frequent abnormalities resulting from treatment on days 18 to 20, whereas only 1 of 226 control offspring was abnormal.

Edwards (1967, 1969a, 1969c) examined effects of hyperthermia applied early during gestation on reproduction and fetal development in guinea pigs. He noted that resorptions appeared to be most common following hyperthermia on about days 11 to 15, whereas abortions, occurring at a

mean of 32.4 days of gestation, appeared most frequently (83%) following hypothermia on days 11 to 18. Of 251 offspring recovered at delivery, the following malformations were noted: microencephaly (41%), hypoplastic digits (13%), exomphalos (7%), talipes (4%), hypoplastic incisors (4%), cataract (3%), renal agenesis (2%), and amyoplasia (2%). A detailed evaluation of prenatal retardation of brain growth at various times during gestation was also conducted by Edwards (1969b). The incidence of reduced brain weight and microencephaly increased most markedly following hyperthermia for 4 or 8 days during days 15 to 32 of gestation. Following 2 successive days of hyperthermia, the effects were most marked on days 20 to 23 of gestation.

Common Protocols

As presented in table 5.12 the guinea pig is used in a wide variety of studies in toxicology. The most common are the various sensitization and photosensitization studies. The rationale for the use of guinea pigs in these designs has previously been reviewed (Gad and Chengelis 1988). Typical or prototype protocols for these more common designs are as follows.

Modified Buehler Procedure

The modified Buehler procedure is a closed patch procedure for evaluating test substances for potential delayed contact dermal sensitization in guinea pigs. The procedure, based on that described by Buehler (1965), is practical for test substances that cannot be evaluated by the traditional intradermal injection procedure of Landsteiner and Jacobs or by the guinea pig maximization test (GMPT) for skin sensitization testing. The closed patch procedure is performed when a test substance either is highly irritating to the skin by the intradermal injection route of exposure or it cannot be dissolved or suspended in a form allowing injection. It is also the method of choice for some companies. This procedure, which is one version of the Buehler test, complies with the test standards set forth in the Toxic Substances Control Act (TSCA) and other regulatory test rules. There are other versions that also comply.

Table 5.12 Dosage Tables for Pharmaceuticals Commonly Used in Guinea Pigs

Antibiotics	
Penicillin—Do not administer	
Cephaloridine—25 mg/kg IM daily	
Sulfamethazine—333 mg/liter drinking water for 300 g animal	
Tetracycline—255 mg/liter drinking water for 300 g animal	
Antihistamines	
Diphenhydramine (Benadryl)	5.0 mg/kg SC
Tripelennamine	5.0 mg/kg PO or IM
Tranquilizers	
Chlorpromazine	0.5 mg/kg IM
Promazine HCl	0.5–1.0 mg/kg IM
Meprobamate	100 mg/kg IM
Anesthetics	
Pentobarbitol sodium	30 mg/kg IV; 40 mg/kg IP
Thiopental sodium	20 mg/kg IV; 55 mg/kg IP
Ketamine	22–44 mg/kg IM
Droperidol-fentanyl	0.66–0.88 mg/kg IM
Urethane	1,500 mg/kg IP
Analgesics	
Aspirin	269 mg/kg IP
Meperidine	2 mg/kg IM
Miscellaneous	
Atropine	0.05 mg/kg SC, IM
Heparin	5 mg/kg IV

Animals

1. Young albino female Hartley strain guinea pigs, weighing between 300 g and 400 g, are currently the standard animals used. Studies sponsored by the Charles River Laboratories have shown that their hairless guinea pigs are just as responsive, however.
2. Although several proposed test rules suggest the use of male guinea pigs, the female sex is preferred because the aggressive social behavior of males could result in considerable skin damage that might interfere with the interpretation of challenge reactions. This concern occurs because animals are commonly group housed (Marzulli and Maibach 1987).
3. Animals that show poor growth or are ill in any way are not used, because illness can markedly decrease the response. Animals with skin marked or scarred from fighting are avoided. The guinea pigs are observed for at least 2 weeks prior to test to detect any illness before starting a study.
4. The guinea pigs are identified by a cage card and marking pen or any other suitable method. There is no regulatory requirement, however, for the identification of individual animals.
5. The guinea pigs are randomly assigned to test and negative control groups consisting of at least 15 and 6 animals each, respectively. If a pretest group is necessary, as many animals as needed for that group are randomized also.

Pretest Screen

1. If practical, the dermal irritation threshold concentration should be established for the test substance prior to the first induction application. A concentration of the test substance that produces minimal or no irritation (erythema or edema formation) is determined. The highest concentration that produces no irritation is preferred for the dermal sensitization study challenge dose.
2. Those animals randomly assigned to the pretest group are used.
3. Each animal is prepared by clipping a 1-in.-square area of hair from the left upper flank using a small animal clipper with a size 40 blade.
4. The test substance is diluted, emulsified, or suspended in a suitable vehicle. Vehicles are selected on the basis of their solubilizing capacity for the test substance and on their ability to penetrate the skin.
5. Different concentrations of the test substance are tested on the pretest group of guinea pigs; a few animals are used for each concentration tested.
6. A volume of 0.5 ml is applied to a patch consisting of a cotton pad (1 in. $\times$ 1 in.) occluded with impermeable surgical tape, or placed in a Hilltop-style occlusive "chamber."
7. The patch is applied to the shaved left flank of a guinea pig. The patch is held firmly in place for 24 hr by wrapping the trunk of the animal with a 3-in.-wide elastic bandage. A 2-in.-wide strip of tape is used to line the center adhesive side of the bandage to prevent skin damage from the adhesive.
8. After 24 hr of exposure, the wrappings and patches are removed.
9. Observations of skin reactions (erythema or edema formation) are recorded 48 hr after application.
10. A judgment is made as to which concentration will be used for the dermal sensitization study based on the dermal irritation data that have been collected. The highest concentration that produces minimal or no dermal irritation is selected.

Induction Phase

1. Test group and control group guinea pigs are weighed at the beginning of the study and weekly thereafter.
2. Test control group guinea pigs are shaved as described earlier.
3. If the test substance is a liquid (solution, suspension, or emulsion), a volume of 0.5 ml of the highest concentration found to be nonirritating in a suitable vehicle (as determined in the pretest portion of this procedure) is applied to a patch consisting of a cotton pad (1 in. $\times$ 1 in.) occluded with impermeable surgical tape. If the test substance is a solid or semisolid, 0.5 g* is applied. If

* When the test substance is in flake, granule, powder, or other particulate form, the weight of the test substance that has a volume of 0.5 ml (after compacting as much as possible without crushing or altering the individual particles, such as by tapping the measuring container) is used whenever this volume weighs less than 0.5 g.

the test substance is a fabric, a 1-in. square is moistened with 0.5 ml of physiological saline before application.

4. The first induction patch is applied to the clipped left flank of each test group guinea pig. The patch is held firmly in place for 24 hr by wrapping the trunk of each animal with a 3-in.-wide elastic bandage. A 2-in.-wide strip of tape is used to line the center adhesive side of the bandage to prevent skin damage from the adhesive. A 2-in. length of athletic adhesive tape is placed over the bandage wrap as a precautionary measure to prevent unraveling.
5. After 24 hr of exposure, the wrappings and patches are removed and disposed of in a plastic bag.
6. Each dermal reaction, if any, is scored on the basis of previously designated values for erythema and edema formation such as the Draize scale (Draize 1959; see table 5.13). Observations are made 48 hr after initiation of the first induction application. Resulting dermal irritation scores are recorded.
7. After the initial induction application, subsequent induction applications (two to nine) are made on alternate days (three times weekly) until a total of 10 treatments have been administered. Each of these patches is removed after 6 hr of exposure. It should be noted that some investigators use a modification that calls for one application per week for 3 weeks.
8. Observations are made 24 and 48 hr after initiation of each subsequent induction application. Dermal scores of the remaining nine induction applications are recorded.
9. Clipping the hair from the left flank induction sites of test group animals and corresponding sites on negative control group animals is performed just prior to each subsequent induction application. Only the test group guinea pigs receive the induction applications.

Table 5.13 Evaluation of Skin Reactions

Skin Reaction	Value
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in-depth)	4
Necrosis (death of tissue)	+N
Eschar (sloughing)	+E
Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well-defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond the area of exposure)	4

Source: Draize (1959).

Challenge Phase

1. Fourteen days after the tenth induction application, all 10 test group and 3 of 6 control group guinea pigs are prepared for challenge application by clipping a 1-in. square of hair from the right side (the side opposite that clipped during the induction phase).
2. A challenge dose, using freshly prepared test substance (solution, suspension, emulsion, semisolid, solid, or fabric), is applied topically to the right side (which had remained untreated during the induction application) of test group animals. The left side, which had previously received induction applications, is not challenge dosed.
3. The concentration of the challenge dose is the same as that used for the first induction application. (It must be a concentration that does not produce dermal irritation after one 24-hr application.)
4. Each of three negative control group guinea pigs is challenge dosed on the right flank at approximately the same time that the test group guinea pigs are challenge dosed. This is, in effect, a check for unexpected primary irritation.

5. All patches are held in contact with the skin for 24 hr before removal.
6. The skin sites are evaluated using the previously selected scoring system for erythema and edema formation, such as that presented in table 5.13. Observations are made 48, 72, and 96 hr after initiation of the challenge application and the skin reactions are recorded.

Rechallenge Phase

1. If the test substance is judged a nonsensitizing agent after the first challenge application, or causes dermal sensitization in only a few animals, or causes dermal reactions that are weak or questionable, then a second and final challenge application will be performed on each test animal 7 days after the initiation of the first challenge dose.
2. Controls from the first challenge application are not rechallenged because they have been exposed to the test substance and are no longer true negative controls. The three remaining naive control group animals (not used for the first challenge) are challenged for comparison to the test group animals.
3. The procedure used for the first challenge applications will be used for the second challenge application (including reclipping, patching method, and duration of exposure). Either the same concentration or a new concentration (higher or lower) of test substance can be used, depending on the results of the first challenge. Observations are made 48, 72, and 96 hr after initiation of the rechallenge application and skin reactions are recorded.
4. When a rechallenge application is performed, the data from both challenges are compared. If neither challenge produces a positive dermal reaction, the classification of the test substance is based on both challenge applications. If one challenge application (whether it is the first or second) produces a greater number of positive dermal reactions than the other, the classification of the test substance is based on the challenge with the most positive responses.
5. Two or more unequivocally positive responses in a group of 15 animals should be considered significant. A negative, equivocal, or single response probably assures that a substance is not a strong sensitizer, although this is best confirmed by further testing with human subjects (NAS 1977).

Interpretation of Results

1. Judgment concerning the presence or absence of sensitization is made for each animal. The judgment is made by comparing the test animal's challenge responses to its first induction treatment response as well as to those challenge responses of negative control animals.
2. Challenge reactions to the test substance that are stronger than challenge reactions to negative controls or to those seen after the initial induction application should be suspected as results of sensitization (NAS 1977). A reaction that occurs at 48 hr but resolves by 72 hr or 96 hr should be considered a positive response as long as it is stronger than that which is displayed by controls at the same time interval.

Strengths and Weaknesses

There are a number of both advantages and disadvantages to the Buehler methodology, which has been in use for more than 20 years. The relative importance and merits of each depend on the intended use of the material. The four advantages are as follows:

1. Virtually no false positives (in fact, in the experience of the author, when the pretest is properly conducted, there are no false positives), compared to human experience, are generated by test.
2. The techniques involved are easy to learn and very reproducible.
3. The Buehler-style test does not overpredict the potency of sensitizers. That is, materials that are identified as sensitizers are truly classified as very strong, weak, or in between—not all (or nearly all) as very strong.
4. There is a large database in existence for the Buehler-style test. Unfortunately, the vast majority of this information not in the published literature.

Likewise, there are three disadvantages associated with the Buehler-style test.

1. The test gives a high rate of false negatives for weak sensitizers and a detectable rate of false negatives for moderate sensitizers. That is, the method is somewhat insensitive—particularly if techniques for occlusive wrapping are inadequate.
2. The test takes a long time to complete. If animals are on hand when started, the test is 5 to 6 weeks long. As few laboratories keep a "pool" of guinea pigs on hand (especially as they are the most expensive of the common lab species), the usual case is that 8 to 10 weeks is the minimum time required to get results from this test.
3. The test uses a relatively large amount of test material. In the normal acute battery, the guinea pig test systems use more material than any other test systems unless an acute inhalation study is included. With 10 induction applications, this is particularly true for the Buehler-style test.

Guinea Pig Maximization Test

The GPMT was developed by Magnusson and Kligman (1969, 1970; Magnusson 1975) and is considered a highly sensitive procedure for evaluating test substances for potential dermal sensitization.

Animals

1. Young adult female guinea pigs weighing between 250 g and 350 g at the initiation of the study are used.
2. Animals that show poor growth or are ill in any way are not used, as illness could markedly decrease the response. Animals with skin marked or scarred from fighting are avoided. The guinea pigs are observed for at least 2 weeks to detect any illness before starting a study.
3. The guinea pigs are randomly assigned to two groups: a test group consisting of 15 animals, and a control group consisting of 6 animals. If a pretest group is necessary, as many animals as needed for that group also are randomized.
4. Test and control group guinea pigs are weighed 1 week prior to dosing (day 7), on the day of dosing (day 0), and weekly thereafter.

Pretest

1. Several animals are used to pretest the test substance and vehicles to determine the topical dermal irritation threshold concentration.
2. These animals are shaved on the left flank, to which is applied a 2×2 cm filter paper patch that contains 0.1 ml of the test concentration.
3. The trunks of the animals are wrapped for 24 hr with a 3-in.-wide elastic bandage to hold the patch in contact with the skin.
4. Wrappings are removed after the 24 hr exposure and, based on skin reactions at 48 hr, a concentration of the test substance to be used on the test is determined. Dermal irritation values are recorded for future reference.
5. In addition, several guinea pigs are utilized to determine a concentration (generally between 1% and 5%) of test substance in vehicle and in Freund's Complete Adjuvant (FCA) emulsion that can be injected intradermally without eliciting a strong local or systemic toxic reaction.
6. The hair is clipped in an area of approximately 4×6 cm from the upper shoulder region of these animals.
7. Several concentrations of the test substance (ranging between 1% and 5%) can be injected in the same animal to compare local dermal reactions produced by the different concentrations.
8. However, if systemic toxicity is suspected, each concentration should be tested in separate animals to determine local and systemic effects.

9. The dermal reactions (erythema, edema, and diameter) are recorded 24 hr after the intradermal injections.

Induction Stage 1 (Day 0)

1. The hair in an area of 4×6 cm is clipped from the shoulder region of each test and control group guinea pig on day 0.
2. Three pairs of intradermal injections are made with a glass 1-ml tuberculin syringe with a 26-gauge needle, each pair flanking the dorsal midline.
3. The three pairs of intradermal injections for test group animals are as follows:
   a. 0.1 ml test substance in appropriate vehicle
   b. 0.1 ml FCA emulsion alone
   c. 0.1 ml test substance in FCA emulsion
4. The three pairs of intradermal injections for control group animals are as follows:
   a. 0.1 ml vehicle alone
   b. 0.1 FCA emulsion alone
   c. 0.1 ml vehicle in FCA emulsion
5. Injections (a) and (b) in the preceding two steps are given close to each other and nearest the head; injection (c) is given most posteriorly.
6. The date, time, and initials of those individuals performing the injections are recorded.
7. Immediately before injection, an emulsion is prepared by blending commercial FCA with an equal volume of house distilled water or other solvent as appropriate.
   a. Water-soluble test materials are dissolved in the water phase prior to emulsification.
   b. Oil-soluble or water-insoluble materials are dissolved or suspended in FCA prior to adding water.
   c. Paraffin oil, peanut oil, or propylene glycol can be used for dissolving or suspending water-insoluble materials.
   d. A homogenizer is used to emulsify the FCA alone and the test substance in either FCA or vehicle prior to the intradermal injections.
   e. The concentration of the test substance for intradermal injections is adjusted to the highest level that can be well tolerated locally and generally.
8. The adjuvant injection infiltration sometimes causes ulceration, especially when the injection is superficial. This ulceration lasts several weeks. These lesions are undesirable but do not invalidate the test results except for lowering the threshold level for skin irritation.

Induction Stage 2 (Day 7)

1. Test substance preparation:
   a. The concentration of the test substance is adjusted to the highest level that can be well tolerated.
   b. If the test substance is an irritant, a concentration is chosen that causes a weak to moderate inflammation (as determined by the pretest).
   c. Solids are micronized or reduced to a fine powder and then suspended in a vehicle, such as petrolatum or propylene glycol.
   d. Water- and oil-soluble test substances are dissolved in an appropriate vehicle.
   e. Liquid materials are applied as such, or diluted if necessary.
2. The same area over the shoulder region that received intradermal injections on day 0 is again shaved on both test and control guinea pigs.
3. A volume of 0.3 ml of a mildly irritating concentration (if possible) of the test substance (determined by the pretest) is spread over a 1×2 in. filter paper patch in a thick, even layer.
4. The patch is occluded with surgical tape and then secured to test group animals with an elastic bandage, which is wrapped around the torso of each test group animal.
5. The control group animals are exposed to 0.3 ml of 100% vehicle using the same procedure.

6. The date, time, and initials of those individuals performing the second induction should be recorded.
7. The dressings of both groups are left in place for 48 hr before removal.

Challenge Stage (Day 21)

1. An area of hair (1.5 × 1.5 in.) on both flanks of the guinea pigs (15 test and 3 controls) is shaved.
2. A 1 × 1 in. patch with a nonirritating concentration of test substance in vehicle (as determined by the pretest) is applied to the left flank and a 1 × 1 in. patch with 100% vehicle is applied to the right flank.
3. The torso of each guinea pig is wrapped in an elastic bandage to secure the patches for 24 hr.
4. The date, time, and initials of those individuals performing the challenge dose are recorded.
5. The patches are removed 24 hr after application.

Rechallenge (Day 28)

1. If the first challenge application of test substance does not cause dermal sensitization, causes dermal sensitization in only a few animals, or causes dermal reactions that are weak or questionable, then a second challenge application of test substance to the 15 test group guinea pigs should be conducted on day 28 (a week after the first challenge). The three remaining naive control group animals (not used for the first challenge) are challenged for comparison to the test group animals.
2. The three negative control group animals used on day 21 will not be rechallenged. These animals will be discontinued from the study because they were exposed to the test substance during the first challenge and are no longer negative controls.
3. A 1 × 1 in. patch with a nonirritating concentration of test substance in vehicle is applied to the right flank of test and control group animals. The left flanks are not dosed.
4. The date, time, and initials of those individuals performing the rechallenge dose should be recorded.
5. Steps 3 and 5 are followed as for the challenge stage (day 21).

Observations—Challenge and Rechallenge Readings

1. Twenty-one hours after removing the patch, the challenge area on each flank is cleaned and shaved, if necessary.
2. Twenty-four hours after removing the patch, the first reading of dermal reactions is taken.
3. The dermal reactions are scored on this 4-point scale:
   0—No reaction
   1—Scattered and mild redness
   2—Moderate and diffuse redness
   3—Intense redness and swelling
4. Forty-eight hours after removing the patch, the second reading is taken and the scores are recorded.

Interpretation of Results

1. Both the intensity and duration of the test responses to the test substance and the vehicle are evaluated.
2. The important statistic in the GPMT is the frequency of sensitization and not the intensity of challenge responses. A value of 1 is considered just as positive as a value of 3 (as long as the values for controls are zero).
3. The test agent is a sensitizer if the challenge reactions in the test group clearly outweigh those in the control group. A reaction that occurs at 24 hr but resolves by 48 hr after removal of patches should be considered a positive response, as long as it is stronger than that which is displayed by controls. The sensitization rate (percentage of positive responders) is based on the greatest number of animals showing a positive response, whether it is from the 24-hr data or the 48-hr data after removal of patches.

4. When a second challenge application is performed, the data from both challenges are compared. If neither challenge produces a positive dermal reaction, the classification of the test substance is based on both challenge applications. If one challenge application (whether it is the first or second) produced a greater number of positive dermal reactions than the other, the classification of the test substance is based on the challenge with the most positive responses.
5. Under the classification scheme of Kligman (1966; see table 5.14) the test substance is assigned to 1 of 5 classes, according to the percentage of animals sensitized, ranging from a weak grade I to an extreme grade V.

Table 5.14 Sensitization Severity Grading Based on Incidence of Positive Responses

Sensitization Rate (%)	Grade	Classification
0–8	I	Weak
9–28	II	Mild
29–64	III	Moderate
65–80	IV	Strong
81–100	V	Extreme

Source: Kligman (1966).

The advantages and disadvantages of the GPMT can be summarized as follows. First, these are the advantages:

1. The test system is sensitive and effectively detects weak sensitizers. It has a low false-negative rate.
2. If properly conducted, there are no false positives; that is, materials that are identified as potential sensitizers will act as such at some incidence level in humans.
3. There is a large database available on the evaluation of compounds in this test system, and many people are familiar with the test system.

The disadvantages, meanwhile are as follows:

1. The test system is sensitive; it overpredicts potency for many sensitizers. There is no real differentiation among weak, moderate, and strong sensitizers; virtually all positive test results identify a material as strong.
2. The techniques involved (particularly the intradermal injections) are not easy. Some regulatory officials have estimated that as many as 35% of the laboratories that try cannot master the system to get it to work reproducibly.
3. The test, although not as long as the Buehler-style test, still takes a minimum of 4 weeks to produce results.
4. The test uses a significant amount of test material.
5. One cannot evaluate fibers or other materials that cannot be injected (e.g., either solids that cannot be finely ground or suspended, or that are highly irritating or toxic by the IV route).
6. The irritation pretest is critical. Failure to detect irritation in this small group of animals does not guarantee against irritation in test animals at challenge.

Split Adjuvant Test

The guinea pig split adjuvant dermal sensitization procedure for detecting contact allergenicity is based on that developed by Maguire (1973a, 1973b, 1975; Maguire and Chase 1967, 1972), and is sensitive and effective for the detection of substance and products with weak allergic potential and will serve as a useful alternative for testing materials that cannot be injected intradermally (e.g., fabrics, nonsoluble solids, and extremely irritating or toxic materials). A concise outline of the split adjuvant technique has been published (Klecak 1983).

Animals

1. Young adult female guinea pigs weighing between 250 g and 350 g at the initiation of the study are used.
2. Animals that show poor growth or are ill in any way are not used, as illness markedly decreases the response. Animals with skin marked or scarred from fighting are avoided. The guinea pigs are observed for at least 2 weeks to detect any illness before starting a study.
3. The guinea pigs are randomly assigned to two groups: a test group consisting of 10 animals, and a control group consisting of 6 animals. If a pretest group is necessary, as many animals as needed for that group also are randomized.
4. Test and control group guinea pigs are weighed and the weights are recorded 1 week prior to dosing (day 7), on the day of dosing (day 0), and weekly thereafter.

Pretest

1. Several animals are used to pretest the test substance and vehicles to determine the dermal irritation threshold concentration.
2. These animals are shaved on the left flank (2×2 cm).
3. Then 0.2 ml of ointment (semisolid) or 0.1 ml of liquid is spread onto a 1.5×1.5 cm Whatman No. 3 filter paper patch, which is occluded on the opposite side with surgical tape.
   a. Solid test substances are micronized or reduced to a fine powder and then suspended in a vehicle, such as petrolatum or propylene glycol.
   b. Water- and oil-soluble test substances are dissolved in an appropriate vehicle.
   c. Liquid test substances are applied as such (100%), or diluted if necessary.
4. The pretest patch is then applied to the left flank. The trunk of each animal is wrapped for 24 hr with a 3-in.-wide elastic bandage to hold the patch in contact with the skin.
5. Wrappings are removed after 24 hr of exposure. Based on skin reactions at 48 hr, a concentration of the test substance to be used on test is determined. Dermal irritation values are recorded for later reference.

Induction Stage

The date, time, and initials of those individuals performing the induction applications should be recorded.

Day 0

1. The hair in an area 1×1 in. is clipped behind the shoulder girdle of each test and control group guinea pig.
2. Dry ice is applied for 5 sec to the skin site of each test and control animal. Dry ice is used only for the day 0 induction application.
3. Then 0.2 ml of ointment (semisolid) or 0.1 ml of liquid is spread onto a 0.5×0.5 in. Whatman No. 3 filter paper patch, which is occluded on the opposite side with surgical tape.
   a. The test substance is tested at a concentration that is minimally irritating (if possible), as determined by the pretest.
   b. If the substance is mixed in petrolatum or ointment, 0.2 ml is dispensed onto the patch.
   c. In the case of liquids, 0.1 ml is used.
   d. If a fabric is to be tested, a 1.5×1.5 cm sample is cut, moistened with 0.2 ml of physiological saline, and then is applied under a filter paper patch.
4. Control group animals are dosed with vehicle only, not test substance.
5. The trunk of each animal is wrapped for 48 hr with a 3-in.-wide elastic bandage to hold the patch in contact with the skin.

Day 2

1. The wrapping and patch is removed from each test and control group animal 48 hr after the initial induction application.
2. A fresh patch is applied to the same site using the same procedure as described for induction day 0 (without dry ice). Test group animals receive test substance in vehicle, and control group animals receive vehicle alone for a 48-hr period.

Day 4

1. The wrapping and patch is removed from each test and control group animal 48 hr after the application of the day 2 induction patch.
2. An FCA emulsion is prepared by blending commercial FCA with an equal volume of house distilled water.
3. Two volumes of 0.1 ml of FCA emulsion are injected intradermally into the induction site of each test and each control group animal with a glass 1-ml tuberculin syringe and a 26-gauge needle. These two injections flank the dorsal midline.
4. A fresh patch is applied to the same site using the same procedure as described on induction day 2. Test group animals receive test substance in vehicle, and control group animals receive vehicle alone for a 48-hr period.

Day 6

The wrapping and patch are removed from each test and control group animal 48 hr after the application of the day 4 induction patch.

Day 7

A fresh patch is applied to the induction site using the same procedure as described on induction day 2. Test group animals receive test substance in vehicle, and control group animals receive vehicle alone for a 48-hr period.

Day 9

The wrapping and patch are removed from each test and control group animal 48 hr after the application of the day 7 induction patch.

Challenge Stage (Day 21)

1. An area of hair (1×1 in.) on both flanks of the guinea pigs (10 test and 3 controls) is shaved.
2. A 0.5×0.5 in. filter paper patch with the highest nonirritating concentration of test substance in vehicle (as determined by the pretest) is applied to the left flank and 1.5×1.5 cm patch with 100% vehicle is applied to the right flank.
   a. If the test substance is liquid, 0.1 ml is applied to the patch.
   b. If the test substance is mixed in petroleum or ointment, 0.2 ml is dispensed onto the patch.
   c. If the test substance is a fabric, a 0.5×0.5 in. sample is cut, moistened with physiological saline, and then is applied under a patch.
3. The torso of each guinea pig is wrapped with an elastic bandage to secure the patches for 24 hr.
4. The date, time, and initials of those individuals performing the challenge dose should be recorded.
5. The patches are removed on day 22 and the challenge area on each flank is cleaned and clipped atraumatically.

Challenge Readings

1. On day 23, 24 hr after removing the patch, the first reading of dermal reactions is taken, and results are recorded.
2. Readings of the challenge site are taken again 48 hr after removing the patch, and results are recorded.
3. The intensity of the skin reaction is classified according to the following rating scale used by Maguire (1973b):

0	= Normal skin
+	= Very faint, nonconfluent pink
+	= Faint pink
++	= Pale pink to pink, slight edema
+++	= Pink, moderate edema
++++	= Pink and thickened
+++++	= Bright pink, markedly thickened

Interpretation of Results

1. The frequency, intensity and duration of the test responses to the test substance and the vehicle are evaluated.
2. The test substance is a sensitizer if the challange reactions in the test group clearly outweigh those in the control group.
3. Two or more unequivocally positive responses (at least a + on the rating scale) in a group of 10 animals should be considered significant. A negative, equivocal, or single response probably assures that a substance is not a strong sensitizer, although this is best confirmed by further testing with human subjects (NAS 1977).

Rechallenge (Day 28)

1. If the first challenge application does not cause dermal sensitization, then a second application of the 10 test group guinea pigs will be conducted on day 28 (1 week after the first challenge). The remaining naive control group animals (not used for the first challenge) are challenged for comparison to the test group animals.
2. The three negative control group animals used on day 21 will not be rechallenged. These animals will be discontinued from the study because they were exposed to the test article during the first challenge and are no longer negative controls.
3. A 0.5×0.5 in. patch with the highest nonirritating concentration of test substance in vehicle is applied to the right flank of test and control group animals. The left flanks are not dosed.
4. The date, time, and initials of those individuals performing the rechallenge dose are recorded.
5. Steps 3 and 5 of the challenge procedure are repeated here.

Strengths and Weaknesses

The advantages and disadvantages of the split adjuvant test can be summarized as follows. The advantages are these:

1. The test system has a lower false-negative rate for moderate and weak sensitizers than does the Buehler design.
2. If properly conducted, there are no false positives.
3. Fibers and other materials that cannot be injected intradermally can be evaluated here.

As elsewhere, there is also a list of disadvantages. These include the following:

1. The techniques involved (particularly the intradermal injection) are not easy ones.
2. The sensitivity of the test system comes at the expense of making relative hazard predictions not necessarily accurate. The test system tends to overpredict potency.
3. The test still both takes a relatively long time to complete and uses a significant amount of test material.
4. There is a limited published database on test system performance, and relatively few people have experience with it.

Photosensitization Tests

There are at least five *in vivo* photosensitization test methods. Only two of these (Harber and Shalita method and the Armstrong assay) are presented here. The other three (the Vinson and Borselli method, the Guillot et al. method, and a method using mice) are beyond the scope of this text.

Although the pattern of evolution of predictive animal tests is not as clear as that of dermal sensitization tests, the two methods presented here each represent a distinct phase of that development.

Harber and Shalita Method

This is the older of the two methods and uses dermal exposure without any adjuvant to increase the response. This method was originally published by Harber and Shalita (1975).

Animals.

1. Young adult female Hartley strain guinea pigs weighing between 300 g and 400 g at the start of the study are used.
2. Animals that show poor growth or are ill in any way are not used, as illness markedly decreases the response. Animals with skin marked or scarred from fighting are avoided. The guinea pigs are quarantined and observed for at least 2 weeks to detect any illness before starting a study.
3. The guinea pigs are randomly assigned to a test group of 10 animals and negative control group of 6 animals. If a pretest group is necessary, as many animals as needed for that group also are randomized.
4. Test and control group guinea pigs are weighed 1 week prior to dosing (day 7), on the day of dosing (day 0), and weekly thereafter.

Pretest (If Necessary).

1. Several animals are used to pretest different concentrations of test substance in vehicle (usually acetone) to determine the topical dermal irritation threshold concentration on skin that is exposed to ultraviolet B (UVB) and ultraviolet A (UVA) irradiation sequentially and on skin that is exposed to UVA irradiation alone.
2. The hair of these animals is shaved over the whole dorsal region.
3. A volume of 0.2 ml of each test concentration is applied twice to each guinea pig: once to the nuchal region and once to the dorsal lumbar region.
4. Thirty minutes after application, the treated nuchal sites are irradiated with sunlamp emissions (UVB) for 30 min and the lumbar sites are shielded with elastoplast tape.
5. After the UVB exposure, the tape is removed from the lumbar region, and both the treated nuchal sites and lumbar sites are irradiated with black light emissions (UVA) for 30 min.
6. The animals are returned to their respective cages after the UVA exposure.

7. Twenty-four hours after the initial exposure to the test substance, the nuchal and lumbar skin sites are scored for erythema formation.
8. A concentration is chosen for induction applications that causes a mild or weak erythema response at the nuchal sites. If the test substance does not cause an erythema response, then the highest concentration level that is practical should be used for induction.
9. The highest concentrate of the test substance that is barely irritating sites is used for challenge application. Two lower concentrations of the test substance, prepared by serial dilution from the hint concentration are also used for the challenge application.

Induction Stage (Days 0, 2, 4, 7, 9, 11).

1. The hair in an area of approximately 1×1 in. is clipped from the nuchal region of each test and control group guinea pig.
2. A volume of 0.2 ml of a relatively high concentration of the test substance in either acetone or ethanol is applied to the shaved nuchal region of each test group guinea pig. The concentration will be the highest level that can be well tolerated locally, and generally by the guinea pig, as determined by a pretest for dermal irritation.
3. A volume of 0.2 ml of solvent (acetone or ethanol) is applied to the shaved nuchal region of each control group guinea pig.
4. Thirty minutes after application, the treated nuchal sites of test and control guinea pigs are irradiated with sunlamp emissions for 30 min and black light emissions for 30 min, successively. The lumbar region of the back is shielded from the light sources during the irradiation procedures with an elastic bandage that is wrapped around the torso of each animal.
5. The clipping, topical exposure to test substance, and irradiation procedures are repeated 6 times during a 12-day period (typical study days are 0, 2, 4, 7, 9, and 11).

Challenge Stage (Day 32).

1. Elicitation of contact photosensitivity is performed 21 days from the last sensitizing (induction) exposure.
2. The hair of the dorsal lumbar region of each of 10 test group and 3 of 6 control group guinea pigs is clipped for the first time.
3. Three different concentrations of test substance using the solvent used for induction, as determined from the pretest, are applied topically to this region; test and control animals are treated alike. Each concentration is applied to the right and left side of the dorsal midline.
4. The torso of each test and control guinea pig is wrapped in plastic wrap (one layer thick) after the test chemical is applied. The plastic wrap is held in place at the ends with athletic adhesive tape. The same tape is used to shield the left side of each animal from the UVA light source.
5. Thirty minutes after application, the right side of each animal is exposed to nonerythrogenic (> 320 nm) UVA emissions for 30 min. The radiation is passed through a pane of window glass 3 mm thick to eliminate passage of radiation lower than 320 nm.
6. After the black light exposure, all animals are unwrapped, returned to their respective cages, and placed in a darkened room for 24 hr.

Challenge Readings.

1. If the test substance leaves a colored residue, the excess test material is removed by washing with a suitable solvent at 24 hr so that the area of challenge skin can be evaluated accurately.
2. All test sites, both irradiated and nonirradiated, are scored and interpreted 24 and 48 hr after the initial test substance application and subsequent exposure to black light irradiation.
3. Erythema is scored as follows:
   0—No erythema
   1—Minimal, but definite erythema
   2—Moderate erythema

3—Considerable erythema
4—Maximal erythema

4. Erythema scores are recorded.

Rechallenge.

1. If the test substance is judged a nonphotosensitizing agent after the first challenge application, a second and final challenge application will be performed on each test group animal 7 days after the initiation of the first challenge dose.
2. Controls from the first challenge application are not rechallenged because they have been exposed to the test substance and are no longer true negative (naive) controls. The three remaining naive control group animals (not used for the first challenge) are challenged for comparison to the rechallenge of test group animals.
3. The procedure used for the first challenge application will be used for the second application, either the same or a new concentration of test substance, including reshaving, the same patching method, and the same duration of exposure. Observations are again made 24 and 48 hr after the second challenge application and skin reactions are recorded.

Interpretation of Results.

1. The negative control group of animals, having received no previous photosensitive (induction) exposures, serves to identify any phototoxic or primary irritant (nonphototoxic) substances.
2. An erythema score of 1 or more is considered a positive response.
3. Interpretation of data is based on the dermal score for erythema (see Gad and Chengelis 1988, for details).

Armstrong Assay

This method, originally published by Ichikawa et al. (1981), introduced the use of adjuvents in a photosensitization test system.

This assay has been recommended by the Cosmetic, Toiletries and Fragrances Association. It is of interest that the EPA has not made public a concern about photoallergens, as several pesticides have similar chemical structures to fragrances and numerous pesticides are known to form reactive species in the presence of UV light.

Lights. The Armstrong assay uses UVA light (320–100 nm) in the induction and challenge phase. The UVA lights are commonly known as black lights and can be purchased as BLB fluorescence-type bulbs from major lighting manufacturers. However, the selection of the light source is critical, as the range of wavelengths emitted by the bulb is controlled by the phosphor coating, and different manufacturers use different phosphors to produce BLB lights. There might even be different phosphors used by the same manufacturer, and there is no code on the bulbs to indicate which phosphor is being used. The General Electric BLB emits effective energy only at wavelengths longer than 350 nm, whereas the entire spectrum between 315 and 400 nm is covered by the Sylvania BLB bulb. Less than 2% of the total energy emitted by the General Electric BLB light is between 250 and 350 nm, whereas 42% of the energy from the Sylvania BLB light falls in this range. There are known photoallergens that require the energy contained in the spectrum below 345 nm for activation and thus give a false negative if the incorrect light source is used. The best precaution is to determine the emission spectrum of the light sources that are to be used in the assay.

It is necessary to determine the total energy being emitted by the lights to calculate the proper joules per square centimeter (J/cm^2) exposure. An International Light Model 700 provides a relatively inexpensive means of measuring the light energy when fitted with a cosine-corrected UVA detector

(W150s quartz diffuser, UVA pass filter SEE015 detector). The device has a peak sensitivity of 360 nm and a width of 50 nm. A bank of eight bulbs is readily prepared by bolting together two industrial 4-bulb (48-in. long) reflectors. Two sets of these will allow 40 animals to be treated at one time. The lights are allowed to warm 30 min before use. They are turned off just before the animals are placed under them and then turned back on. The light intensity is measured at several locations at the level of the top of the backs of the animals and the correct exposure time is then calculated. The lights are adjusted to be between 4 and 6 in. above the back and 10 J/cm^2 is the proper exposure.

Patching. The Hill Top Chamber (see the earlier description of the Buehler assay) provides a good patching system in this assay. A volume of 0.3 ml is used. The animal restrainers described in the description of the Buehler assay work well for holding the animals during the patching and the exposure to the light as well as in providing excellent occlusion.

Induction Site Preparation. The majority of hair is removed from the intended patching site with a small animal clipper fitted with a number 40 blade. The assay has a frequent requirement for the complete removal of hair using a depilatory that (e.g., that available from Whitehall Laboratories, New York) is applied and left in contact with the skin for no more than 15 min. It must be washed away completely with a stream of warm running water. The animals are dried with a towel and the inside of the cages wiped clean of any depilatory before returning the guinea pigs.

When required, the epidermis is partially removed by tape stripping. The skin must be completely dry or the stripping will be ineffective. A length of tape approximately 8 in. long is used. Starting at one end of the tape, it is placed against the skin and rubbed with the finger a few times to cause good adhesion. It is then peeled away, taking with it some dry epidermal cells. A new section of the tape is then applied to the skin and the procedure is repeated four or five times. The skin will have a shiny appearance owing to the leakage of moisture from the dermis. The tape should not be jerked away from the skin as this can cause the rupture of dermal capillaries.

The potential of the animal to respond to a sensitizer is enhanced by the injection of FCA (Calbio-chem, San Diego, California, or Difco, Detroit, Michigan). The adjuvant is diluted 1:1 with sterile water before using. The injections must be intradermal. In the Armstrong assay, a pattern of four 0.1-ml injections are given just prior to the first induction patching in the nuchal area. All four injections should fit under the edge of the area to be covered by the Hill Top Chamber. It is advisable to perform the skin-stripping operation before the injections, because adjuvant can leak onto the skin and prevent effective removal of the epidermis.

The occlusion of the patches is done in the same manner as described for the Buehler assay. The test sites are exposed to the UVA light after 2 hr of occlusion. The animal is left in the restrainer and the dental dam above the test site to be exposed is cut and the patch removed. Sites not to be exposed are left patched. Excess material is wiped from the site to be exposed and the remaining parts of the animal are covered with aluminum foil. All patches are removed after the light-exposure step, patched areas are wiped free of excess material, and the animal is returned to its cage.

Grading. The grading is the same as used in the Buehler assay.

Vehicles. With the exception of water, it is desirable to use a vehicle for the inductions that is different from the one used at the challenge (see Buehler assay). Because the control animals in the Armstrong assay are sham treated (including any vehicle), one can patch the test and control animals with vehicle at the time of challenge if the same vehicle must be used for both the induction and the challenge. It is advantageous to use a vehicle that dissolves the test material, although suspensions might not be avoidable in all cases.

Irritation Screens. The irritation screen is used to determine acceptable concentrations for the induction phase (i.e., one which does not produce eschar with repeated exposure or systemic

toxicity) and the challenge phase (no more than slightly irritating). Each concentration must be tested with and without exposure to UVA light, as both conditions are used in the challenge. Thus, to evaluate four concentrations requires that eight animals be used. Each animal receives a pair of patches, with each pair being a different concentration (i.e., each concentration is patched on four animals). One of each pair of patches is placed on the left side and the corresponding concentration on the remaining patch is placed on the right side. The hair is removed by depilation on the day of patching. The patches on the right side are removed after 2 hr of occlusion, the remaining parts of the animal are covered with foil, and the right side is exposed to 10 J/cm^2 of UVA light. Animals are returned to their cages after the exposure. If different solvents are being used in the induction and challenge phase, two separate screens need to be run.

Conducting the Armstrong Assay. Combining the discussed techniques in a specific regimen yields the assay as follows:

1. Irritation/toxicity pretest (eight animals)
   Day 0: Remove the hair from the lumbar region by clipping and depilitation. Apply two concentrations on each animal on adjacent left side and right side locations for a total of four dose concentrations. Occlude the patches for 2 hr (± 15 min). Expose the right side to 10 J/cm^2 of UVA light after removing the patches on the right side. Remove the remaining patches and excess material after the exposure to light.
   Day 1: Grade all test sites 24 hr (± 1 hr) after removal of all patches (24-hr grade).
   Day 2: Repeat the grading 48 hr (±2 hr) after removing the patches (48-hr grade).
2. Induction (20 test + 10 sham controls + any rechallenge controls)
   Day 0: Weigh all test and control animals. Remove the hair from the nuchal area with clippers and depilatory. Remove the epidermis by stripping four or five times with tape. Make four 0.01-m1 intradermal injections of a 1:1 dilution of FCA in an area to be covered by the patch. Cover this area on the test animals with a Hill Top Chamber that has 0.3 ml of test material preparation in it. Patch the sham controls with water or solvent on the patch. Occlude with dental dam and restrain in a holder for 2 hr (±15 min). Remove the patches, cover the nonpatched areas with foil, and expose to 10 J/cm^2 of UVA light for 30 min.
   Days 2, 4, 7, 9, 11: Repeat the activity of day 0 with the following exceptions: Do not weigh animals and do not inject adjuvant. Move the patch back when the original induction site becomes too damaged but remain in the nuchal area. Dipilation might not be needed at each induction.
3. Challenge (20 test + 10 sham control animals 9–13 days after last induction exposure)
   Day 0: Weigh all animals, clip the lumber region free of hair and dipilate. Do not strip the skin. Patch each animal with a pair of adjacent patches (one on the left side and one on the right side) containing 0.3 ml of a nonirritating concentration of test material in a Hill Top Chamber. Occlude the patches and restrain the animal for 2 hr (± 15 min). Remove the patches from the right side and cover the rest of the animal's body with foil. Expose the right side to 10 J/cm^2 of UVA light. Remove the remaining patch and any excess material.
   Day 1: Grade all challenge sites, keeping separate the grades of the site exposed to light and those not exposed to light 24 hr (± 1 hr) after removal of the patches (24-hr grade).
   Day 2: Repeat the grading 48 hr (±2 hr) after removal of the patches (48-hr grade).
4. Rechallenge
   All or selected animals can be rechallenged with the same or a different test material 7 to 12 days after the challenge. Use 10 new sham-treated controls and naive test sites on all animals following the same procedure as used in the challenge.
5. Interpretation of results
   Determine the number of positive responders (number of animals with a score > 1 at either the 24- or 48-hr grading or with a score 1 unit higher than the highest score in the control). Determine the average score at 24 hr and at 48 hr for the test and control groups using face values. Keep the data for the sites exposed to light separate from the data from sites not exposed to light.

Strengths and Weaknesses. The Armstrong assay was found to give responses in the guinea pig that were consistent with what has been observed in humans: positive responses for 6-methyl coumarin and musk ambrette. One major disadvantage is that the procedure is time consuming with six induction exposures; additional work might demonstrate that fewer exposures will yield the same results.

The procedure is very stressful on the animals because of the injection of adjuvant and the multiple skin strippings and depilation.

As with any assay involving the intradermal injection of adjuvant, there is often a problem with using the results of the irritation screen in naive animals to accurately predict the results that will be seen in the sham controls at the challenge. If the material being tested is a nonirritant, or if one selects a concentration of an irritant that is far below the irritating concentration, then the screen does an adequate job of predicting the background irritation level in the challenge controls. However, if a slightly irritant concentration of an irritant is used, then the screen often underpredicts the irritation response and a high background level of irritation is observed at the challenge in the sham controls. The interpretation of the results of the challenge becomes difficult. The use of animals in the irritation screen that have had a prior injection of adjuvant might provide a viable alternative and reduce the number of times that rechallenges must be run because of high background levels of irritation.

The Armstrong assay was designed to evaluate materials for their photoactivated sensitization potential and not their potential to be nonphotoactivated dermal sensitizers. At this time, there is no background data that will allow for properly positioning results of the Armstrong assay with regard to human risk if the assay indicates that a test material is a sensitizer or that a material is both a sensitizer and a photoallergen. Thus, it is highly recommended that a "standard" sensitization assay that can be related to humans be run before or in conjunction with the photosensitization assay. The use of a subjective grading system can be a source of significant verification.

Host Resistance Test

The guinea pig can also be used for the host resistance assay, a primary immunotoxicity screen. A protocol for such an assay is as follows.

Test System

1. Male Hartley guinea pigs (Charles River Breeding Laboratories, Portage, Michigan) are used. The guinea pigs should be 7 to 8 weeks old at the time of dosing. Animals will be acclimatized for at least 14 days prior to dosing.
2. Animals that appear abnormal should be rejected prior to dosing or assignment to a test group.
3. Animals are randomly selected from the pool of available animals and assigned to each dosage group.
4. Each animal is identified by a cage card.

Challenge Organism

1. *Pseudomonas aeruginosa (P. aeru.)* culture, from American Type Culture Collection, 27853, is grown in tryptic soy broth. This broth will be centrifuged down, and the resulting pellet washed and resuspended in 0.9% saline. Plate counts should be performed to determine the concentration of the bacteria; the solution can be adjusted to give the desired concentration. Once prepared, samples of the bacterial solution will be repeated to confirm the concentration of the bacteria.
2. Administration of the test organism is done intravenously to cause septicemia. Based on experimental data, a dosage of 2.3 colony-forming units (CFU) $\times$ 108/kg should be expected to challenge the immune system of the guinea pig and provide a minimally lethal response. Each animal should

receive a single exposure. They should be lightly anesthetized with ether and the inoculum should be administered into an ear vein. The dose volume should be 1 ml/kg of body weight.
3. The concentration (determined on solution preparation) of the *P. aeru*. is confirmed by the replating of the samples taken after preparation.
4. Once prepared, *P. aeru*. cultures should be stored in a refrigerator for no more than 3 days.
5. All materials that come into contact with the organism should be placed in plastic bags, marked as a biohazard, and incinerated as soon as possible.

Study Design

Details for the study design are shown in table 5.15.

Table 5.15 Host Resistance Test Study Design

Group	No. in Group	Test Material (mg/kg/Day)	*P. aeru.*
1	10	—	Yes
2	10	—	Yes
3	10	—	No

The guinea pig should be treated by the appropriate route with test material daily for 7 days. Guinea pigs should receive an IV dosage of *P. aeru.* on day 8 at a dosage of approximately 2.3 CFU $\times$ 108/kg. The experiment is then terminated on day 15.

Observations

1. Prior to *P. aeru*. challenge guinea pigs are observed at least twice daily. On the day of challenge, they should be observed periodically during the first 4 hr after challenge and then at least three times daily for the next 7 days for obvious signs of treatment, including death. Body weights of all animals will be determined prior to dosing on study days –3, 1, 8, and 15. Rectal temperatures are determined on days –1, 1 (2 hr postdosing), 2, 3, and 4, after *P. aeru*. challenge.
2. Animals found dead should be discarded. Necropsies are not conducted. Animals killed by design should be asphyxiated with carbon dioxide and discarded. Surviving animals are killed on study day 15 (7 days after dosing of *P. aeru*.).

Analysis and Interpretation

1. Mortality data will be analyzed by probit analysis or minimum normit chi-square analysis. Means and standard deviations are prepared for survival time body weights and rectal temperatures. Time-to-death analysis is also done.
2. A significant increase in mortality should be interpreted as a positive finding in this test system.

PATHOLOGY

Usefulness: Strengths and Weaknesses

Respiratory System

The sensitivity of the respiratory system of the guinea pig has provided animal models for numerous studies of inhalation phenomena, including bronchospasms, asthma, other respiratory allergies, hypersensitivity reactions to dust, and other air pollutants, and development of antihistamines.

Digestive System

Guinea pigs are fastidious eaters. As they mature they develop rigid habit patterns that must be accommodated if the animal is to thrive. Any changes in feed (taste, odor, texture, and form), water, feeder, or watering device might cause the guinea pig to stop eating or drinking (Harkness and Wagner 1989). Starvation with clinical or subclinical illness from vitamin deficiencies could result.

The behavior of coprophagy and anatomical structure of a glandular stomach with a large cecum might modify the effects of experiments involving nutritional factors (Navia and Hunt 1976).

Urogenital System and Fetal Tissues

Although the guinea pig has been employed infrequently in the past, it is unique among the animals available for reproductive and teratological studies. It occupies a position intermediate between the laboratory rodents, rats and mice, and the more evolved subhuman primates and man. For example, the endocrine control of its reproduction is similar to that of humans, even to the trimesteric characteristics, yet pregnancy is preceded by a well-defined estrus. Its placenta appears to be capable of endocrine activity, but it is labyrinthine, and its transfer functions are supplemented by an everted yolk sac exposed to uterine secretions. The elements of the reproductive cycle that precede pregnancy (i.e., estrus, ovulation, and fertilization) can be accurately determined as with all rodents, but its relatively long gestation allows the assessment of the effect of potentially harmful agents applied late in development on organ functions or behavior patterns that develop after birth in other rodents (Hoar 1976b, 1976c). Their long gestation period of 68 days provides an opportunity for separating toxic or teratogenic effects on the embryo from those on the fetus and allows investigation of a fetus with an essentially mature central nervous system prior to delivery (Hoar 1976c). In many instances, pregnancy is maintained following ovariectomy at 25 or more days. The guinea pig is, therefore, an animal of choice for studying the effects of hormones and endocrine glands on pregnancy (Harkness and Wagner 1989). The circulatory form of pregnancy toxemia in guinea pigs has been identified as a possible model for preeclampsia in women (Percy and Barthold 2001).

Malformations have been induced in guinea pigs by a variety of agents, including drugs, dyes, pesticides, vitamin excess, hyperthermia, adrenocortical hormone excess, synthetic steroids, ionizing radiation, and mineral-deficient diets. Spontaneous malformations of genetic and unknown origin also occur (Hoar 1976b, 1976c).

Nervous System and Special Sense Organs

Because of the anatomy of the guinea pig ear and associated structures, it is an important animal model for ototoxicity studies. A postauricular surgical approach to the middle ear presents no major blood vessels or muscles. The petrous bone (otic capsule) is easily entered and dissected away without drilling to expose the inner ear structures in much less time than the cat and monkey. The guinea pig has been used to elucidate the pathophysiology of drugs that cause deafness and vestibular disorders, including the aminoglycosidic antibiotics such as neomycin, gentamicin, and other members of the streptomycin family, the diuretic ethacrynic acid, quinine, and salicylates (McCormick and Nuttall 1976). In addition to ototoxicity, the guinea pig provides animal models for studies of noise, proprioception, Meniere's disease, amyotropic lateral sclerosis, experimental allergic encephalomyelitis, spinal cord injury, epilepsy, and multiple sclerosis, as well as development of antianxiety and anti-inflammatory compounds (Hanes 2003).

Integumentary System and Soft Tissues

Guinea pigs have been used extensively for the study of immunological and inflammatory reactions involving the skin and subcutis. They are frequently used to test the safety of skin lotions

and ointments. The dermal response of the guinea pig to irritation by test materials has been shown in many instances to be more like that of man than the rabbit; therefore, the guinea pig is the species of choice for dermal irritation tests (Gilman 1982). In addition, physiological characteristics of its skin are similar to those of humans, which have led to other areas of investigation such as that of wound repair and thermal burns (Hoar 1976a). Also, the guinea pig has provided animal models for studies of psoriasis and chemical burns such as mustard gas (Hanes 2003).

The capability of chemical compounds to cause skin sensitization was first demonstrated in guinea pigs by Landsteiner and Jacobs (1935, 1936). Using substituted benzene compounds, they demonstrated development of a true allergic reaction following sensitization of the skin. Subsequently, experiments proved that skin could be sensitized by the administration of a chemical either topically, intradermally, or intraperitoneally. Skin sensitization tests are designed to determine if a chemical compound will cause an allergic reaction after there has been a previous contact to the same or a similar compound. The Hartley strain of guinea pig is the laboratory animal most commonly used for these tests (Gilman 1982). Individual guinea pigs have been demonstrated to inherit differing skin susceptibilities to some compounds such as 2, 4-dinitrochlorobenzene and poison ivy (Chase 1941).

The female has one pair of inguinal mammary glands. Despite the apparent shortage of nipples, adult females have successfully raised litters of three, four, and more offspring (Harkness and Wagner 1989).

Hematopoietic and Lymphoid Systems

The bone marrow of the guinea pig has been studied extensively because of its similarity to that of humans, its ease of dispersion to give uniform cell suspensions, and the ease of staining and identification of its cells (Sisk 1976). Also, hormonally and immunologically the guinea pig more nearly resembles the human than do rats and mice (Ernstrom 1970). Unlike many species that are very immature at birth, the neonatal guinea pig possesses very mature myeloid and lymphoid tissues. There are considerable data on cellular elements, physiological properties, and biochemical characteristics of circulating blood and bone marrow (Sisk 1976). As a corticosteroid-resistant species, treatment with steroids does not markedly affect thymic physiology or peripheral lymphocyte counts (Sisk 1976).

Mature females are an excellent source of serum complement used in a variety of immune and endotoxin serological reactions. Guinea pigs produce antibodies to specific proteins and are used to test for the presence or absence of small amounts of antigens by the production of anaphylaxis. Unlike the rabbit or chicken, injected antibodies protect the guinea pig from anaphylaxis (Harkness and Wagner 1989).

Nutrition and Metabolic Diseases

The guinea pig is uniquely susceptible to vitamin C (ascorbic acid) deficiency, which results in bone and collagen disease and increased susceptibility to infections with inflammation, especially of the lungs and cervical lymph nodes.

The vitamin C requirement makes the guinea pig a useful model for nutritional studies pertaining to vitamin C metabolism, collagen studies, skin studies, bone studies, atherosclerosis studies, adrenal-pituitary studies, and hydroxylating reactions where ascorbic acid seems to play a role (Navia and Hunt 1976). The guinea pig was used for the biological assay of vitamin C before the chemical assays were developed (Fenner 1986).

Bacterial, Viral, and Rickettsial Diseases

Guinea pigs were once used extensively for disease diagnosis and isolation of pathogenic agents such as in tuberculosis (Jolly and Heywood 1979). Guinea pigs are highly susceptible to both

human- and bovine-type tubercle bacilli and have long filled an important place in tuberculosis research and diagnosis. A guinea pig model of low-dose aerogenic tubercular infections has been developed (Chambers et al. 2001). Guinea pigs infected with the Legionnaires' disease bacillus, *Legionella pneumophila*, have been used to evaluate new therapeutic agents (Edelstein et al. 2001; Edelstein et al. 2003). They are also susceptible to rickettsial infections, and a number of rickettsiae pathogenic to humans were first studied in the guinea pig (Fenner 1986). Guinea pigs are susceptible to the Q fever agent *Coxiella burnetii* and provide an animal model of this disease (Heggers et al. 1975). The guinea pig provides animal models for development of a wide variety of therapeutics including antibiotic, antifungal, and antiviral compounds (Hanes 2003).

Neoplastic Diseases

Although guinea pigs have been used in large numbers as experimental laboratory animals, they have not been used as frequently in carcinogenesis studies. This has been in part because of a prevailing view that guinea pigs are particularly resistant to induced neoplasia. This genetic susceptibility to spontaneous neoplasia appears to vary (Percy and Barthold 2001).

Guinea pigs were considered to be resistant to chemical carcinogens until 1962 because exposure to chemicals known to be carcinogenic in rats failed to produce tumors (Argus 1971; Mosinger 1961a, 1961b). This observation led to investigation of tumor-resistant factors in guinea pigs. Guinea pig serum was found to have a factor that inhibited the growth of several lymphomas in mice. This factor was termed tumor inhibitory principle (TIP), but it could not be isolated. The existence of this principle is still uncertain (Manning 1976). A serum factor (probably asparaginase) in normal guinea pig sera has been demonstrated to have antitumor activity. Splenic preparations containing large numbers of Kurloff cells have shown inhibition of transformed human epithelial cells *in vitro* (Percy and Barthold 2001). Since 1962, guinea pigs have been shown to be susceptible to the carcinogenic activity of a variety of agents, including physical and chemical irritants, hydrocarbons, nitrosamines, and hormones, particularly estrogens (Mosinger 1961b; Blumenthal and Rogers, 1967; Manning 1976).

The refractoriness of the guinea pig to the carcinogenic action of aromatic amines and amino azo dyes possibly resides in its limited ability to metabolize these agents to their N-hydroxy derivatives. Miller et al. (1964) showed that N-hydroxy-2-acetylaminofluorene in guinea pigs induced adenocarcinomas of the small intestine (on feeding) and sarcomas (on injection), whereas 2-acetylaminofluorene is inactive. Very rapid elimination of N-hydroxy derivatives from guinea pig tissues has been proposed as another mechanism (Kiese and Wiedemann 1968). Berenblum (1949) found that increasing the dosage of 9, 10-dimethyl-1, 2-benzanthracene to 20-fold that of the rat resulted in tumors in guinea pigs. Rogers and Blumenthal (1960) induced tumors in 57% of 735 guinea pigs with methylcholanthrene injected subcutaneously and intramuscularly.

Guinea pigs are susceptible to many different chemical carcinogens, including ethyl carbamate, polycylic hydrocarbons (7, 12-dimethylbenz [a] anthracene, 3-methylcholanthrene, benzopyrene, 1,2,5,6-dibenzanthracene, and others), nitrosamine-type alkylating agents (diethylnitrosamine, dimethylnitrosamine, N-methyl-N-nitrosourea, nitrosomethyldodecyclamine, nitroso-2,6-dimethylmorpholine, dinitroso-2,6-dimethylpiperazine, di-N-butylnitrosamine), methylazomethanol, aflatoxin, and dioxan (Argus 1971; Squire et al. 1978; Cardy and Lijinsky 1980).

Animals used in carcinogenicity studies must be reasonable in cost to allow adequate numbers of both test and control animals, have a life span within both the financial capabilities of the sponsor to maintain them and the public health and political time constraints for determining an answer, and be well adapted to the laboratory environment without serious interfering infectious diseases. Although mice and rats meet the first two requirements better than guinea pigs, guinea pigs should be considered if the metabolism of the compound and its availability at the expected target site are similar to that in humans, and if the value or extended use of the test product warrants the extra cost (Robens et al. 1982).

Table 5.16 Organ and Body Weights for Guinea Pigs

Organ	Weight (g)	Organ/Body Weight Ratio[a] (× Adjustment Factor)		Organ/Brain Ratio
Brain	4.300	4.778	(×1,000)	—
Spinal cord	14.500	1.611	(×100)	3.372
Pituitary	0.022	0.024	(×1,000)	0.005
Thyroid	0.134	0.149	(×1,000)	0.031
Adrenal	0.725	0.806	(×1,000)	0.169
Liver	42.500	4.722	(×100)	9.884
Lung	5.000	5.556	(×1,000)	1.163
Kidneys	6.120	6.800	(×1,000)	1.423
Pancreas	2.500	2.778	(×1,000)	0.581
Testes	4.300	4.778	(×1,000)	1.000
Epididymis	0.660	0.733	(×1,000)	0.153
Ovaries	0.192	0.213	(×1,000)	0.045
Stomach	0.420	0.467	(×1,000)	0.098
Intestine	28.000	3.111	(×100)	6.512
Urinary bladder	4.250	4.722	(×1,000)	0.988
Stomach and intestine (with contents)	120.000			
Eyes	1.250			
Skeleton and ligaments	64.000			
Musculature	320.00			

[a] Based on 900-g guinea pig.

It is important for the testing laboratory to establish the background tumor incidence and other lesions of aging guinea pigs to help determine if the incidence of tumors among the controls in any specific test is representative. The laboratory incidence should be compared with reported tumor incidence when the latter are available (Robens et al. 1982).

Organ Weights and Ratios

Organ weights have rarely been reported by investigators because of the infrequency of guinea pigs used in acute, subchronic, and chronic toxicity tests by the chemical, cosmetic, and pharmaceutical industries. The organ and body weights shown in table 5.16 were calculated based on data reported by Breazile and Brown (1976).

For additional information see the other portions of this chapter, and see Turton, Shaw, Bleby, et al. (1977) and Turton, Shaw, Tucker, et al. (1977).

Spontaneous Lesions: Nonneoplastic and Neoplastic

General Considerations: Nonneoplastic Findings

Guinea pigs are nervous in temperament and respond to sudden noises, unfamiliar surroundings, and sudden movements by freezing in place or a random stampede, which can result in injuries. They might refuse to eat or drink for some time following any significant change in location, feed, or other management practice. They require a constant source of water and must be trained to lick sipper tubes. Males and some females are prone to fight cagemates, which can result in severe injuries, and even deaths. Their large size at birth contributes to dystocia. The major diseases affecting guinea pigs are scurvy, respiratory tract infections, and enteritis (Percy and Barthold 2001).

Anorexia usually accompanied with weight loss can result from many conditions including changes in housing or mechanical failure, water deprivation, unfavorable room temperature, changes

in available food, unpalatable or contaminated food, malocclusion, oral lacerations, obesity, ketosis, renal failure, urolithiasis, vitamin C deficiency, protein deficiency, metastatic calcification, pododermatitis or infections at various other sites, neoplasia, ectoparasitism, loss of a cagemate, or antagonism of a dominant cage mate (Ediger 1976; Hanes 2003).

Deaths can occur from excessive cold or heat, septicemia, toxemia, Salmonella infection, enteritis, pregnancy toxemia, antibiotic toxicity, pneumonia, volvulus of cecum or stomach, dystocia, dehydration, fractured limb, or being caught in the cage floor (Hanes 2003).

General Considerations: Neoplastic Findings

Considering only animals of comparative ages, guinea pigs have a lower incidence of spontaneous neoplasms than rats or mice (Morgan 1969). Accurate estimates of the incidence of spontaneous neoplasia in any species must be based on complete necropsies and thorough microscopic examination of tissues from animals allowed to live one-half or more of the natural life span (Manning 1976; Peckham 1980). The breeding life of laboratory guinea pigs is from 18 months to 4 years, and they have been known to live 8 years; however, they rarely survive in the home longer than 5 years (Harkness and Wagner 1989; Ruf 1984). The life span of the laboratory guinea pig is 4 to 6 years (Wallach and Boever 1983). An occasional animal might reach 9.0 or 9.5 years of age (Kunstyr and Naumann 1984). There are few definitive reports in the literature that meet the criteria of complete necropsy examinations with histopathology and full life spans. Shimkin and Mider (1941) reported no neoplasms in 15,000 guinea pigs of inbred strains born and observed for up to 5 years between 1916 and 1937. Papanicolaou and Olcott (1942) observed about 100 tumors in more than 7,000 guinea pig necropsies for an incidence of 1.4%; tumors were rare in animals less than 4 to 5 years of age.

Rogers and Blumenthal (1960) examined 6,000 guinea pigs of two inbred strains over a 10-year period. These animals, which had not been used for experimental purposes, had a spontaneous tumor incidence of 0.4% for all ages and 14.4% (14 of 97) for animals surviving 3 years or more. All 14 tumors occurred in the R9 strain. The incidence of tumors in random-bred Hartley strain guinea pigs from necropsies of 8,400 animals (estimated male and female ratio 1:3) at ages of less than 27 months was 0.75% and of 34 retired breeders (6 males and 28 females) greater than 27 months of age was 29.4%.

Cardiovascular System

Generalized cardiovascular system septicemias sometimes occur with bacterial infections such as staphylococcosis and those caused by *Pseudomonas aeruginosa, Pasteurella multocida,* and *P. haemolytica*. Pregnancy toxemia, a common problem in guinea pigs, has a circulatory form (Percy and Barthold 2001). The guinea pig provides animal models for studies of implants, septic shock, vasoconstriction, and antiarrhythmic compounds (Hanes 2003).

Heart

Nonneoplastic Lesions: Spontaneous. Cardiac rhabdomyomas, also termed rhabdomyomatosis, are commonly observed circumscribed accumulations of glycogen that might be confused with neoplastic lesions. Rhabdomyomatosis is considered to be an incidental finding resulting from a congenital disease in glycogen metabolism (Hoch-Ligeti, Restrepo et al. 1986; Manning 1976; Percy and Barthold 2001; Vink 1969; Takahashi and Iwata 1985).

Spontaneous interstitial lymphocytic myocarditis of unknown pathogenesis has been observed as an incidental finding in guinea pigs that is similar to that described in mice, rats, and rabbits (Miller 1924). Vegetative endocarditis can be caused by *Streptococcus spp.* infections.

A colony of guinea pigs with myocardial necrosis and mineralization was investigated for the possible roles of vitamin E or selenium deficiencies. Vitamin E and selenium levels were within normal ranges, suggesting that these heart lesions resulted from inbreeding within the colony (Griffith and Lang 1987).

Neoplastic Lesions: Spontaneous. Spontaneous cardiovascular tumors are uncommon. Benign mesenchymal mixed tumors (mesenchymomas or myxomas) have been observed in the heart of 12 females of the Hartley strain (Manning 1976; McConnell and Ediger 1968). These tumors included well-differentiated mesenchymal components, such as cartilage, bone, and fat. They should not be confused with rhabdomyomatosis, congenital glycogen lesions (Percy and Barthold 2001). Also, a fibrosarcoma of the heart and a cavernous hemangioma of the liver have been reported (Manning 1976; Rogers and Blumenthal 1960).

Aorta and Arteries

Nonneoplastic Lesions: Spontaneous. The circulatory form of pregnancy toxemia in guinea pigs is characterized by uteroplacental ischemia due to compression of the aorta caudal to the renal blood vessels by the gravid uterus. This results in reduced blood pressure in the uterine vessels and tissue anoxia, with subsequent placental necrosis, uterine hemorrhage, thrombocytopenia, ketosis, and death. The disease has been considered a possible model for preeclampsia in women (Percy and Barthold 2001; Seidel et al. 1979).

Nonneoplastic Lesions: Induced. Medial calcification of the major elastic arteries and soft tissue calcification have been associated with hypervitaminosis D (Wallach and Boever 1983).

Respiratory System

Lung

General Considerations. Clinical respiratory signs can result from bacterial or viral pneumonia, heat stress, diaphragmatic hernia, pregnancy toxemia, or gastric torsion (Hanes 2003).

Nonneoplastic Lesions: Spontaneous. Susceptibility to acute anaphylaxis is related to the quantity of histamine available for release in the lung. In highly susceptible Hartley animals, this amount can be more than 10 times greater than in the resistant strain 2. Strain 2 and Hartley guinea pigs are equally susceptible to histamine toxicity (Stone et al. 1964).

Adenomatosis or alveolar epithelial hyperplasia is a frequent lung lesion in guinea pigs that must be differentiated from alveolar or bronchogenic adenomas (Hoch-Ligeti et al. 1982). Proliferations of alveolar epithelium diagnosed as tumors were often associated with interstitial pneumonia, foreign bodies, or other inflammatory changes, which suggest many of these lesions are in reality hyperplasia (Manning 1976).

Pneumonia is caused by one of several bacteria or viruses in guinea pigs. The most important of these bacteria include *Bordetella bronchiseptica, Klebsiella pneumoniae, Pasteurella multocida* or *P. pneumotropica,* and *Streptococcus (Diplococcus) pneumoniae* (Saito et al. 1983). Other microorganisms associated with pneumonia in guinea pigs are *Streptobacillus moniliformis* (Kirchner et al. 1992), *Corynebacterium kutscheri,* other *Streptococcus spp., Pseudomonas aeruginosa, Citrobacter freundii* (Ocholi et al. 1988), and *Mycoplasma pulmonis.* Signs in affected guinea pigs include rough hair coat, anorexia, emaciation, hunched posture, dyspnea, abdominal breathing, rales, sneezing, and sometimes a mucosanguineous to purulent discharge from the eyes and nose. At necropsy, *B. bronchiseptica* has been shown to cause partial consolidation of the lungs. Histologically, a marked purulent bronchitis and bronchiolitis or fibrinous bronchopneumonia can be observed. *S. pneumoniae* commonly causes

a fibrinopurulent pleuritis or peritonitis (Parker et al. 1977). *S. zooepidemicus* can cause septicemia and pneumonia. *P. aeruginosa* can cause a focal necrotizing and granulomatous pneumonia with "sulfur" granules or green exudate. Pneumonias caused by *Escherichia coli* and *Klebsiella pneumoniae* are usually secondary and accompanied by pleuritis, pericarditis, and peritonitis (Ganaway 1976; Ruf 1984; Wagner 1979; Wallach and Boever 1983).

Guinea pigs that are latent carriers of *Streptococcus (Diplococcus) pneumoniae* frequently die of fibrinopurulent peritonitis, pericarditis, pleuritis, pneumonia, or meningitis following the injection of irritating substances into or removal of fluids from body cavities (Wagner 1976). Vitamin C deficiency results in an increased susceptibility to pneumonia and pleuritis from bacterial infections.

Adenoviral pneumonia was fatal to 11 of 1,600 preadult guinea pigs. The only clinical sign was dyspnea. Grossly partial consolidation of the anterior lobes was observed in the lungs. Lesions were limited to the respiratory system, and included emphysema, petechiation, hydrothorax, bronchiolitis, bronchial epithelial sloughing, and focal parenchymal necrosis and inflammation. The desquamated bronchial epithelium frequently contained large basophilic intranuclear inclusion bodies. Numerous viral particles of an adenovirus were found (Brennecke et al. 1983; Kaup et al. 1984; Kunstyr et al. 1984; Naumann et al. 1981; Richter 1986). Adenoviral infections are probably more prevalent than currently recognized. Frequently outbreaks have occurred in animals subjected to experimental procedures that might have resulted in impaired immunity (Percy and Barthold 2001).

Adiaspiromycosis caused by *Emmonsia parva* and *E. crescens* has been reported in guinea pigs. It is a benign self-limiting granulomatous pulmonary disease (Wallach and Boever 1983).

Perivascular lymphoid nodules in the lungs of guinea pigs occur around smaller branches of the pulmonary arteries and veins. They have been reported as occurring in 14% to 85% of guinea pigs of both sexes and all ages from seven strains, including a germ-free animal (Thompson et al. 1962). The nodules appear to enlarge with age and might be visible grossly as pinpoint, subpleural foci. These normal lymphoid nodules must be differentiated from focal granulomatous pulmonary lesions seen after treatment with Freund's adjuvant (Percy and Barthold 2001).

Pulmonary arteries and arterioles are greatly thickened as a result of prominent smooth muscle layers in the tunica media. This is not an abnormal finding (Percy and Barthold 2001).

Nonneoplastic Lesions: Induced. Histamine administration results in lethal bronchiolar smooth muscle contraction. When prostaglandin IR-PGF20 generation was studied, the peripheral lung responses closely resembled those of the peripheral human lung to histamine H1 stimulation (Steel et al. 1979).

Experimental airborne tuberculosis in the guinea pig provides a useful model of human tuberculosis with a naturally occurring bacillemia and cavitary stages of the disease (Smith and Harding 1977). A guinea pig model of low-dose, aerogenic tubercular infections might be suitable for virulence, vaccination, and immunological studies (Chambers et al. 2001).

Induction of pneumonic pasteurellosis in guinea pigs provides a model for studing bovine pneumonic pasteurellosis, shipping fever pneumonia (Morck et al. 1990). Pulmonary lesions have been reported after experimental infections with *Actinobacillus pleuropneumoniae* (Perfumo et al. 1999).

Eosinophilic granulomas were observed in the lungs as a result of visceral larval migrans due to *Baylisascaris procyonis* eggs in wood shavings bedding contaminated by raccoon feces (Van Andel et al. 1995).

Pneumoconiosis (foreign body pneumonias) are incidental findings in the lungs, especially in young guinea pigs. They are associated with aspirated food or bedding materials. They have been observed as foci or circumscribed nodules of granulomatous or chronic active inflammation in the bronchioles or alveoli with mononuclear cells and foreign body giant cells. Plant fibers or other foreign materials might be identified. These findings could complicate respiratory research studies. Lung lesions in guinea pigs induced by perlite and fir bark dusts have been described (McMichael et al. 1983). Pulmonary multifocal granulomas might also occur following subcutaneous injection with Freund's adjuvant (Percy and Barthold 2001).

Bony spicules (osseous metaplasia) are occasionally observed in the lung. They consist of dense lamellar bone, with varying degrees of calcification, and sometimes include well-differentiated bone marrow. They have been observed in the interstitium of alveolar septa of the lungs of guinea pigs fed commercial diets (Kaufmann 1970). Large numbers of these metaplastic osseous foci have been observed following X-irradiation (Percy and Barthold 2001).

Neoplastic Lesions: Spontaneous. Spontaneous lung tumors constitute some of the most important tumor types in guinea pigs and have been the most frequently reported solid tumors, approximately 35% in one survey. Most of the tumors were benign papillary adenomas and appeared to have bronchiogenic origin (Percy and Barthold 2001). Other reports include a number of alveologenic tumors, an adenoma, papillary or bronchogenic adenomas, and bronchogenic adenocarcinomas (Manning 1976) as well as hemangiosarcoma, lymphangioma, and intrabronchial papilloma (Hoch-Ligeti et al. 1982).

Rogers and Blumenthal (1960) found that 64 papillary adenomas (2.6%) and one adenocarcinoma of the lungs had been reported in the literature. Eighteen adenomas and 17 adenocarcinomas were reported by Mosinger (1961a). These bronchoalveolar or alveologenic tumors are common in animals over 3 years of age. In some studies, the proliferations of alveolar epithelium that were considered to be tumors were associated with interstitial pneumonia, foreign bodies, or other inflammatory changes, which suggest that some of these lesions were hyperplastic, not neoplastic (Manning 1976). Primary malignant tumors of the lung are rarely observed (Percy and Barthold 2001).

Neoplastic Lesions: Induced. Irradiation resulted in the earlier occurrence of alveologenic tumors and an increased number of tumor nodules than in untreated controls. The number of alveologenic tumors in guinea pigs that survived more than 20 months was not statistically significantly different between irradiated and untreated groups. The tumors observed in the lungs were alveologenic tumors (synonyms: papillary adenomas, alveolar adenomas, adenomas), hemangiosarcomas, lymphangiomas, intrabronchial papillomas, and adenomatosis (Hoch-Ligeti and Argus 1970; Hoch-Ligeti et al. 1982).

Of 111 strains 2 and 13 guinea pigs injected intravenously with either 20-methylcholanthrene or 1,2,5,6-dibenzathracene, pulmonary tumors occurred in 32 animals with a similar frequency in both strains (Heston and Deringer 1952).

Digestive System

Guinea pigs have a long colon (60% of the length of the small intestine vs. only 16% in the rat) with a characteristic large, thin-walled cecum with numerous lateral pouches that occupies the left side of the abdominal cavity. The tenia coli have served as a preferred source of smooth muscle for physiologists (Breazile and Brown 1976).

Teeth

Nonneoplastic Lesions: Spontaneous. Malocclusion and loss of opposing teeth lead to overgrowth of the teeth. Because all guinea pig teeth are open-rooted and erupt continuously, all teeth have a tendency to overgrow, especially the premolars or the anterior cheek teeth (Ediger et al. 1975; Harkness and Wagner 1989). Calcium deficiencies can cause soft maxillary and mandibular bone with misalignment of the teeth and overgrowth of the incisors and molars. Soft foods aggravate the problem of malocclusion. Clinical signs include excessive salivation (slobbers), loss of weight (wasting), anorexia (the animal is usually hungry but cannot eat), licking movements, and loss of the ability to close the mouth (Wallach and Boever 1983). Dental abnormalities also occur with scurvy and exposure to fluorides (Percy and Barthold 2001). In addition to improper diet, a genetic predisposition involving more than one gene has been reported in a high incidence in strain 13. Excessive salivation also can occur with heat stress (Hanes 2003).

Salivary Gland

Nonneoplastic Lesions: Spontaneous. Cytomegalovirus is a well-described salivary gland disease of guinea pigs. It is a herpesvirus infection characterized by eosinophilic intranuclear and rarely by intracytoplasmic inclusion bodies in ductal epithelium of the salivary glands and the epithelium of the proximal and distal convoluted tubules of the kidneys. In the salivary glands, affected cells are markedly enlarged (Cook 1958; Percy and Barthold 2001; Van Hoosier and Robinette 1976). Disseminated cytomegalovirus disease has been reported with involvement of the spleen, kidney, liver, and lung (Van Hoosier et al. 1985). The experimental disease is considered a useful animal model for infections in other species, including human patients (Percy and Barthold 2001).

Neoplastic Lesions: Spontaneous. A benign mixed tumor of a sublingual salivary gland was reported in a 1.5-year-old guinea pig (Koestner and Buerger 1965).

Esophagus

Neoplastic Lesions: Induced. Spontaneous tumors of the esophagus are apparently extremely rare; however, a squamous cell papilloma and carcinomas were produced by methylcholanthrene and nitrosopiperidine, respectively (Squire et al. 1978).

Stomach

Nonneoplastic Lesions: Spontaneous. Gastric bloat occurs in guinea pigs when excesses of fresh green forage are fed. The clinical signs of bloat include distended abdomen, abdominal pain, depression, and cyanosis (Wallach and Boever 1983). Acute gastric dilatation associated with gastric volvulus was observed in multiple animals of one colony. Frequently affected animals were found dead without previous signs of illness (Lee et al. 1977). Gastric ulcers are fairly common; most are secondary to other diseases, especially ketosis (Wagner 1979).

Neoplastic Lesions: Spontaneous. Papanicolaou and Olcott (1940, 1942) reported five benign mesenchymal neoplasms in the stomach of guinea pigs. The tumors were a fibromyoma, three leiomyomas, and a lipoma.

Neoplastic Lesions: Induced. Adenocarcinomas and sarcomas of the glandular stomach have been produced by methylcholanthrene (Squire et al. 1978).

Intestine

Nonneoplastic Lesions: Spontaneous. Guinea pigs are particularly prone to acute enteritis although peracute, subacute, and chronic forms also occur. Diarrhea might or might not be present. Nonspecific signs include anorexia, weight loss, depression, and conjunctivitis. Latent forms of the disease can be precipitated by stressful environmental changes such as shipping, pregnancy, and research procedures. Acute enteritis has been also associated with subclinical scurvy (Clarke et al. 1980).

A wasting syndrome associated with viral enteritis has been reported in young guinea pigs following their arrival at a research facility. This syndrome is characterized by wasting, anorexia, and diarrhea. It has a low morbidity and mortality (Jaax et al. 1990; Marshall and Doultree 1996).

Enterocolitis, acute necrotic cecitis, or typhlocolitis is a highly lethal disease that resembles antibiotic "toxicity." It is characterized by sudden death and acute necrosis of the intestinal mucosa and adjacent submucosa, especially in the cecum. This disease occurs sporadically in guinea pigs of all ages. No specific cause or causes have been identified. A thermolabile toxin has been found

in some animals (Wagner 1976, 1979). *Clostridium perfringens* has been isolated from some affected guinea pigs. Also in other affected animals, *Clostridium difficile* has been demonstrated in the absence of prior antibiotic treatment (Percy and Barthold 2001).

Among the most common forms of enteritis are those of salmonellosis and the fatal necrolytic enterocolitis. The *Salmonella spp.* most commonly found are *S. typhimurium* and *S. enteritidis*; however, other salmonellae including *S. dublin* and *S. limete* have been isolated. Other bacteria associated with enteritis include pseudotuberculosis caused by *Yersinia (Pasteurella) pseudotuberculosis* and *Y. enterocolitica;* colibacillosis caused by *Escherichia coli, Klebsiella pneumoniae, Citrobacter freundii* (Ocholi et al. 1988), and *Arizona spp.*; Tyzzer's disease caused by *Clostridium piliforme* (formerly *Bacillus piliformis*; Waggie et al. 1986); and *Pseudomonas spp.* Typical findings in cavian Tyzzer's disease are necrotizing ileitis and typhlitis that can be accompanied by characteristic focal coagulative necrosis of liver (Percy and Barthold 2001). Common necropsy findings of salmonellosis include an enlarged red spleen, sometimes with pale foci of necrosis; enlarged liver with necrotic foci; enlarged lymph nodes; and congested intestines (Habermann and Williams 1958; Jayasheela et al. 1985; John et al. 1988; Wagner 1979; Wallach and Boever 1983). In a young guinea pig with Tyzzer's disease, the *Clostridium piliforme* bacilli were associated with cryptosporidiosis and unclassified intestinal spirochetes (McLeod et al. 1977). Naturally acquired and expeimentally induced colonic spirochetosis caused by Brachyspira (formerly Serpulina) species were compared (Duhamel 2001).

Segmental duodenal hyperplasia associated with proliferation of intracellular bacteria was observed in a female guinea pig, 1 of 140 obtained from a commercial colony. Two cagemates died with acute enteritis without the mucosal hyperplasia (Elwell et al. 1981). The hyperplastic lesions have been attributed to *Lawsonia (Campylobacter) intracellularis* infections. These lesions and bacteria were associated with a spontaneous outbreak of diarrhea with weight loss and mortalities (Moto et al. 1983). Also, the disease has been observed during steroid treatment (Percy and Barthold 2001).

Although a large number of protozoan parasites are observed in guinea pigs, protozoan diseases are rare. The most important protozoan disease is coccidiosis, which is caused by *Eimeria caviae.* Coccidia are sometimes seen in histological sections of cecum and colon, but rarely cause clinical disease. The clinical signs are anorexia, diarrhea, emaciation, dry hair coat, and rarely death. *Cryptosporidium wrairi,* a small coccidium of the small intestine, can be associated with chronic enteritis, diarrhea, weight loss, and emaciation (Vetterling et al. 1971). Clinical infections are observed most frequently in juvenile animals. Infection rates of 30% to 40% are common in conventional colonies. Cryptosporidiosis is often accompanied by *Escherichia coli* infections (Jervis et al. 1966; Percy and Barthold 2001). Numerous other protozoa, including *Balantidium coli,* have been observed in the intestinal tract of guinea pigs with little evidence of clinical disease (Vetterling 1976; Wagner 1979).

The only intestinal nematodiasis reported in guinea pigs with any degree of frequency is the cecal worm *Paraspidodera uncincata.* It rarely causes clinical disease (Wagner 1979; Wescott 1976). Infections can result in diarrhea, lethargy, and anorexia (Wallach and Boever 1983). No lesions are associated with these infections (Habermann and Williams 1958).

Cecal torsion occasionally results in guinea pig deaths. In affected animals, the twisted cecum is edematous, hemorrhagic, and distended with fluid and gas (Percy and Barthold 2001).

Germ-free animals have disproportionately large cecum. The cecum is increased from about 10% in a conventional animal to 25% to 30% of the body weight in germ-free animals. This large cecum predisposes animals to cecal ruptures, herniation, torsion, volvulus, and uterine prolapse (Hanes 2003).

Prolapse of the colon into the rectum resulting from colon intussusceptions is associated with stress, dehydration, and excessive straining (Hanes 2003).

Intestinal hemosiderosis, enterosiderosis, is the result of accumulations of hemosiderin-laden macrophages in the lamina propria of the intestine, particularly the large intestine. This is a common finding in guinea pigs. The cause is unknown but is suggested to arise from the normally zealous iron binding of herbivores, with uptake of excessive dietary iron (Percy and Barthold 2001). A study of enterosiderosis by light and electron microscopy found the pigments in macrophages were mixtures of iron and lipofuscin granules associated with red blood cell phagocytosis (Takahashi et al. 1985).

Nonneoplastic Lesions: Induced. Antibiotics should be used with caution. The abrupt changes in intestinal microflora following the use of several antibiotics, especially those with antibacterial activity against gram-positive organisms, causes high mortality rates from enteritis and what is thought to be endotoxic shock due to overgrowth of gram-negative bacteria, with their accompanying toxins, in the large intestine (Wagner 1979; Wallach and Boever 1983). Other reports suggest a role of gram-positive *Clostridium difficile* in this syndrome (Percy and Barthold 2001). Penicillin "toxicity" is a well-recognized fatal disease in guinea pigs. Other antibiotics, including bacitracin, chlortetracycline, oxytetracycline, streptomycin, aureomycin, lincomycin, methicillin, erythromycin, and chloromycetin, also have been reported to be toxic to guinea pigs. Penicillin was not toxic for germ-free guinea pigs. Profuse diarrhea with high mortality can develop in up to 50% or more of the guinea pigs within 1 to 5 days following the antibiotic treatment. Enterotoxins of *Clostridium difficile* have been demonstrated after treatment with penicillin (Percy and Barthold 2001). The toxicity of intramuscular injections of chloromycetin was attributed to glycols used as vehicles (Altemeier et al. 1950; Hoar 1976c).

Hartley guinea pigs infected with *Entamoeba histolytica* provide an animal model for enteric amebiasis, with the same type flora found in the original human host. The infection was produced by introducing *E. histolytica* trophozoites and the accompanying enteric flora obtained from humans into germ-free guinea pigs (Jervis and Takeuchi 1979).

Inflammatory bowel disease (ulcerative colitis and Crohn's disease) can be induced by feeding degraded carrageenan (Anver and Cohen 1976), or by immunization with an initial sensitization by dinitrochlorobenzene followed with multiple intrarectal instillations of this same chemical (Rabin 1980). Grossly, multiple pinpoint ulcers and hemorrhages were present in the cecal and colonic mucosae. Cecal and colonic lymphoid tissues were enlarged. Microscopically, the colonic and cecal mucosae have multiple crypt abscesses, superficial mucosal ulcerations, depletion of mucus in cells lining the crypts, edema infiltration of the lamina propria by a variety of inflammatory cells, mucosal distortion craterous ulcers, and mucosal and submucosal granulation tissue resembling features of the human ulcerative disease (Anver and Cohen 1976; Rabin 1980). Acute duodenal ulceration can result after fasting (Jervis et al. 1973). The enterotoxicity of Autumn Crocus was compared with colchicine in guinea pigs and mice. The guinea pigs developed diarrhea as seen in cattle and horses. The mice did not (Yamada et al. 2000).

Neoplastic Lesions: Spontaneous. Spontaneous tumors of the intestine are apparently infrequent. Two tumors have been reported, a fibroid and a liposarcoma of the intestine (Manning 1976; Rogers and Blumenthal 1960).

Abdominal Cavity

Nonneoplastic Lesions: Spontaneous. Abscesses in the visceral organs and peritonitis have been observed with staphylococcosis, *Pseudomonas spp.* (green exudate), and *Corynebacterium spp.* infections including *C. pyogenes, C. krutscheri,* and *C. pseudotuberculosis* (grayish and caseous exudate). Ultrasonography provides a new method for the diagnosis of abdominal diseases including abscesses (Beregi et al. 1999; Beregi, Molnar, et al. 2000; Beregi, Zorn, et al. 2000).

Neoplastic Lesions: Spontaneous. Two primary tumors have been reported in the peritoneal cavity. One tumor was a fibrosarcoma (Manning 1976) and the other was a mesothelioma involving multiple abdominal locations (Wilson and Brigman 1982).

Pancreas

Nonneoplastic Lesions: Spontaneous. The portion of exocrine pancreas decreases, whereas the portion of fat increases with age. There is no apparent impairment of function. Histologically, large areas of adipose tissue separate normal appearing pancreatic acini and ducts (Hanes 2003; Wagner 1976).

Neoplastic Lesions: Spontaneous. Adenomas or nodules of acinar cells are frequently observed in older animals (Squire et al. 1978).

Liver and Gallbladder

Nonneoplastic Lesions: Spontaneous. Multifocal hepatic necrosis and enteritis occur together in a variety of conditions. These include salmonellosis, Tyzzer's disease (*Clostridium piliforme*, formerly *Bacillus piliformis*), pseudotuberculosis (*Yersinia pseudotuberculosis* and *Y. enterocolitica*), listeriosis (*Listeria monocytogenes*), and toxoplasmosis (*Toxoplasma gondii*; Boot and Walvoort 1984; Sparrow and Naylor 1978; Zwicker et al. 1978). Hepatic multifocal necrosis is often accompanied by similar lesions in the spleen. Also, hepatic necrosis has been observed after infections by cytomegalovirus and possibly other herpeslike viruses (Percy and Barthold 2001). Multifocal coagulation necrosis in livers is occasionally seen at necropsy. Affected areas are usually subcapsular with minimal or no inflammation, suggesting that they arise as terminal events and secondary to localized impaired blood flow (Percy and Barthold 2001). Similar focal lesions were reported in clinically normal guinea pigs (Cuba-caparo et al. 1977) and have been produced by repeated administration of halothane (Hughes and Lang 1972). A marked fatty liver often is associated with ketosis.

Hemorrhage into the peritoneal cavity from tears of the liver capsule is occasionally observed at necropsy. These traumatic lesions can be caused by falls or mishandling (Percy and Barthold 2001).

Periportal fibrosis associated with chronic hepatopathy occurs occasionally in adult guinea pigs as an enzootic problem. The lesions are usually concentrated around portal triads and characterized by hepatocyte degeneration, proliferation of cholangioles, and interstitial fibrosis. These findings are suggestive of a toxin-induced change (Percy and Barthold 2001).

Nonneoplastic Lesions: Induced. Liver lesions have been produced by ingestion of pyrrolizidine alkaloids in *Crotalaris spectabilis* seeds. Clinical signs include ascites, distended abdomen, hepatic necrosis, thrombosis of portal veins, and focal hepatic fibrosis (Carlton 1967; Chesney and Allen 1973).

Neoplastic Lesions: Spontaneous. Spontaneous hepatobiliary neoplasms in the guinea pig are very rare. Reports have included a hepatocellular adenoma, a hepatic cavernous hemangioma, and a papilloma of the gallbladder (Manning 1976).

Adenocarcinomas of the gallbladder developed in 17 of 68 guinea pigs of strains 2 and 13 over 20 months of age, but none developed in nine noninbred guinea pigs. Significantly more females than males developed tumors (Hoch-Ligeti et al. 1979).

Nonneoplastic Lesions: Induced. The biliary system of guinea pigs is exquisitely susceptible to hyperplastic or neoplastic stimuli. Benign and malignant neoplasms of the biliary tract were readily induced in guinea pigs by placement of foreign bodies (choleliths, pebbles, string sutures) in the gallbladder and by administration of chemical irritants (Pityrol, lanolin, pitch pellets; Manning 1976). Whole body exposure to γ- or x-ray irradiation increased both the number of adenocarcinomas and metastases in male inbred guinea pigs, but not in females (Hoch-Ligeti et al. 1979).

Hepatocellular carcinomas were produced by oral administration of diethylnitrosamine in 14 of 15 guinea pigs (Argus and Hoch-Ligeti 1963). Hepatocellular tumors were also induced by N-nitroso-N-methylurea (Yoshida et al. 1977).

Urinary System

Kidney

Nonneoplastic Lesions: Spontaneous. Chronic interstitial nephritis, also termed segmental nephrosclerosis, is a commonly observed lesion of aged guinea pigs and ascribed to be the cause

of wasting disease in old pet guinea pigs (Wagner 1976). In this disease, the animal seems to waste away. It drinks a lot of water, loses weight, hunches up, loses condition, and gradually becomes weaker until death. Its mouth often has a strong odor (Ruf 1984). The lesions of nephrosclerosis were accelerated in guinea pigs fed an unusually high-protein diet. Clinical findings in animals with advanced kidney lesions include high blood urea nitrogen (BUN) and serum creatinine values, nonregenerative anemia, and low urinary-specific gravity (Percy and Barthold 2001). Gross findings are characterized by irregularly pitted renal cortices. Possible causes include autoimmune diseases, infectious agents, and vascular diseases (Hanes 2003). Nephrosclerosis with increased blood pressure, resembling that in hypertensive humans, has been described in Abyssinian and Hartley guinea pigs (Takeda and Grollman 1970).

Kidneys heavily infected by the coccidium, *Klossiella cobayae,* might have an irregular surface with gray mottling, but in most cases, gross lesions are inapparent. Finding sporocysts in the kidney tubules is diagnostic. Subacute nephritis can accompany the protozoan infection (Vetterling 1976). Pyelonephritis has been associated with *Corynebacterium spp.* infections, including *C. pyogenes, C. krutscheri,* and *C. pseudotuberculosis.*

Osseous metaplasia is occasionally observed in the kidneys (Hanes 2003).

Nonneoplastic Lesions: Induced. Studies with lead acetate have shown kidney lesions with aminoaciduria that are typical of lead poisoning (Bielecka 1972). Autoimmune tubulointerstitial nephritis can be induced by rabbit tubular basement membrane in both strain 2 and strain 13, but the nephropathy develops more quickly in strain 13 (Milgrom et al. 1979). NIH Hartley strain guinea pigs are also susceptible to experimental autoimmune tubulointerstitial nephritis (Hyman et al. 1976; Steblay 1979).

Neoplastic Lesions: Spontaneous. Only four renal tumors have been reported; two adenocarcinomas (Mosinger 1961a), a round cell sarcoma (lymphosarcoma?; Ball and Pagnon 1935), and an osteogenic sarcoma (Twort and Twort 1932).

Urinary Bladder and Urethra

Nonneoplastic Lesions: Spontaneous. The most common urinary problem in guinea pigs is cystitis, evidenced by pus and blood passed when the animal's bladder is pressed (Roach 1983). Older females are particularly affected probably because of the proximity of the urethral orifice to the anus and the likelihood of infection from fecal bacteria such as *Escherichia coli* (Percy and Barthold 2001). Cystitis in males is sometimes secondary to occlusion of the penile urethra by coagulum from the vesicular glands (Wagner 1979). Obstruction of the urethra with proteinaceous concretions, probably of seminal vesicular origin, is a contributing cause of death in aged males (Wagner 1976). Urinary calculi occur primarily in females and vary from sandlike to large concentric bladder stones. Age, sex, and immunosuppression are related to development of urinary calculi (Peng et al. 1990; Hanes 2003).

Neoplastic Lesions: Spontaneous. Prior to 1976, five urinary bladder tumors, four of which were malignant, had been reported. These included two transitional cell carcinomas, an epidermoid papilloma, an epidermoid carcinoma, and an anaplastic tumor (Heston and Deringer 1952; Manning 1976). In 1980, an additional seven tumors were described in untreated control animals; two were hemangiopericytomas and the others were not specifically identified (Hoch-Ligeti et al. 1980).

Neoplastic Lesions: Induced. Evans (1968) reported an unspecified number of guinea pigs fed bracken fern developed transitional cell, glandular, and squamous cell tumors. A single animal developed an unspecified urinary bladder tumor after 26 guinea pigs received a single injection of 7, 12-dimethylbenz (a) anthracene (Toth 1970). Hemangiopericytomas, transitional cell carcinomas, squamous cell carcinomas, and adenosquamous carcinomas developed in untreated controls and

guinea pigs exposed to γ- and x-ray irradiation. Irradiation was associated with significantly earlier tumors, but the increase in numbers of tumor-bearing animals was not statistically significant (Hoch-Ligeti et al. 1980).

Reproductive System and Fetal Tissues

General Considerations

Infertility and fetal or newborn deaths can result from many causes including age, stress, the type of flooring, estrogen in the feed, bedding adherent to genitals, increased room temperature, nutritional deficiencies, metritis, preputial dermatitis, segmental aplasia of the uterus, or cystic ovaries. Perinatal deaths, abortion, and stillborn young are usually associated with dystocia, bacterial infections such as Bordetella, Salmonella, or Streptococcus, cytomegalovirus infections, birth asphyxiation, and pregnancy toxemia (Hanes 2003).

Ovary

Nonneoplastic Lesions: Spontaneous. Ovarian and paraovarian cysts are very common in older breeding females (Quattropani 1977, 1978, 1981; Shi et al. 2001). Cystic rete ovarii and serous cysts were found at necropsy in 54 of 71 (76%) female guinea pigs between 18 and 60 months of age. Fertility was markedly reduced in affected females over the age of 15 months. Cystic endometrial hyperplasia, mucometra, endometritis, inappropriate placental tissue, fibroleiomyomas, and alopecia frequently accompanied the cystic ovaries (Keller et al. 1987; Percy and Barthold 2001). Ultrasonography provides a new method for the diagnosis of ovarian cysts and abdominal abscesses (Beregi et al. 1999; Beregi, Molnar, et al. 2000; Beregi, Zorn, et al. 2000; Burns et al. 2001).

Nonneoplastic embryonal malformations within the ovary, also termed embryonic placentomas, were described by Loeb (1932). These structures could be misinterpreted as neoplasms. They represent parthenogenetic development of ova within the ovary, resulting in the formation of placental and embryonal structures, and are thought to occur frequently in guinea pigs. They were seen in females less than 6 months of age and most were in guinea pigs less than 4 months of age (Loeb 1932). These malformations can become fibrotic in mature ovaries (Hanes 2003). Estrogens appear to enhance their development (Mosinger 1961b).

Neoplastic Lesions: Spontaneous. True tumors of the ovary are rare and most (80%) of those reported are teratomas (Frisk et al. 1978; Willis 1962). Nineteen of these tumors had been reported as of 1970 (Vink 1970). The low incidence is indicated by the finding of only 3 in 4,200 necropsied animals of the R9 strain (Blumenthal and Rogers 1965) and 10 in 13,000 random-bred animals (Vink 1970). A malignant ovarian teratoma was described by Gupta and Sarmah (1985) and a stromal sarcoma by Olson and Anver (1980). Five cystadenomas and a granulosa cell tumor have been reported (Jain et al. 1970; Manning 1976; Squire et al. 1978). Another granulosa cell tumor was reported by Burns et al. (2001) and a cystadenocarcinoma by Hong (1980). Tumors of the ovaries should not be confused with cystic rete tubules seen commonly in old guinea pigs (Percy and Barthold 2001).

Oviduct, Uterus, and Vagina

The adult female has an intact, epithelial vaginal closure membrane except for the few days of estrus and at parturition. Both these events are signaled by the perforation of this membrane.

Trophoblastic giant cells derived from the fetal placenta can migrate into the myometrium and adjacent blood vessels (Hanes 2003).

Nonneoplastic Lesions: Spontaneous. Guinea pigs appear to be predisposed to high perinatal mortality (Eveleigh et al. 1987). These deaths are often associated with dystocia and subclinical ketosis (pregnancy toxemia).

The circulatory form of pregnancy toxemia in guinea pigs is characterized by uteroplacental ischemia due to compression of the aorta caudal to the renal blood vessels by a large gravid uterus containing a near-term fetus. This results in reduced blood pressure in the uterine vessels and tissue anoxia, with subsequent placental necrosis, uterine hemorrhage, thrombocytopenia, ketosis, and death. The tissue anoxia and necrosis leads to the toxemia. Pregnancy toxemia is manifested by depression, acidosis, ketosis, proteinuria, ketonuria, and a lowered urinary pH from 9 to 5 to 6 (Percy and Barthold 2001).

Dystocias are common problems in guinea pigs and usually occur either in young females because of the relatively large fetus or in older females because of delayed breeding (Wallach and Boever 1983). If the first breeding of female guinea pigs is delayed past 7 or 8 months, the pubic symphysis separates with increased difficulty at parturition and fat pads occlude the pelvic canal, which lead to dystocia and death (Harkness and Wagner 1989). Also, dystocias appear to be common sequelae of subclinical ketosis (pregnancy toxemia). Vaginal prolapse is occasionally associated with parturition (Wagner 1976).

Metritis, or pyometra, and pyosalpinx can be caused by a variety of organisms including *Staphylococcus spp., Streptococcus spp., Corynebacterium pyogenes, C. krutscheri, C. pseudotuberculosis,* and *Escherichia coli.* The clinical signs are a mucopurulent to sanguinopurulent discharge from the vulva and a distended abdomen (Wallach and Boever 1983).

The first deciduoma in a laboratory animal was reported in the guinea pig (Loeb 1908). These lesions consist of proliferating decidual tissue of the uterus, and were originally regarded as tumors.

Simple cysts are common in the female reproductive tract of guinea pigs. These probably represent remnants of the Wolffian duct system (Squire et al. 1978). Cyst endometrial hyperplasia, mucometra, endometritis, inappropriate placental tissue, and fibroleiomyomas were seen in 21 of 54 (39%) guinea pigs with cystic ovaries, but in only 1 of 17 (6%) guinea pigs without cystic ovaries (Keller et al. 1987). Cystic endometrial hyperplasia has been described by Ong (1987).

Nonneoplastic Lesions: Induced. Clinical and pathological features of the acute and recurrent genital herpes disease, which are similar to those seen in human genital disease, can be produced in females by inoculation with low dosages of herpes simplex virus type 2. This includes complete healing of genital lesions, histological changes in the genital epithelium and nerve tissues, latent viral infection in the dorsal root ganglia, and transmission of the virus from mother to newborn through an infected birth canal (Hsiung et al. 1984). The cervical dysplasia seen in guinea pigs is similar to that seen in humans, but none of the guinea pigs has been kept long enough for cancer to develop (Lucia et al. 1983).

Neoplastic Lesions: Spontaneous. One oviduct tumor, an adenoma, has been reported (Manning 1976). Uterine tumors are usually benign and have mesenchymal origin. Although a variety of tumors have been observed in the uterus, most are fibromas or leiomyomas (Field et al. 1994). Malignant uterine tumors are rare and often consist of poorly differentiated mesenchymal cells, with extension into the peritoneal cavity (Percy and Barthold 2001). Seven tumors were described in the uterus of R9 strain animals by Rogers and Blumenthal (1960). These included two leiomyomas, an adenomyoma, a leiomyosarcoma, a fibrosacoma, a myxosarcoma, and a mixed mesenchymal tumor. Other tumors reported include a sarcoma, a fibromyoma, a myxofibroma, a fibroma, and two adenocarcinomas (Manning 1976).

Neoplastic Lesions: Induced. Prolonged estrogen administration has induced benign tumors in the uterus of guinea pigs, including fibromas, fibromyomas, and leiomyomas (Manning 1976).

Prepuce

Nonneoplastic Lesions: Spontaneous. Wood shavings or sawdust used for bedding can become lodged in the prepuce or around the anus in the male guinea pig and form a hard mass that prevents erection (Harkness and Wagner 1989). Male guinea pigs occasionally develop infections due to these foreign materials in the preputial folds (Lee et al. 1978). Long-standing infections can cause adhesions and infertility (Wagner 1976).

Vesicular Glands

Nonneoplastic Lesions: Spontaneous. The vesicular glands of the adult male are bilateral, smooth, and transparent and extend approximately 10 cm into the abdominal cavity from their origin in the accessory sex glands. They can be confused with the uterus by an unexperienced observer (Harkness and Wagner 1989). Their large size and the normal presence of open inguinal canals contributes to their herniation into the scrotum.

Testes

Nonneoplastic Lesions: Induced. Strain 13 guinea pigs are susceptible to the development of experimental autoimmune orchitis by immunization with isogenic and allogenic spermatozoa and with sperm autoantigens (Carlo et al. 1976; Toullet and Voisin 1979). Isoimmunization with testicular material in FCA resulted in a reduction in subsequent chronic orchitis (Parsonson et al. 1971). Testicular atrophy has been reported to result from analgesic drugs (Boyd 1970).

Neoplastic Lesions: Spontaneous. Testicular tumors of any type are extremely rare in the guinea pig (Squire et al. 1978). There has been a single report of an embryonal carcinoma of the testis (Blumenthal and Rogers 1965).

Musculoskeletal System

Bone and Joints

General Considerations. When guinea pigs are reluctant to move, malnutrition, scurvy, vitamin E deficiency, osteoarthritis, spinal trauma, fractures, myopathy, muscular dystrophy, or bacterial infections such as Bordetella and Salmonella might be the cause (Hanes 2003).

Nonneoplastic Lesions: Spontaneous. Broken limbs often occur when guinea pigs not raised in wire-bottomed cages are placed in such cages (Harkness and Wagner 1989), or are incorrectly handled (Roach 1983). Vertebral subluxation has been reported (Hammons 1979). Bone lesions of scurvy (vitamin C deficiency) include hemorrhage of the epiphyseal plate accompanied by subcutaneous and periarticular hemorrhages (Gleiser 1974). Osteodystrophy fibrosa was reported in two guinea pigs (Schwartz et al. 2001). Osteoarthritis in guinea pigs was described by Silverstein and Sokoloff (1958). Spontaneous cartilage degeneration, osteoarthrosis, or osteoarthritis of the femorotibial joint resembles that of osteoarthritis in humans and provides a useful model for this disease (Bendele and Hulman 1988; Bendele et al. 1999; Bendele et al. 1989; Gupta et al. 1972). Septic arthritis has been associated with *Streptococcus spp., Corynebacterium spp.,* including *C. pyogenes, C. krutscheri,* and *C. pseudotuberculosis,* and *Streptobacillus moniliformis.*

Metastatic calcification is often seen as an incidental finding especially in the hind legs (Hanes 2003; Sparschu and Christie 1968). Characterised by calcium deposits in joints, muscles, and various soft tissues including those of internal organs, such as the heart, aorta, lungs, kidneys, stomach, and colon, it occurs most often in adult males over the age of 1 year. The guinea pig loses weight, has stiff joints, and usually dies. The causes are considered imbalances of magnesium, potassium, calcium, and phosphorus, aggravated by too much vitamin D (Ruf 1984). It is believed

that hyperphosphatemia results from the inability of the guinea pig to conserve fixed bases by excreting ammonia in the urine; thus, the low-base reserve impairs normal urinary excretion of phosphorus (Fraser et al. 1986).

Nonneoplastic Lesions: Induced. Progressive chronic osteoarthritis has been induced experimentally in the femorotibial joints of guinea pigs by partial medial meniscectomy (Bendele 1987; Bendele et al. 1999; Bendele and White 1987).

Neoplastic Lesions: Spontaneous. Spontaneous tumors of bones are also rare. Manning (1976) cited five tumors that included an enchondroma, an osteogenic sarcoma (osteosarcoma), a chondrosarcoma, an osteochondrosarcoma, and a neurogenic fibrosarcoma. An osteosarcoma was described by Jolivet (1988) and two osteogenic sarcomas were described by Hong and Lui (1981). Multiple extraskeletal osteogenic sarcomas were reported in a 2-year-old female (Cook et al. 1982). A chondrosarcoma with metastasis was reported by Olcott and Papanicolaou (1943).

Neoplastic Lesions: Induced. A few osteosarcomas and chondrosarcomas were produced at injection sites by methylcholanthrene in the subcutaneous and muscular tissues (Blumenthal and Rogers 1965).

Skeletal Muscle

Nonneoplastic Lesions: Spontaneous. Muscular dystrophy is characterized by lameness, stiffness, and refusal to move due to vitamin E deficiency (Ruf 1984). In a colony of 150 animals, 54 were affected with a myopathy resembling nutritional muscular dystrophy. Fifty percent of those affected died. Major clinical signs were depression, conjunctivitis, and reluctance to move. Lesions were widespread throughout skeletal and cardiac musculature. There was pallor or pale longitudinal streaks in otherwise normal muscle. Microscopically, the lesions were characterized by widespread degeneration and coagulation necrosis with myositis and regeneration in various muscle groups (Ward et al. 1977; Webb 1970).

Many cases of myopathy have been observed and reported in guinea pigs in a number of different experimental regimens that have no apparent common etiology. The large muscles of the rear legs are most involved. The lesions are characterized by general muscle degeneration having a varied degree of swelling, loss of cross striations, fragmentation of fibers, and vacuolation (Wagner 1976).

Myositis was characterized clinically by swelling and pain in the large muscle groups of the hind legs, followed by front leg involvement, prostration, and death in newly purchased animals. Gross ecchymotic hemorrhages and edema were observed in affected muscles. Microscopically, hemorrhage and edema of the skeletal muscles were the prominent changes with infiltration by acute- to subacute-type inflammatory cells. A virus was suspected but could not be isolated (Saunders 1958). Guinea pig poxlike virus was isolated from young guinea pigs that spontaneously developed a fibrovascular proliferation in the thigh muscles (Van Hoosier and Robinette 1976).

Spontaneous arthrogryposis has been reported in a guinea pig. The disease was tetramelic and apparently due to the reduced size of skeletal muscles and their individual fibers. There was an associated myelodysplasia characterized by focal duplication of the spinal canal and no central canal in other areas of the cord (Doige and Olfert 1974).

Neoplastic Lesions: Spontaneous. Primary tumors of the skeletal muscle are very rare. Kroning and Wepler (1938) reported a lipomyxofibroma invading the psoas muscle in a 3-year-old male guinea pig.

Nervous System and Special Sense Organs

Brain, Spinal Cord, and Nerves

Nonneoplastic Lesions: Spontaneous. Lymphocytic choriomeningitis is a naturally occurring arenaviral disease of the nervous system. The clinical signs include ruffled fur, huddled posture, palpebral edema, conjunctivitis, tremors, convulsions, and hind leg paralysis. Gross findings can be minimal or consist of interstitial pneumonia, pulmonary edema, pleural exudate, fatty liver, and enlarged spleen. Microscopically, marked lymphocytic infiltrations are observed in the meninges, especially at the base of the brain and in the choroid plexus, as well as the liver, adrenals, kidneys, and lungs (Van Hoosier and Robinette 1976).

A poliovirus-like disease, called guinea pig lameness, was associated with a flaccid paralysis and loss of weight. Microscopic changes consisted of a meningomyeloencephalitis affecting the lumbar spinal cord and medulla oblongata. A median and ulnar neuropathy has been reported (Fullerton and Gilliatt 1967).

Alpha-mannosidosis is a lysosomal storage disorder resulting from deficient activity of lysosomal alpha-mannosidase. The disease is characterized by neuronal storage leading to progressive mental deterioration. The affected guinea pigs closely resemble the human disease and will provide a convenient model for investigation of new therapeutic strategies for neuronal storage diseases, such as enzyme replacement and gene replacement therapies (Crawley et al. 1999).

Cerebral larval migrans with multifocal malacia and eosinophilic granulomatous inflammation was observed in a colony due to *Baylisascaris procyonis* eggs in wood shavings bedding contaminated by raccoon feces. The affected guinea pigs manifested cachexia, stupor, hyperexcitability, lateral recumbency, and opisthotonus (Van Andel et al. 1995).

Spontaneous encephalomyelitis caused by *Encephalitozoon (Nosema) cuniculi,* a microsporidian organism (Moffatt and Schiefer 1973) and encephalitis caused by *Toxoplasma gondii,* the tissue form of the feline coccidia, have been observed in guinea pigs (Vetterling 1976). Meningoencephalitis is sometimes associated with *Streptococcus (Diplococcus) pneumoniae.*

Nonneoplastic Lesions: Induced. NIH Hartley strain guinea pigs were susceptible to experimental allergic encephalomyelitis (Lisak et al. 1975). Chronic demyelinating optic neuritis can be produced in juvenile strain 13 guinea pigs by intradermal injections of the spinal cord emulsified with FCA. After several months, the optic neuritis is followed by encephalomyelitis. Injections of adult strain 13 animals resulted in acute optic neuritis and allergic encephalomyelitis with death within 4 to 6 weeks (Rao et al. 1979).

Segmental demyelination and axonal degeneration of the peripheral and cranial nerves in guinea pigs have been produced by lead acetate (Fullerton 1966; Gozdzik-Zolnierkiewicz and Moszynski 1969). Asphyxia neonatorum in fetal animals resulted in signs of neural damage and a variety of histopathological changes (Windle and Becker 1943). Guinea pigs have proven valuable experimental animals for the study of viruses capable of causing encephalitis such a Hendra virus which affects humans and horses (Williamson et al. 2001).

Neoplastic Lesions: Spontaneous. Tumors of the nervous system are apparently very rare (Squire et al. 1978). Only one tumor was reported in the central nervous system, a teratoma of the pons (Lutz 1910). Interestingly, no gliomas, which are the most frequent tumors in most other species, were observed (Squire et al. 1978). Three tumors of the nerve sheaths were described. All three appeared to be neurilemmomas, although one of the inner abdominal wall had a diagnosis of glioma. The other two occurred in the mesentery (Manning 1976).

Ear

Nonneoplastic Lesions: Spontaneous. Suppurative ear infections (otitis media) are seen occasionally with torticollis, incoordination, and circling behavior if the infection extends to the inner ear. A number of bacterial agents can be involved. Among the most common are those that are also capable of causing respiratory disease such as *Streptococcus zooepidemicus, S. pneumoniae,* and *Bordetella bronchiseptica* (Boot and Walvoort 1986; Roach 1983; Wagner 1979; Wagner et al. 1976).

The waltzer strain of guinea pig is characterized by a tendency to "waltz" or whirl and loss of hearing. Rotation does not elicit a nystagmus response and tilting does not evoke counter-rolling of the eyes. There is a loss of the Preyer reflex by 4 to 6 weeks. They have an impaired swimming ability and righting reflexes. The hearing loss, which affects the high-frequency range first, is complete by 42 days. Vestibular capacity is decreased at birth and progresses to complete loss of function. The hair cells of the organ of Corti degenerate, followed by supporting cell degeneration and depopulation of neurons in the spiral ganglion (Ernston 1970, 1971a, 1971b, 1972a, 1972b, 1972c).

Nonneoplastic Lesions: Induced. The guinea pig ear and associated structures provide an important animal model for ototoxicity and acoustic trauma studies. The petrous bone (otic capsule) is easily entered and dissected away. The guinea pig has been used to elucidate the pathophysiology of drugs that cause deafness and vestibular disorders, including the aminoglycosidic antibiotics such as neomycin, gentamicin, and other members of the streptomycin family; the diuretic ethacrynic acid; quinine; and salicylates (McCormick and Nuttall 1976). A variety of protocols continue to be used to study mechanisms of ototoxicity especially by compounds such as the anticancer drug cisplatin and other potential therapeutics (Ekborn et al. 2003; Guneri et al. 2001; Sergi et al. 2003).

Eye

Nonneoplastic Lesions: Spontaneous. The most common eye disorders in guinea pigs are conjunctivitis, corneal ulcers, and keratitis (Cullen et al. 2000; Roach 1983). A wide variety of microorganisms have been isolated from eye lesions, including coliforms, *Streptococcus zooepidemicus,* hemolytic *Streptococcus spp., Micrococcus spp., Staphylococcus aureus, Pasteurella multocida, P. hemolytica, Salmonella typhimurium, S. enteritidis, S. dublin, Mycoplasma pulmonis,* and *Proteus spp.* (Wagner 1976). Guinea pig inclusion conjunctivitis is caused by *Chlamydophila caviae,* formerly termed *Chlamydia psittaci* (Eidson 2002) and usually is a mild, self-limiting ocular disease in animals 4 to 8 weeks old (Deeb et al. 1989).

Congestion of the lower conjunctival sac following irritation results in a protrusion termed red or pea eye. Protrusion of the lower conjunctival sac also can be observed in some guinea pigs as a result of excess retrobulbar fat (Richardson 2000). Anophthalmos has been reported as an inherited trait in some guinea pigs (Komich 1971).

Cataracts are occasionally seen in young and adult animals as incidental findings, irrespective of the diet (Wagner 1976). An autosomal dominant trait possibly associated with diabetes or L-tryptophan deficiency has been identified (Hanes 2003). Senile cataracts develop in old pet guinea pigs at ages 6 to 8 years (Ruf 1984). Opacities of the cornea can result from drying of the eye during long-term anesthesia (Hanes 2003).

Bilateral osseous choristomas of the ciliary body were observed in 3 guinea pigs of a research colony of about 200 animals. One of these animals had also bilateral cataracts (Griffith et al. 1988).

Osseous metaplasia of the eye has been observed (Hanes 2003). Formation of heterotropic bone was reported in the ciliary body of an aged guinea pig (Brooks et al. 1990).

Nonneoplastic Lesions: Induced. Optic disk swelling, a protrusion of the optic nerve head into the vitreous with varying degrees of vascular and peripapillary retinal changes, can be produced in guinea pigs with β, β-iminodipropionitrile (IDPN; Parhad et al. 1982). Optic disk swelling is seen also in association with optic neuritis and papilledema induced by the intradermal injection of the spinal cord emulsified with Freund's adjuvant (Rao et al. 1979). NIH Hartley strain guinea pigs were susceptible to experimental autoimmune uveitis (McMaster et al. 1976).

The guinea pig model of allergic conjunctivitis is used to study the effectiveness of therapeutic agents (Schoch 2003).

Neoplastic Lesions: Spontaneous. A lymphosarcoma of the choroid and two intraocular reticulosarcomas were reported in guinea pigs by Congdon and Lorenz (1954). Widespread systemic involvement in malignant lymphoma can accompany or precede recognizable intraocular lesions. Bilateral involvement of the eyes by a disseminated T-cell lymphoma was reported by Steinberg (2000). A corneal dermoid was observed in a hairless guinea pig (Otto et al. 1991). Scleral dermoids have been observed in four animals (Gupta 1972).

Endocrine System

Pancreas

Nonneoplastic Lesions: Spontaneous. Acute spontaneous diabetes mellitus was characterized by polydipsia, polyuria, hyperglycemia, glycosuria, elevated glucose tolerance tests, and elevated plasma triglycerides in Abyssinian guinea pigs. It resembled human juvenile diabetes mellitus and spread from animal to animal possibly caused by an unidentified virus (Lang and Munger 1976; Lang et al. 1977; Munger and Lang 1973). Diabetes represents a possible cause of infertility (Percy and Barthold 2001).

Fatty deposits in the pancreas increase markedly with age. This increase is associated with a proportional increase in islet tissue and a decrease in exocrine pancreatic tissue (Wagner 1976).

Neoplastic Lesions: Spontaneous. Very few endocrine tumors have been reported. The most frequent were islet cell adenomas. These benign pancreatic islet cell tumors were found in 0.7% of animals of the N: Hart NIH Hartley strain guinea pigs and in 3.5% of strain 13 animals that survived longer than 2 years (Yoshida et al. 1979).

Neoplastic Lesions: Induced. Pancreatic adenocarcinomas have been induced by N-methyl-N-nitrosourea (Reddy and Rao 1975; Reddy et al. 1974).

Adrenal

Guinea pigs have normally large prominent adrenal glands, which must be considered when evaluating this organ at necropsy.

Nonneoplastic Lesions: Spontaneous. Occasional cortical adenomas or hyperplastic nodules are seen in older guinea pigs (Squire et al. 1978). A number of these lesions were reported in male guinea pigs some years after castration. Manning (1976) considered these lesions most likely to be castration-induced hyperplastic nodules. Enlarged adrenal glands with hemorrhage and cortical necrosis can result from ketosis.

Neoplastic Lesions: Spontaneous. Occasional cortical adenomas or hyperplastic nodules are seen in older guinea pigs (Squire et al. 1978). A number of these lesions were reported in male guinea pigs some years after castration. Manning (1976) considered these lesions to most likely to be castration-induced hyperplastic nodules. Other reports included three cortical adenomas, a cortical carcinoma, and an unclassified tumor (Manning 1976).

Thyroid and Parathyroid

Nonneoplastic Lesions: Induced. NIH Hartley strain guinea pigs (McMaster and Lerner 1967; McMaster et al. 1967) and strain 2 (Braley-Mullen et al. 1975) guinea pigs are highly susceptible to experimental autoimmune thyroiditis.

Neoplastic Lesions: Spontaneous. Spontaneous thyroid tumors are also rare. One papillary adenoma was observed in a Hartley strain animal (LaRegina and Wightman 1979). One carcinoma, possibly of parafollicular origin, was described by Zarrin (1974). No parathyroid tumors were reported (Manning 1976).

Pituitary

Neoplastic Lesions: Spontaneous. Spontaneous pituitary tumors are very rare, if they occur. None have been reported (Manning 1976; Squire et al. 1978).

Integumentary System and Soft Tissues

Skin

Nonneoplastic Lesions: Spontaneous. Guinea pigs born in wire-bottom cages learn to walk without mishap, but naive animals placed in such cages often fall through the mesh and break or lacerate their limbs. The latter animals might also exhibit hair loss, footpad ulcers, decreased reproduction, and effects from stress (Harkness and Wagner 1989).

The most common foot problem is peeling of the skin and thickening of the hock with or without ulceration. It seems to be associated with unsuitable bedding and the presence of moisture. Hard, damp straw will cause the problem (Roach 1983). Marked hyperkeratosis of the footpads is occasionally observed. In some cases, the horny growths approached 2 cm in length. All cases were aged animals confined to laboratory cages (Wagner 1976).

Abscesses are most commonly found around the jaw, neck, and feet. Abscesses near the throat are of two types. One type is the result of streptococcal infections, and usually involves the cervical lymph nodes. The other type results from inflammation around food materials caught in little pockets or indentations in the cheeks (Ruf 1984). Abscesses of the skin and feet are often associated with *Staphylococcus aureus* infections (Taylor et al. 1971). A chronic ulcerative pododermatitis can result in which the foot lesions persist indefinitely, periodically ulcerating and bleeding and producing abscesses in the draining lymph nodes, osteoarthritis, and generalized amyloidosis (Gleiser 1974). Wounds and abscesses on the back could be the results of fighting between boars with a group of overcrowding (Roach 1983).

Dermatitis occurs as result of a variety of conditions in guinea pigs, and can be caused by vitamin C deficiency, bacterial infections, external parasites, or ringworm. External parasites include fleas, ticks, lice (*Gliricola porcelli, Gyropus ovalis,* or *Tremenopon hispidium [jenningsi]*), and mites (*Trixacarus caviae, Chirodiscoides caviae,* or *Demodex caviae*; Fuentealba and Hanna 1996). Heavily louse-infested animals can have a rough, dry hair coat, and might scratch excessively. The signs of ringworm include patchy loss of hair and scaling of the hair or dark spots around the eyes and other parts of the body. Usually there is mild to severe itching, often leading to secondary

scabbing and bleeding. The most common causes of ringworm are *Trichophyton mentagrophytes* and *Microsporum gypseum* (Fraser et al. 1986; Pollock 2003; Roach 1983; Ruf 1984; Wagner 1979). A case of dermal cryptococcosis caused by *Cryptococcus neoformas* involved the nose (Van Herck et al. 1988).

Alopecia is a manifestation of several conditions. Alopecia, usually bilateral, develops to a degree in all guinea pigs in late pregnancy and is considered a normal phenomenon in aged breeding females. The condition appears to be due to reduced anabolism of maternal skin associated with fetal growth. The hair loss frequently occurs over the back and rump. Also alopecia can accompany cystic rete ovarii (Percy and Barthold 2001). Adequate zinc levels in the diet are required for hair growth (Wallach and Boever 1983). Protein deficiency and other nutritional or genetic factors might be involved (Hanes 2003). Thinning of the hair around the time of weaning is common in young guinea pigs during the period of transition as the baby fur is lost and the coarse guard hairs of adult fur appear (Wagner 1976, 1979).

Loss of hair from pulling, stripping, barbering, or hair chewing occurs in some colonies, especially among animals reared in wire cages. It is thought to be mainly due to boredom. It can be self-inflicted or mutually inflicted (Roach 1983).

Ear chewing occurs as a vice associated with excessive aggressiveness of one or more individuals in a cage or primary enclosure (Wagner 1976). Severe trauma and amputation of the pinnae can result (Percy and Barthold 2001). This practice can lead to skin infections as well as serious interference in the identification of individual animals from loss of ear notches or ear tags.

Dense populations of sebaceous glands, marking glands, are located circumanally and on the rump. Activity of these glands can result in excessive accumulation of sebaceous secretions in the folds of the circumanal and genital region of adult male guinea pigs. These folds must be cleaned periodically in older males to preclude infections and unpleasant odors.

Nonneoplastic Lesions: Induced. Guinea pigs are used as models for studies of phototoxicity, thermal burns, chemical burns, and wound healing. For example, the guinea pig provides a model for experimental hypertrophic scars (Aksoy 2002). These studies are useful in developing products for treatment of ulcers, burns, wounds, and scars (Tan 2002; Wormser et al. 2000a, 2000b). A hairless strain of guinea pig with normal thymic activity provides a useful dermal research subject. Their hair follicles produce rudimentary hairs. Their skin is nonpigmented and fragile (Hanes 2003). Athymic or hypothymic hairless guinea pigs also have been observed. Animals with the thymic mutations were agammaglobulinemic and died of infections usually associated with immunodeficiencies (Reed and O'Donoghue 1979). Injections of a mixture of droperidol and fentanyl citrate (Innovar-Vet) have resulted in self-mutilation, distal necrosis at the injection site, and amputation of the leg (Leach et al. 1973; Newton et al. 1975; Wagner 1979). Infections by T. mentagrophytes provide useful models for mycotic dermatitis (Pollock 2003; Treiber et al. 2001).

Neoplastic Lesions: Spontaneous. Prior to 1965, spontaneous skin tumors were considered rare in guinea pigs (Blumenthal and Rogers 1965, 1967); however, examinations of Hartley strain animals at Fort Detrick, Maryland have revealed 29 trichofolliculomas (Mosinger 1961a; Manning 1976). Twenty-one trichofolliculomas were noted at necropsy of 7670 guinea pigs (Ediger et al. 1971). Other skin and subcutaneous tumors reported include cutaneous papillomas, penile papillomas, trichoepitheliomas, sebaceous adenomas, an undifferentiated adenocarcinoma, an undifferentiated carcinoma, a fibroma, a fibrolipoma, lipomas, fibrosarcomas, a schwannoma, and an undifferentiated sarcoma (Manning 1976; Steele 2001; Zwart et al. 1981; Percy and Barthold 2001). A complex adnexal tumor with both sebaceous and apocrine differentiation has been reported (Allison and Moeller 1993).

Neoplastic Lesions: Induced. Epidermal neoplasms have proven very difficult to induce by topical application. The few that have been reported required a minimum of 2 years of application and large doses of carcinogen (Stenbeck 1970).

Mammary Gland

Nonneoplastic Lesions: Spontaneous. Bacterial mastitis is a fairly common disease of guinea pigs, particularly in sows during early lactation. It is characterized by red to purple enlarged, firm, congested, edematous mammary glands and caused by infections from a variety of organisms, including *Staphylococcus spp., Streptococcus spp.,* and *Corynebacterium spp.* (Kinkler et al. 1976; Wagner 1979).

Neoplastic Lesions: Spontaneous. Tumors of the mammary gland are relatively common and occur in both males and females (Hoch-Ligeti, Liebelt et al. 1986; Percy and Barthold 2001). Blumenthal and Rogers (1965) reported 12 of the 140 tumors reported in guinea pigs were mammary gland lesions. Tumors that have been described are adenomas, a cystadenoma, fibroadenomas, an adenofibroma, fibrocytadenomas, papillary adenomas, papillary cystadenomas, adenocarcinomas, a liposarcoma, a carcinosarcoma, and a malignant mixed tumor. The most common mammary tumors are adenocarcinomas, several of which have been reported in males. Nearly all the carcinomas were believed to be of ductal origin (Andrews 1976; Manning 1976; Squire et al. 1978). Although many are of low-grade malignancy and remain localized, metastases have been observed in the adjacent regional lymph nodes (Hoch-Ligeti, Liebelt et al. 1986; Percy and Barthold 2001).

Subcutis

Nonneoplastic Lesions: Spontaneous. Subcutaneous abscesses are common lesions in guinea pigs from a variety of injuries. The microorganisms involved vary considerably, but can include *Staphylococcus spp., Streptococcus spp., Corynebacterium spp., Sphaerophorus necrophorus,* and *Escherichia coli* (Wallach and Boever 1983).

Soft tissue calcification is a disease of nutritional origin characterized by metastatic calcification in various soft tissues, including the muscles, myocardium, joints, stomach, aorta, lungs, kidneys, uterus, sclera and, most striking, at the colonic flexure. In some animals, the soft tissue mineralization might be confined to the elbows and ribs (Percy and Barthold 2001). Microscopically, mineralization can be seen in almost any organ, most often in adult males over the age of 1 year. The condition occurs when there is an imbalance in magnesium, calcium, and phosphorus interrelationships and is corrected by providing an adequate amount of magnesium (Lane-Petter et al. 1967).

Nonneoplastic Lesions: Induced. Soft tissue calcification is a toxic effect of the Argentinian plant *Solanum malacoxylon* (Camberos et al. 1970).

Neoplastic Lesions: Induced. Subcutaneous sarcomas, including fibrosarcomas, a fibromyxoliposarcoma, and liposarcomas, were produced at the site of injection by methylcholanthrene (Shimkin and Mider 1941).

Hematopoietic and Lymphoid System

Thymus

The thymus in immature animals is located subcutaneously in the neck on either side of the trachea, where it is easily removed by surgery (Harkness and Wagner 1989). Degeneration of thymocytes adjacent to Hassall's corpuscles is a common finding, especially in young animals, unrelated to treatment (Percy and Barthold 2001).

Kurloff cells, also termed Foa-Kurloff cells, are mononuclear leukocytes containing intracytoplasmic inclusions (Kurloff bodies) of a glycoprotein composition. These cells proliferate during estrogenic stimulation and are found in highest numbers in the placenta, where they might have a

function protecting fetal antigens from sensitized maternal lymphocytes and immune globulins. These cells are most prominent during late pregnancy. They are commonly seen in histological sections of the bone marrow, thymus, spleen, and placenta and might originate from the thymus gland (Harkness and Wagner 1989; Wagner 1979). Kurloff cells could be the counterpart to natural killer cells in other species and play a role in the low frequency of neoplasia in guinea pigs (Percy and Barthold 2001). It is important to be aware of these special lymphoid cells and not mistake them for abnormalities such as lupus erythematous cells in blood samples.

Lymphoid Tissues, Lymph Nodes, and Spleen

Nonneoplastic Lesions: Spontaneous. Cervical lymphadenitis primarily caused by β-hemolytic group C *Streptococcus zooepidemicus* results in abscesses of the cervical lymph nodes and occasionally other nodes. The lymph nodes are enlarged and full of pus, from which the organisms can readily be isolated. Other organisms that have been isolated are *Yersinia (Pasteurella) pseudotuberculosis, Streptobacillus moniliformis,* and *Sphaerophorus necrophorus.*

Sick guinea pigs should be palpated for enlarged mesenteric lymph nodes, which might indicate mesenteric lymphadenitis resulting from infections caused by *Yersinia pseudotuberculosis* or phycomycosis caused by a fungus in hay. Pseudotuberculosis can spread through the blood to the liver and spleen. Young guinea pigs with swollen mesenteric lymph nodes should be held for 30 days before being discarded, as phycomycosis usually resolves within a month of infection with no ill effects (Hime and O'Donoghue 1979). Phycomycosis (mucormycosis) is caused by *Mucor spp.* and *Absidia spp.,* and results in a characteristic lymphadenitis and granulomatous inflammation of the viscera. This mycotic infection of the mesenteric lymph nodes must be differentiated microscopically from pseudotuberculosis. Other causes of lymphadenitis in various parts of the body include *Staphylococcus spp.; Pasteurella spp.; Pseudomonas spp.;* and *Corynebacterium spp.* infections, including *C. pyogenes, C. krutscheri,* and *C. pseudotuberculosis,* and toxoplasmosis.

Enlargement and focal necrosis of the spleen often accompanies bacterial diseases such as salmonellosis (*Salmonella typhimurium, S. enteritidis, S. dublin,* and *S. limete*) and pseudotuberculosis (*Yersinia pseudotuberculosis* and *Y. enterocolitica*).

Nonneoplastic Lesions: Induced. Cervical lymphadenitis in guinea pigs was induced experimentally with Lancefield's group C streptococci but not with group E streptococci (Olson et al. 1976).

Feeding dried plant material of *Swainsona galegifolia* produces vacuolation of circulating lymphocytes as well as vacuolar lesions in the kidneys, liver, and neurons of the central nervous system. This vacuolation is a form of the lysosomal storage disease alpha-mannosidosis caused by the indolizidine alkaloid swainsonine, a potent inhibitor of lysosomal α-D-mannosidase (Huxtable 1969; Huxtable and Dorling 1982; Huxtable and Gibson 1970). Spontaneous alpha-mannosidosis occurs in guinea pigs with deficient lysosomal alpha-mannosidase activity. Affected guinea pigs have neural and visceral changes that closely resemble the human disease (Crawley et al. 1999; Muntz et al. 1999).

Germ-free guinea pigs have hypoplastic lymph nodes along the GI tract. White blood cell counts are lower than in conventional animals. The differential white cell counts are also different (Hanes 2003).

Neoplastic Lesions: Spontaneous. Disseminated lymphomas and lymphocytic leukemias occur in middle-aged to old guinea pigs (Blumenthal and Rogers 1965; Ediger and Rabstein 1968; Steinberg 2000). Blumenthal and Rogers (1965) reported an incidence of 7%, including the cases found in the literature. Lymphatic leukemia had an occurrence of 3.6% in the strain 2 guinea pig and an occurrence of 6.7% in strain 13 (Congdon and Lorenz 1954). The clinical signs are anemia, anorexia, and enlargement of peripheral lymph nodes. Leukocytosis with counts of 50,000 to 180,000/mm^3 or greater with a preponderance of lymphoblastic cells is typical of leukemic blood samples (Percy and Barthold 2001). A needle aspirate can be an aid for the diagnosis of the disease

in guinea pigs (McEwan and Callanan 1993). At necropsy, grossly enlarged lymph nodes, spleen, and liver are usually present. Microscopically, lymphoblastic cells infiltrate many tissues, especially perivascularly, such as in the kidney, liver, interstitium of the lung, heart, kidney, thymus, eyes, and adrenals. The spleen, cervical and mesenteric lymph nodes, Peyer's patches, and bone marrow are all heavily infiltrated (Van Hoosier and Robinette 1976). Leukemia occurs on rare occasions as a spontaneous disease in various inbred and noninbred strains in young adult animals (Day and Briggs 1997; Debout et al. 1987; Green et al. 1973; Hong et al. 1980; Percy and Barthold 2001).

A virus has been isolated that produces acute lymphoblastic or stem cell leukemia in strain 2 or F_1 hybrid guinea pigs (Rhim and Green 1977; Kaplow and Nadel 1979). Four distinct morphological types of tumors developed in animals injected with the Snijders transplantable leukemia: lymphoblastic leukemia, aleukemic leukemia, lymphosarcoma, and "lymphosarcoma resembling that of man" (Manning 1976). Similarities of strain 2 leukemias to human leukemias include origin from bone marrow, rapidly fulminating course with lymphocyte counts of 25,000 to 100,000, frequent infiltration of practically all organ systems, and response to therapeutic agents (Kaplow and Nadel 1979; Murphy and LoBuglin 1977; Opler 1971).

A histiocytic lymphosarcoma was reported by Kitchen et al. (1975). Robinson (1976) reported two primary spleen tumors: a splenoma and a sarcoma. An additional eight primary splenic tumors were reported in untreated control animals of both sexes. They were three glomerular vascular tumors, three sinusoidal hemangioendothelioma, one hemangiosarcoma, and a chrondromatous tumor (Hoch-Ligeti et al. 1981).

Neoplastic Lesions: Induced. Whole body irradiation with γ- or x-rays increased the rate of occurrence and number of splenic tumors after the age of 30 months. Lipomatous tumors occurred in addition to the types observed in untreated control animals. A significantly greater number of tumors occurred in inbred strains than in the noninbred strains (Hoch-Ligeti et al. 1981).

Nutrition and Metabolic Diseases

Nonneoplastic Lesions: Spontaneous

Vitamin C (Ascorbic Acid). Guinea pigs, like primates, are genetically deficient in the enzyme L-gulonolactone oxidase in the glucose to vitamin C pathway. They are unable to convert L-gulonolactone to L-ascorbic acid. Also, ascorbic acid turnover is rapid and tissue storage is insufficient for periods of inadequate intake. Therefore, they require adequate dietary ascorbic acid, which is approximately 10 mg/kg body weight per day for maintenance and 30 mg/kg body weight per day during pregnancy. The ascorbic acid can be supplied in the feed, added to the water, or by fresh vegetables such as cabbage, kale, or oranges. Carrots and lettuce are not good sources of vitamin C. Ascorbic acid should be placed in water daily because of the loss of the vitamin's activity. Feed containing ascorbic acid should be properly stored and used within 90 days of milling. Guinea pigs should not be fed diets indicated for other species. Rabbit food, for example, contains no ascorbic acid, and for guinea pigs, excess levels of vitamin D (Harkness and Wagner 1989; Navia and Hunt 1976). Guinea pigs fed feed accidentally autoclaved, which destroyed the vitamin C, have developed scurvy. Vitamin C (ascorbic acid) deficiency results in increased susceptibility to infectious agents such as *Streptococcus zooepidemicus, S. pneumoniae, Bordetella bronchiseptica,* and *Klebsiella pneumoniae* with inflammation especially of the lungs and cervical lymph nodes.

Signs of vitamin C deficiency are unsteady gait, painful locomotion, hemorrhage from gums, swelling of costochondral junctions, and emaciation. Deaths are common. Lesions include serosal, subcutaneous, and periarticular hemorrhages. Microscopically, there are hemorrhages of the epiphyseal plate, disarray of the cartilage columns, and fibrosis of the marrow in areas of active osteogenesis (Fraser et al. 1986; Navia and Hunt 1976). Many guinea pigs die of acute bacterial infections before they can die of classic scurvy (Gleiser 1974).

Nine episodes of subclinical scurvy were observed in 28 guinea pigs. The clinical signs were diarrhea, weight loss, and dehydration. The classic lesions of hemorrhage in the subperiosteum, skeletal muscles, joints, and intestine were not seen. Microscopically, the epiphyses were attenuated and irregular. The amount of osteoid was less than normal. Many guinea pigs had acute enteritis. All episodes were associated with either autoclaving food without adequate supplementation or other inadequate feed management practices (Clarke et al. 1980).

Vitamin D. Hypervitaminosis D is characterized by medial calcification of the major elastic arteries and soft tissue calcification.

Vitamin E. Guinea pigs are very sensitive to α-tocopherol deficiency. The clinical signs are stiffness, reluctant movement, and hind limb weakness. Affected sows might have marked reduction in reproductive performance. Testicular degeneration is observed after longer periods of deficiency. Severely affected animals can die within a week of the onset of clinical signs. Microscopically, the skeletal muscle has coagulation necrosis (Percy and Barthold 2001; Wagner 1979). When fed a diet with a combined deficiency of vitamins E and C, weanling guinea pigs developed a distinct clinical syndrome in which they became paralyzed and died (Hill et al. 2003).

Vitamin A. Hypervitaminosis A is characterized by a high incidence of gross structural malformations in fetuses and newborn animals (Shenefelt 1972).

Zinc. Zinc deficiency results in alopecia, especially in female guinea pigs at about the 50th day of gestation. The hair will usually regrow in 2 or 3 weeks following parturition if the animals are on an adequate zinc diet (Wallach and Boever 1983).

Ketosis. Ketosis is a common metabolic abnormality in obese guinea pigs after stress factors such as fasting, shipping, or changes in feeding routines. It primarily affects animals in late pregnancy fed low-energy diets and usually occurs during the first or second pregnancy, but can be induced in obese virgin females. Ketosis is characterized clinically by rapid onset, inappetence, depression, and death within 2 to 5 days. Other features include hypoglycemia, lipemia, ketonemia, ketouria, aciduria, and proteinuria. Lesions are nonspecific and include usually a markedly fatty liver and enlarged adrenal glands. Some adrenals have hemorrhages and cortical necrosis (Navia and Hunt 1976).

Because ketosis usually affects females in the last 2 to 3 weeks of pregnancy, it is often considered the metabolic form of pregnancy toxemia. Both the circulatory and metabolic forms of pregnancy toxemia are manifested by the same clinical signs of depression, acidosis, ketosis, proteinuria, ketonuria, and a lowered urinary pH (Percy and Barthold 2001).

Starvation and Water Deprivation. Starvation and water deprivation should not be underestimated as causes of death in guinea pigs. They are notoriously fixed in their eating habits and do not readily adapt to diets different from those to which they are accustomed. Likewise, they are quite inadept at finding new sources of drinking water in their environment (Wagner 1979).

Other Diseases, Spontaneous. Alpha-mannosidosis is a lysosomal storage disorder resulting from deficient activity of lysosomal alpha-mannosidase. The affected guinea pigs have neural and visceral changes that closely resemble the human disease and will provide a convenient model for investigation of new therapeutic strategies for neuronal storage diseases, such as enzyme replacement and gene replacement therapies (Crawley et al. 1999).

Bacterial, Viral, and Rickettsial Diseases

Guinea pigs scatter their bedding into feeders and water crocks. They are notorious for chewing on and otherwise blocking sipper-tubed waterers. They mix dry feed and water in their mouths and

pass the slurry into the sipper tube, thereby blocking the tube or causing it to drip. They will also defecate into their feed and water crocks if the feeders and crocks are not suspended above the bedding (Harkness and Wagner 1983). Because of these behavioral characteristics, infectious agents can become problems in research studies. Good general husbandry and disease prevention procedures are essential when using guinea pigs.

Bacterial Diseases

Infections: Spontaneous. Guinea pigs are susceptible to a wide variety of microorganisms. At least 19 genera of bacteria, mycoplasmas, and rickettsia-like agents have been recovered from laboratory guinea pigs with spontaneous diseases. The most frequently reported bacterial infections causing epizootic disease were *Salmonella spp., Bordetella bronchiseptica, Streptococcus spp.* (Lancefield group C), and *Yersinia pseudotuberculosis.* These infections have been associated often with high mortality in the guinea pig colony. Less frequently reported causes of epizootic disease have been *Streptococcus pneumoniae, Klebsiella pneumoniae, Pasteurella multocida, Actinobacillus spp.,* and *Pseudomonas spp.* After the epizootic disease subsides, carriers persist, resulting in enzootic disease. Predisposing factors that contribute to the severity of the illness or deaths in the colony are poor husbandry practices such as unhealthy sanitation, overcrowding, mixing of animal species in the same room, improper ventilation, incorrect temperature and humidity control, inadequate diet, transporting the guinea pigs, and experimental procedures (Boot et al. 1983; Ganaway 1976).

Because they are susceptible to Bordetella pneumonia, guinea pigs should not be housed with rabbits, cats, dogs, and other species that carry Bordetella subclinically (Harkness and Wagner 1989). *Lawsonia (Campylobacter) jejuni* was isolated from the intestines of guinea pigs with no clinical disease (Meanger and Marshall 1989).

Infections: Induced. *Salmonella typhi* introduced into the gallbladder was recovered from bile and feces of infected, but apparently normal, animals of the NIH Hartley strain for up to 5 months. These animals provide a model for the asymptomatic human typhoid carrier (Lavergne et al. 1977).

Experimental infection of guinea pigs with pathogenic *Serpulina (Treponema) hyodysenteriae* can be used as an animal model for swine dysentery (Joens et al. 1978; Joens et al. 1993).

A guinea pig model of low-dose aerogenic tubercular infections has been developed (Chambers et al. 2001). Guinea pigs infected with the Legionnaires' disease bacillus, *Legionella pneumophila*, have been used to evaluate new therapeutic agents (Edelstein et al. 2001; Edelstein et al. 2003).

Viral, Rickettsial, and Chlamydial Diseases

Infections: Spontaneous. Evidence of more than 16 viral and chlamydial agents has been reported in guinea pigs. These agents belong to a variety of groups including enterovirus (poliovirus), reovirus, paramyxovirus (Sendai virus, simian virus, parainfluenza type 1), leukovirus (leukemia), arenavirus (lymphocytic choriomeningitis virus), herpesvirus, pox virus, and psittacosis-lymphogranuloma venereum-trachoma (Chlamydia). Serological tests revealed antibodies against poliovirus (16%), reovirus type 3 (4%), pneumonia virus mice (23%), simian virus (25%), and Sendai virus (9%). Leukemia, lymphocytic choriomeningitis, cytomegalovirus (salivary gland), guinea pig herpeslike virus (Connelly et al. 1987), guinea pig poxlike virus, and guinea pig inclusion conjunctivitis chlamydophia, *Chlamydophila caviae,* formerly termed *Chlamydia psittaci* (Eidson 2002), have been isolated and described. Viruses are suspected to be involved in myositis (Saunders 1958) wasting disease, hepatoenteritis, and pneumonia. Viral, rickettsial, and chlamydial diseases and their agents are described in more detail by Van Hoosier and Robinette (1976).

Infections: Induced. In addition to the naturally occurring or spontaneous diseases, the guinea pig has been proven to be a valuable experimental animal in the study of a wide variety of viral, rickettsial, and chlamydial diseases (Van Hoosier and Robinette 1976). An example is the adaptation

of Pichinde virus, an arenavirus, to inbred strain 13 guinea pigs to produce infections similar by virological, pathological, and clinical features of the human disease Lassa fever. Infections by this arenavirus are a potentially useful animal model of human disease (Lucia et al. 1989; Lucia et al. 1990; Jahrling et al. 1981). Guinea pigs infected with guinea pig cytomegalovirus develop a mononucleosis syndrome that is similar to that seen in immunocompetent humans (Griffith et al. 1981, 1983; Lucia et al. 1985). Genital herpesvirus infections in guinea pigs are useful models of primary and recurrent infections for studies of prophylactic, antiviral, and vaccine therapies (Bernstein et al. 2001; Bourne et al. 2002; Lucia et al. 1983; Simms et al. 2000; Stanberry et al. 1985).

They are also susceptible to rickettsial infections, and a number of rickettsiae pathogenic to humans were first studied in the guinea pig (Fenner 1986). Guinea pigs are susceptible to the Q fever agent, *Coxiella burnetii*, and provide an animal model of this disease (Heggers et al. 1975).

Mycotic Diseases

The vast majority of spontaneous fungal diseases in guinea pigs are dermatophytoses limited to the skin. Systemic mycoses such as caused by *Mucor spp.* and *Absidia spp.* are sporadic and described in detail by Sprouse (1976). A case of dermal cryptococcosis caused by *Cryptococcus neoformas* involved the nose (Van Herck et al. 1988).

Parasitic (Protozoan and Metazoan) Diseases

Protozoan Infection: Spontaneous

Although a large number of protozoan parasites are observed in guinea pigs, protozoan diseases are rare. The most important protozoan diseases are intestinal coccidosis caused by *Eimeria caviae* and renal coccidosis caused by *Klossiella cobayae* (Vetterling 1976). Three protozoa can cause generalized infections during their acute phases: *Toxoplasma gondii,* the tissue form of the feline coccidia; *Sarcocystis caviae;* and *Encephalitozoon (Nosema) cuniculi.* Toxoplasma infections might produce encephalitis, visceral granulomas, lymphadenitis, splenomegaly, or myocarditis (Wallach and Boever 1983). Sarcocystis is found in skeletal muscle, and Encephalitozoon has been reported in the brain (Yost 1958). Protozoan parasites of guinea pigs have been reviewed in detail by Vetterling (1976).

Protozoan Infection: Induced

Guinea pigs were intradermally infected with *Trypanosoma cruzi,* the infective agent for Chagas' disease to evaluate the role of epidermal Langerhans cells in skin-related immunological events (Nargis et al. 2001). Experimental immunization against *T. cruzi* has been studied in guinea pigs exposed to infected Triatoma bugs (Basombrio et al. 1997). Experimental cryptosporidiosis in guinea pigs is a useful small animal model of this disease (Chrisp et al. 1990).

Metazoan Infection: Spontaneous

The guinea pig might become infested with several different arthropod ectoparasites. Most of these are specific for the guinea pig and well adapted to their host with minimal disease (Ronald and Wagner 1976).

Metazoan Infection: Induced

Experimental infections by the fluke, *Fascioloides magna*, have demonstrated that the guinea pig is a suitable animal model for studing this parasite, an important pathogen in sheep (Conboy et al. 1991; Conboy and Stromberg 1991).

The only helminth of importance reported in guinea pigs is the cecal worm, *Paraspidodera uncincata*. This intestinal nematode rarely causes clinical disease (Wescott 1976).

Immunological Disease

Spontaneous Conditions

An inbred strain of complement 4-deficiency guinea pigs has a total deficiency of the C4 component of complement with no evidence of a C4 inhibitor in the serum (Ellman and Green 1970). Passive anaphylaxis, contact and delayed hypersensitivity, and cellular exudative response to a foreign body are normal, indicating the presence of an alternate complement pathway (Ellman, Green, et al. 1971; Frank et al. 1971). The NIH Hartley strain of guinea pig is susceptible to acute systemic anaphylaxis (Stone et al. 1964). Amyloidosis of multiple organs, particularly the kidneys, liver, spleen, and adrenal glands is rather commonly found in guinea pigs (Wagner 1976). Guinea pigs deficient in the fourth component of complement did not have glomerulonephritis (Foltz et al. 1994). Athymic or hypothymic hairless guinea pigs are agammaglobulinemic and can be used for studies of immunodeficiency diseases (Reed and O'Donoghue 1979).

Induced Conditions

Strain 2 guinea pigs develop an immune response to bovine serum albumin, 2,4-D protamine, human serum albumin, potassium dichromate, beryllium fluoride, hydralazine, insulin, human fibrinopeptide B β1-14, GA, DNP-PLL, PLL, and DNP-PLA (Barcinski and Rosenthal 1977; Ellman, Green, et al. 1971; Ellman, Inman, et al. 1971; Geczy and DeWeck 1977; Rose et al. 1979; Thomas et al. 1979a, 1979b).

Strain 13 guinea pigs develop an immune response to benzyl-penicilloyl bovine γ-globulin, DNP guinea pig skin protein conjugates, 2, 4-DNP guinea pig albumin, mercuric chloride, aspirin, poly (TGAG), GT, insulin, and human fibrinopeptide B β1-14 (Geczy and DeWeck 1977).

The sensitivity of the respiratory system of the guinea pig has provided animal models for numerous studies of inhalation phenomena, including bronchospasms, asthma, latex allergy (Aamir et al. 1996), and hypersensitivity reactions to dust, 2,4-toluene diisocyanate (Sugawara et al. 1993), other air pollutants, and development of antihistamines. A guinea pig model has been developed to assess adverse hypersensitivity reactions to Q fever vaccines (Wilhelmsen and Waag 2000).

Conclusions

The guinea pig has great usefulness as an animal model in toxicology for a broad variety of experimental diseases and test systems in several organ systems. There are relatively few infectious, nutritional, and metabolic diseases that might interfere with toxicology studies. The majority of spontaneous diseases are well described and can be controlled by appropriate husbandry practices. More information on husbandry, pathology, and diseases, including treatment, are given in the works of Melby and Altman (1974, 1976), Wagner and Manning (1976), Benirschke et al. (1978), Hime and O'Donoghue (1979), Wallach and Boever (1983), Rowsell (1984), Manning et al. (1984), Fraser et al. (1986), Anderson (1987), Poole (1987), Harkness and Wagner (1989), Richardson (2000), and Percy and Barthold (2001). Necropsy of the guinea pig is described by Feldman and Seely (1988). The detailed anatomy is presented by Cooper and Schiller (1975).

METABOLISM

The objective behind the use of any animal model in toxicology is almost always to predict what will happen in humans. A necessary and essential part of this is knowing the reasons for any differences in response between the model and the human. A major component of such differences is undoubtedly variations (qualitative and quantitative) in metabolism. CYP-450 isoform activities are not currently

Table 5.17 Major Metabolic Characteristics of the Guinea Pig

Characteristics	Reference
Has higher sulfotransferase and glucuronosyltranferase form 1 in the jejunum than in the liver	Schwenk and Locher (1985)
Has higher substrate specificity for mono-oxygenases (benzo[a]pyrene hydroxylase and N-demethylase) in intestines than in liver	Gregus et al. (1983) Laitinen and Watkins (1986)
Liver size is proportionately (to body size) greater than in the human (2% of total body weight in the human, 3.5% in the guinea pig)	Experimental data
Does not have aromatic hydroxylation capacity for amphetamines	Caldwell (1981)
Whereas rat and dog have more efficient biliary excretion than man, the guinea pig is comparable to man	Levine (1978)

well characterized in guinea pigs. Liu and Chan (2000) have characterized the induction of CYP1A1 with frying oil. Metabolism of variconazole, which is primarily by CYP 3A4 in humans, rats, and dogs, is minimal and not inducible in the guinea pig (Roffey et al. 2003), Successfully identified in the guinea pig have been CYP1A1, 1A2, 3A14, 3A15, 4A13, 11B1, 11B2, and 17.

Table 5.17 summarizes some key factors about guinea pig metabolism of xenobiotics.

Species Peculiarities

As is the case with all other animal models that have been studied for some time, a number of responses to potentially toxic xenobiotics have been identified in the guinea pig that are different from humans or other model species. This section seeks to overview these differences.

Guinea pigs are remarkable in their requirements for vitamin C. The animal maintained on a vitamin C-deficient diet develops scurvylike symptoms. Before chemical methods were developed, guinea pigs were used for vitamin C assay in nutritional studies. The guinea pig is highly susceptible to human and bovine tuberculosis, and it can be used for the diagnosis of human mycobacteriosis. The guinea pig is an excellent model for anaphylaxis and other immunological procedures, but this excellence is founded on the fact that the immune responses in the guinea pig (both cellular and humoral) are exaggerated compared to humans.

Antibiotic Toxicity

Guinea pigs are highly sensitive to antibiotics, particularly those specific for gram-positive organisms. The normal intestinal flora of guinea pigs is predominantly gram-positive. Administration of antibiotics specific for gram-positive bacteria destroys the normal flora of the intestinal tract and permits an overgrowth of gram-negative organisms. Until recently, complications following antibiotic administration were attributed primarily to *Escherichia coli*; but current evidence suggests that *Clostridium difficile* plays an important role in the enterotoxemia that follows antibiotic treatment. These antibiotics are not directly toxic and are not harmful when administered in therapeutic dosages to germ-free animals. Tetracycline, cephaloridine, chloramphenicol, and the sulfonamides are among the less hazardous antimicrobials, but all should be administered with caution, using the minimal effective dosage.

Response to Anti-Inflammatories

The area of anti-inflammatories is (and has been for at least 15 years) a very active one in pharmaceutical research. These efforts have been hampered by the lack of good models for assessing the safety of such compounds, particularly in terms of potential GI and renal toxicity.

Many of the anti-inflammatories (particularly the nonsteroidal anti-inflammatory drugs, or NSAIDs) act at least in part by inhibiting prostaglandin synthesis. Some of the standard models

used for screening for potentially effective compounds include assaying homogenates of the stomach of dosed guinea pigs for prostaglandin synthesis, and inhibition of ultraviolet-induced erythema in the skin of dosed guinea pigs (Birnbaum et al. 1982).

The guinea pig, although still more sensitive than the human to the GI ulceration induced by NSAIDs, is less so than the dog or rat for a range of NSAIDs (Birnbaum et al. 1982; Mariani and Bonanomi 1978). The most common toxic effect reported for anti-inflammatories in the guinea pigs is gastrointestinal ulceration, however, and they are generally more sensitive than humans.

Cardiotoxic Effects of Catecholamines

Waldenstrom et al. (1987) performed extensive evaluations of the cardiotoxicity of catecholamines in both the rat and guinea pig, and have found the guinea pig to be less sensitive than the rat to the local anoxia caused damage induced by catecholamines.

Strains

There are three naturally occurring strains of guinea pigs, which can be distinguished from each other by differences in the length, texture, and direction of their hair growth. These are the short-haired, smooth, and coarse-coated English; the long-haired Abyssinian, which has its hair arranged in whirls; and the long, fine-haired Peruvian.

The English guinea pig, which comes as an albino, bi-, or tricolored animal and as outbred and inbred strains, is the most common and the most frequently used strain in the laboratory. They are alert and well fleshed with smooth, shiny skin. The albino Hartley strain is the variety most familiar to toxicologists, and it is considered the standard animal for skin sensitization studies.

Recently, Charles River Breeding Laboratories has developed and made commercially available a hairless form of the Hartley guinea pig, designated the Crl:IAF/HA(hr/hr)BR. This is a fertile, euthymic animal. There are also athymic and hypothymic varieties available.

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CHAPTER 6

The Rabbit

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CONTENTS

TOXICOLOGY

History

The domestic rabbit, *Oryctolagus cuniculus,* which belongs to the order *Lagomorpha* (and hence is referred to as a Lagomorph) and the family *Leporidae* (which also includes hares) is descended from the wild rabbits of western Europe and northwestern Africa, which were first discovered by the Phoenicians in 1100 B.C. (Fox 1974). The rabbit is one of the most successful mammals of the world; it is both prolific and adaptable and appears to be equally at home on all the continents. Domestic rabbits are similar to rodents in many respects. The principal anatomical difference is that rabbits have two pairs of upper incisor teeth, whereas rodents have only one pair (Flatt 1977). The additional pair of incisors in rabbits is smaller and located directly behind the other pair.

Domestication of the rabbit probably began in monasteries during the sixteenth century (Fox 1974). By the middle of the seventeenth century, the rabbit was completely domesticated and rabbit raising was active in England and continental Europe. This is the only species of lagomorph that has been domesticated and, as such, the species has been introduced into every country of the world as a source of meat or fur, as a pet, or as a laboratory animal.

Early descriptions of research with rabbits date back to 1683 when Leewenhoeck first described the dominance of the wild-type coat ("normal" white-bellied agouti) over albinism, nonagouti, and so on (Sirks 1959). Since then rabbits have been used extensively to investigate the genetics of coat color and hair morphology, and the rabbit is one of the most common species used for biomedical experimentation. In addition, their by-products are utilized for pharmaceutical and vaccine production.

Choice of the Rabbit in Toxicological Research

Justification for Use

A number of size, shape, and color variations derived from centuries of selective breeding constitute the more than 50 well-established breeds recognized by the rabbit breeders' associations. Representatives of the small breeds (under 2 kg or 5 lb) include the American Dutch and Polish breeds, the medium-sized breeds (2–5 kg or 5–10 lb) are the California and New Zealand White (albino) rabbits, and the large breeds (5 kg or 10 lb and over) are the Flemish and Checkered Giants. The New Zealand White albino is the rabbit most commonly used for research purposes and is the focus of this chapter. However, other breeds, such as the American Dutch, Flemish Giant, and Polish, are also used as laboratory animals.

Compared to the high cost of cats, dogs, and monkeys and the problems associated with their proper care and maintenance, rabbits are relatively inexpensive, hardy, small, clean, and more easily housed and handled. Thus, they are readily used for a wide variety of experimental procedures and testing situations, including immunology (they are good antibody producers and blood is relatively easy to collect from their large and accessible ear veins), teratological, dermal, ocular, and implant studies. Specific study designs are discussed later in this chapter.

Because the distribution of intestinal microflora within the gut (and hence, their potential metabolic activity) is more similar in rabbits to that of humans than that of the guinea pig, rat, or mouse (Williams 1972), an orally administered compound is less likely to be metabolized, possibly into a toxic or active metabolite, in the rabbit than in these other laboratory species. However, because gastric emptying time for the rabbit can vary anywhere from 20 min to 20 hr in contrast to the human rate of 1.0 hr to 1.5 hr (Digens et al. 1980) the rabbit is a poor species for evaluating the absorption of orally administered compounds (Maeda et al. 1977).

Although rabbits are frequently used to study dermal toxicity, they might not be the best species. Because human skin has a thicker stratum corneum, it is more resistant to the dermal absorption

of foreign substances and is penetrated much less easily by xenobiotics than the skin of the most widely used animal models, including the rabbit and the rat (Calabrese 1984). *In vitro*, skin permeability to ionic and covalent substances in aqueous solution and organic solutes increases in the following order: human, pig, guinea pig, rat, and rabbit (Tregear 1966). When Bartek et al. (1972) compared the *in vivo* skin permeability of several compounds, they also found that the absolute dermal absorption rate for the rabbit was higher than that of the rat, pig, or human (in descending order). It appears that because the permeability of the skin of miniature swine is close to that of human skin, for studies in which dermal toxicity data are to be used to predict toxicity in humans, in some respects the miniature swine appears to be a more suitable test animal than the rabbit. However, despite the considerable species difference between rabbit and human skin, rabbits are routinely employed in dermal toxicity tests. Thus, when interpreting data generated using rabbits, the researcher must consider the following (Bartek et al. 1972). In general, for a single dermal dose, total exposure of the outer surface of the skin to the applied compound will be of shorter duration in the rabbit than in humans. However, because of the higher penetration rate in the rabbit, temporarily higher concentrations of compound might occur within the rabbit skin as compared to human skin. In addition, the time course of systemic exposure to the compound will also be markedly different in the two species.

Important Physical and Physiological Characteristics

Some general values for selected physical and physiological data in rabbits are given in table 6.1. More detailed information on these characteristics can be found under the appropriate subsections in this chapter.

Table 6.1 Selected Normative Data for New Zealand White Rabbits

General adult body weight	
Male	4–5 kg
Female	4–6 kg
Life span	5–13 years
Surface area	2.5 kg: 1,270cm^2 4.8 kg: 3,040cm^2
Chromosome number (diploid)	44
Water consumption	50–100 mg/kg/day
Food consumption	50 g/kg/day
Rectal temperature	102.0–103.5°F
Basal metabolism	110 cal/kg/day
GI transit time	4–5 hr
Reproduction	
Puberty	3–8 months
Breeding age	
Male	6–10 months
Female	5–9 months
Breeding season	All year (controlled conditions)
Estrus cycle	Polyestrous, induced
Breeding habits	Female brought to male
Time of ovulation	9.75–13.5 hr; postcopulation
Time of implantation	approx. 7 days postcopulation
Length of gestation	29–35 days
Postpartum estrus	None
Litter size	4–10
Birth weight	30–100 g
Weight at 3 days	400–700 g
Weaning age	4–6 weeks
Weaning weight	1–2 kg

Growth and Development

The New Zealand White rabbit grows very rapidly. At weaning these animals weigh 1.0kg to 1.5 kg and the adult animal weighs 4 kg to 6 kg depending on sex. The life span of most strains of rabbits is 5 to 8 years, and some might live to 15 years (Harkness and Wagner 1989). One index of age is the size and appearance of the claws. They do not project beyond the fur until the rabbit approaches maturity, and then they grow and curl with age.

Reproduction

The age at which rabbits reach puberty varies from 4 to 12 months and depends somewhat on breed and strain. It tends to be inversely related to size; that is, smaller rabbits like Polish rabbis can be bred at 4 months, whereas heavier Flemish rabbits reach puberty between 9 and 12 months. The New Zealand White can be bred at 5 to 6 months (female) and 7 to 8 months (male). The male rabbit matures slower than the female. Motile spermatozoa appear in the ejaculate at 4 months and adult levels of sperm production are reached at about 7 or 8 months (Bivin and Timmons 1974).

Although rabbits do not have a definite estrus cycle, there are short periods of 1 to 2 days every 2 weeks when the doe is not receptive to the male. Also, a doe might refuse to mate with one buck but might be receptive to another. Mating behavior consists of tail flagging and enurination, and sometimes chasing. In tail flagging, the buck elevates his hindquarters, walks stiff-legged, and lays his tail flat on his back, providing visual stimulus to the doe and olfactory stimulus from the inguinal glands. In enurination, the buck might turn his hindquarters toward the doe and emit a jet of urine in a display of sexual aggressiveness; sometimes this is followed by a circling of the cage. Enurination might also be directed toward bucks in adjacent cages. If the doe is receptive, she will elevate her hindquarters, permitting the buck to mount.

Rabbits are induced ovulators, which means the female remains in estrus until copulation, which triggers the induction of ovulation. Ovulation can also be induced by an injection of chorionic gonadotropin or other luteinizing hormone, which is done if the researcher wishes to employ artificial insemination. Ovulation occurs 10 to 13 hr after copulation or after the injection of luteinizing hormone.

Rabbits have been used extensively for reproductive studies because of the precise timing that can be obtained for studies of egg maturation, fertilization, cleavage, and implantation. Pregnancy can be confirmed by palpation approximately 10 to 12 days after breeding. Fertile copulations result in pregnancy and a gestation of 30 to 35 days depending on the breed. Parturition is called kindling.

Clinical signs of pregnancy or pseudopregnancy are an increase in the size of the ruff, a large tuft of hair on the ventral neck, and a subsequent increase in hair pulling, especially from the ruff to make a nest.

Pseudopregnancy, which lasts 16 to 17 days, is easily induced in the doe and can be caused by infertile mating, sexual excitement from a doe mounting another doe, injection of lueinizing hormone, or stress due to shipping or experimentation. During pseudopregnancy the doe is not receptive to mating.

Excretion

Rabbits have two types of feces: soft, moist nighttime feces and firm, dry daytime fecal pellets. The nighttime feces, which are covered with mucus and consist primarily of secretion from the cecum, are produced by the initial ingestion of food; hard feces are produced by reingestion of the soft feces. The soft feces are protein and vitamin rich, and their ingestion (coprophagy) directly from the anus improves the utilization of nitrogen (Thacker and Brandt 1955), provides an abundance of certain B vitamins, plays an important role in the incorporation of sulfur in the soft tissues,

and conserves water (Kulwich et al. 1954; Kulwich et al. 1953). Wild rabbits are able to survive up to a week without food because of reingestion of feces.

Because of their diet, rabbit urine is cloudy and ranges in color from light yellow to deep orange or red brown and might resemble purulent discharge. It contains carbonate and phosphate crystals, which cause a scale to accumulate on cage surfaces.

Husbandry

As with other laboratory animals, the facilities, equipment, and husbandry procedure used for rabbits should be designed to afford maximum environmental control (i.e., minimal variation in temperature, humidity, and ventilation); optimal conditions for that animal's comfort, health, and welfare; and minimal exposure to injury and disease.

Facilities

Rabbits should be maintained in an area that is clean and dry, with adequate ventilation, away from excessive noise, and within a specified range of temperature and humidity.

Temperature, Relative Humidity, and Ventilation

Because the thermal environment can influence the severity, duration, and variability of toxic responses to chemicals by influencing the physiology of the animal and the metabolism and disposition of test materials (Clough 1982; Fuhrman and Fuhrman 1961; Weihe 1973), the temperature, humidity, and ventilation of the animal facilities must be strictly controlled (Rao 1986). The thermoneutral zone of resting laboratory animals (and humans) is very small (Weihe 1973). When exposed to temperatures outside their thermoneutral zone, laboratory animals adapt behaviorally (unless they are prevented from doing so by experimentally enforced restrictions) or metabolically (by increasing or decreasing their metabolic rates). Thus, marked variations in the environmental conditions of the animal room can alter the animal's rate of metabolism, and, ultimately, the potential toxicity of a test material.

Ambient relative humidity is also a major factor in maintaining the heat balance of an animal (Clough 1982). It is a very important consideration with dermal absorption studies, particularly when shaved animals are used. In these situations, the relative humidity will directly affect the rate of evaporation, the viscosity of the test material, and the animal's peripheral circulation, thus having a major impact on the potential toxicity of the applied material.

For rabbits, animal room temperatures should be maintained between 60°F and 70°F (16°C–20°C) and relative humidity should be between 40% and 60% (ILAR 1985). This temperature range is lower (vs. 64.4°F–78.8°F or 84.2°F and 18°C–26°C or 29°C) and the relative humidity range is narrower (vs. 30%–70%) than that required for other common laboratory animals (ILAR 1985). With rabbits, elevated temperatures in particular can result in lower male fertility, lower conception rates for the does, and reduced litter sizes.

The recommended ventilation rate is 12 cubic ft/min/animal (up to approximately 5.6 kg)/day (Runkle 1964) or at least 10 complete air changes per hour (ILAR 1985), preferably with 100% fresh air. Recirculation of air is not recommended. If the air is recirculated, more efficient filtration will be required to remove odors and contaminants.

Light and Noise

Most facilities maintain light cycles of 12 hr light and 12 hr dark. However, light cycles of 14hr to 16 hr light for females are recommended when the rabbits are used in reproductive studies.

Situations that might result in elevated noise levels for extended periods of time, such as housing rabbits in the same area as dogs or in the vicinity of noisy equipment, should be regarded with caution and avoided if possible. Nayfield and Besch (1981) have reported indications of stress, such as increased spontaneous activity and adrenal weights and decreased spleen and thymus weights, in rabbits that were exposed to elevated noise (1.5 hr of white noise at intensities of 107–112 dbls/day for 2 weeks). An auditory stimulus also appears to affect plasma cholesterol concentrations (Friedman et al. 1967) and initiates changes in the hypothalamus (Henkin and Knigge 1963) of exposed rabbits.

Caging

Rabbit cages and racks should be constructed of a smooth, corrosion-resistant material, preferably stainless steel, that is impervious to liquids and moisture, and is easily sanitized and sterilized. The floor of the cage is usually made of steel grid or wire mesh with a recommended size of 1 in. × ½ in. or ⅝ in. sq./1 × 2.5 cm. It should be smooth and free of sharp projections. The wire mesh floors allow excrement to fall to excreta trays below the cage. Some type of welded wire (1 in. × 1 in. or 1 in. × 2 in.) or solid metal walls with air vents are generally used on the dies, back and top of the cage, and wire grid is used on the front. Painting of cages and racks is not recommended. The racks and cages should be movable to facilitate transportation to a washing area.

The amount of floor space required per animal (or the cage size for individually housed animals) is determined by the weight of the rabbit. Current minimum space recommendations for rabbits are given in table 6.2 (ILAR 1985). It is recommended that the racks holding the cages be placed at least 2 m apart to minimize the between-rack spread of airborne microorganisms due to convection currents (Teelman and Weihe 1974).

Two standard sizes of cages that are available commercially and provide 3 and 4 ft^2 of floor area have dimensions (width × depth × height) of 24 in. × 18 in. × 16 in. and 24 in. × 24 in. × 15 in., respectively. Young immature rabbits can be group housed by sex, but rabbits that are sexually mature (over 4 months) often attack one another and should always be individually housed. Because wounding, pseudopregnancies, and infertility can occur in groups housed together, mature rabbits should be paired only at mating.

Nest boxes must be provided for does that are expected to deliver and nurse young. Suitable bedding used in nest boxes should be a nonedible material (e.g., wood shavings).

Dropping pans are lined with disposable absorbent liners that should be changed at least three times each week. The animals should be transferred to clean cages at least every 2 weeks. Facilities should be physically cleaned and sanitized at least three times a week.

Rabbit urine, with a pH of 8.2, is very alkaline and contains phosphate and carbonate crystals that accumulate on the cage surfaces and form a scale that is difficult to remove. Detergents,

Table 6.2 Minimum Cage Sizes

Weight		Floor Area/Rabbit	
kg	lb	ft^2	m^2
< 2	5	1.5	0.14
2–4	5–10	3.0	0.28
4–5.3	10–12	4.0	0.37
> 5.4	> 12	5.0	0.46

Note: The cage height should be at least 14 in. (25.56 cm).
Source: ILAR (1985).

disinfectants, and lime-scale removers (acidic solutions such as vinegar or acid products at pH 2) can be applied with a stiff brush in routine cage cleaning. However, acidic materials, which might cause discoloration and damage to the cages, should be used with caution. Flaming might also be necessary to remove hair and manure and kill coccidial oocysts.

Feed and Water

Feed and water systems should be clean and designed so that they cannot become easily contaminated. Metal feeders that attach to the front of the cage and can be filled outside without opening the cage door are commercially available and should be used in preference to crocks or other open containers. Likewise, water bottles (if an automatic water system is not available) are usually mounted on the outside on the front of the cage. Sipper-tube watering devices are preferred.

Rabbits should be provided ad libitum with a plentiful supply of fresh, clean water. Nonpregnant does drink approximately 10 mL/100 g of body weight per day, and lactating does can drink up to 90 mL/100 g/day (Harkness and Wagner 1983). If water bottles are used, they should be filled with fresh water daily and sanitized at least once weekly.

Food hoppers should be constructed of durable material (other than wood) that is resistant to the gnawing of rabbits, is corrosion resistant, and is easily sanitized. They should be mounted 4 in. from the floor of the cage and the design should permit easy access, but not allow the rabbit to enter. They should also be free of sharp edges that could cause cuts or scratches.

The preferred diet is a wholesome, nutritious, pelleted form of feed, free of drugs, hormones, pesticides, and animal and vermin contaminants. Because of the great variability in fiber and nutrient content in commercially available diets (Wise and Gilburt 1980, 1981), and because of the potential presence of contaminants, it is recommended that only diets that have been analyzed for these materials be used. Recommended nutrient levels are presented in table 6.3. The nutrient content of two commercially available diets, Purina Rabbit Diet and Purina Certified High Fiber Rabbit Diet (PMI® Nutrition International), which are made of natural ingredients and purified, is presented in table 6.4.

Fresh feed should be provided at least weekly. However, a once-daily feeding (limited feeding) of approximately 120 g (4 oz or ⅔ cup) of pellets is sufficient to maintain an adult, medium-sized rabbit at a constant weight; this is the amount of feed that can be give to animals that are to be used for acute studies. For longer studies, the animals are provided with approximately 190 g (6 oz or 1 cup) of pellets each day. At peak lactation, a doe can consume up to 450 g (16 oz) of feed per day, and pregnant or lactating does should be provided with feed ad libitum. The feeders should be checked daily and any powdered feed removed.

The diet should contain 16% to 20% crude fiber and 14% to 18% crude protein (Harkness and Wagner 1983). Rabbits have a higher requirement for fiber than other species. Fiber has an important role as bulk, but rabbits do not digest over 18% dietary fiber in a single passage. Diarrhea might result if the fiber concentration is below 6%, and fiber above 20% can lead to lowered feed efficiency (Harkness 1987). The feeing of diets containing a high concentration of fiber (18%–20%) is often utilized to reduce the occurrence of intestinal enteropathies.

Malnutrition is uncommon in rabbits. Other relatively uncommon nutritional problems include vitamin D, calcium, and phosphorus imbalances (atherosclerosis); vitamin A (hydrocephalus, prenatal death) and vitamin E (muscular dystrophy, prenatal mortality, seminiferous tubal degeneration) deficiencies; and some specific mineral or amino acid deficiencies.

Because feed and bedding are generally used in animal facilities without prior sanitization, they are potential sources of contamination for diseases, parasites, and hormones. Therefore, potential problems could occur and caution should be used in purchasing and storing food and bedding.

Table 6.3 Nutrient Requirements[a]

Energy and protein	
Digestible energy (kcal)	2,100–2,500
TDN (%)	55–65
Crude fiber (%)	16–20
Fat (%)	2–4
Crude protein (%)	14–18
Inorganic nutrients	
Calcium (%)	0.4–0.5
Phosphorus	0.22–0.4
Magnesium (mg)	300–400
Potassium	0.6
Sodium (%)	0.2–0.5
Chloride (%)	0.5
Copper (mg)	3–10
Iodine (mg)	0.2
Iron (mg)	100
Manganese (mg)	8.5–40
Zinc (mg)	50
Vitamins	
Vitamin A (mg)	0.33–0.44
Vitamin A as carotene (mg)	0.83
Vitamin E (mg)	20–40
Vitamin K3 (1119)	2
Niacin (mg)	180
Pyridoxine (mg)	39
Choline (g)	1.2[b]
Amino acids	
Lysine	0.65
Methionine + cystine	0.6
Arginine	0.6
Histidine	0.3[b]
Leucine	1.1[b]
Isoleucine	0.6[b]
Phenylalanine + tyrosine	1.1[b]
Threonine	0.6[a]
Tryptophan	0.2[b]
Valine	0.7[b]
Glycine	—[c]

[a] Presented as percentage (%) or mg/kg in the diet.

[b] Might not be minimum but known to be adequate.

[c] Quantitative requirement not determined, but dietary need demonstrated.

Source: Data from Clarke et al. (1977), Hunt and Harrington (1974), and National Academy of Sciences (1977).

Table 6.4 Chemical Composition of Purina Certified Rabbit Diet (A) and Purina Certified High Fiber Rabbit Diet (B)

Nutrients	A	B
Energy and protein		
Protein, min. (%)	16.0	14.0
Fat, min. (%)	2.5	1.5
Fiber, max. (%)	18.0	25.0
Moisture, max.	12.0	—
Ash, max. (%)	8.0	10.0
Added minerals, max. (%)	2.1	1.5
Nutrients		
Protein (%)	16.2	14.5

Table 6.4 Chemical Composition of Purina Certified Rabbit Diet (A) and Purina Certified High Fiber Rabbit Diet (B) (continued)

Nutrients	A	B
Fat (%)	2.5	1.7
Cholesterol (ppm)	61.0	60.0
Fiber, crude (%)	13.0	22.5
Neutral detergent fiber	27.4	40.9
Acid detergent fiber 950	15.8	24.6
Total digestible nutrients	66.0	57.0
Nitrogen-free extract, by difference	52.0	42.4
M		
Gross energy (kcal/g)	4.0	3.9
Physiological fuel (kcal/g)	2.95	2.43
Ash (%)	7.3	8.9
Calcium (%)	0.95	1.2
Phosphorus	0.50	0.5
Potassium (%)	1.15	1.67
Magnesium	0.25	0.30
Sodium	0.25	0.32
Chlorine	0.50	0.79
Iron (ppm)	276.2	315.9
Zinc (ppm)	105.5	122.2
Manganese (ppm)	107.2	127.5
Copper (ppm)	27.8	25.8
Cobalt (ppm)	0.38	0.46
Iodine (ppm)	0.59	0.58
Chromium (ppm)	4.5	5.0
Selenium (ppm)	0.13	0.2
Vitamins		
Vitamin A (IU/g)	20.0	20.0
Carotene (ppm)	27.6	31.2
Vitamin D (IU/g)	2.3	2.2
Ci-Tocopherol (IU/kg)	44.0	33.0
Niacin (ppm)	33.0	33.4
Pyridoxine (ppm)	4.5	4.5
Pantothenic acid (ppm)	19.0	19.1
Thiamine (ppm)	3.5	2.8
Riboflavin (ppm)	5.0	8.6
Choline (ppm x 100)	16.0	16.0
Folic acid (ppm)	2.0	3.3
Biotin (ppm)	0.12	0.14
B_{12} (IUg/kg)	6.6	6.6
Amino acids		
Lyine	0.78	0.61
Methionine	0.35	0.30
Arginine	0.90	0.68
Histidine	0.40	0.32
Leucine	1.3	1.06
Isoleucine	0.82	0.79
Phenylalanine	0.80	0.65
Tyrosine	0.50	0.42
Threonine	0.64	0.56
Tryptophan	0.23	0.19
Valine	0.84	0.68
Glycine	0.77	0.70
Cystine	0.25	0.20

Source: Ralston Purina Co.

Handling and Restraint

Rabbits must be picked up and held correctly to prevent both animal and human injuries. They should be handled firmly but gently. Because they are very shy animals and are easily frightened, they often struggle and try to escape. With the exception of older bucks and primiparous does with strong territorial instincts, rabbits seldom bite people.

However, a rabbit that is picked up incorrectly or is not held securely will kick violently with its hind legs, and it is very easy to cause injury to the rabbit, particularly a broken back. In addition, while trying to escape, rabbits can inflict painful scratches on the handler with their powerful hind legs.

Rabbit ears are very fragile and they should never be used to pick up the animal or as a means for restraint. A safe method to handle or pick up rabbits is by firmly grasping the loose skin at the base of the skull (this area is commonly called the nape or the scruff) with one hand while supporting the rear legs with the other hand. A rabbit can also be held by encircling its body with one arm, with the sternum supported by the hand, while holding the scruff with the other hand. Or, it can be held with one arm holding the rabbit's hindquarters and pressing the animal toward the handler's body. The abdomen and sternum are then supported by the handler's forearm, with the other hand on the scruff. If the hindquarters are not supported, the rabbit might struggle. If the rabbit does struggle, it can be easily calmed by placing it on the forearm with the head concealed in the bend of the handler's elbow.

Various types of restraining devices are available for use with rabbits. They serve primarily to control the body of the rabbit while the head or ears are being manipulated. The most common devices are usually some variation of a restraint box or stocks. If the basic stocks and restraining box are used, the rabbit might need to be trained and calmed to avoid struggling, which could cause a broken back. A squeeze-cage stock is often used for IV injections and a cat bag can be used for gavage administration. The rabbit can also be manually restrained. If manual restraint is used, the rabbit should not be placed directly on a smooth surface because it might flail. Instead it should be placed on a towel or mat.

Dosing Techniques

Compounds can be administered to rabbits by a variety of routes. The more commonly used routes and specific techniques are described next.

Oral Administration

The simplest method of administering compounds orally is by incorporating them in the feed or water. However, as with other species, rabbits might not voluntarily consume the material if it has an unpleasant odor or taste, or if it is in a form that cannot be easily consumed. In addition, rabbits tend to spread their feed around and "play" with their water bottles. Thus, because of potential palatability problems, spillage, and wastage these methods might not be satisfactory if it is important that precise amounts of the material be administered.

Very small volumes of liquid materials can be administered by placing the tip of a ball-tipped syringe in the corner of the rabbit's mouth and slowly introducing the material. However, the most accurate method of administering compounds orally is to deliver the material by gavage. This can be done using a stainless steel ball-tipped needle (13 gauge) or a latex catheter (size 14 French, 16 in. long). The needle is attached to a syringe with a locking end, the head and neck of the animal are manually restrained to avoid injury if the animal should struggle, and the needle is inserted into the back of the mouth and into the esophagus.

When using a latex catheter for gavage, the animal is also restrained. The animal can be manually restrained or a cat bag can be used for restraint. The mouth is held open and the catheter is inserted into the back of the throat, being careful to avoid the teeth, and then gently introduced into the

esophagus and into the stomach. To establish that the catheter is in the stomach and not the bronchi, there should be no air passage in the tube that corresponds to respiratory movements. If the animal struggles, the catheter should be removed and reinserted. After the catheter is inserted, a ball-tipped needle attached to a syringe is inserted into the open end of the catheter, and the plunger of the syringe is gently pushed to administer the dose. Depending on the nature of the material and the dose volume, it might be advisable to flush the catheter by leaving it in place and affixing another syringe with water or the appropriate vehicle. The catheter, attached to the empty syringe, is then removed, again being careful to avoid the teeth. The volume of material administered is based on the vehicle used. In general, rabbits should not be given more than 2 mL/kg of corn oil; water or a water-based solution can be given at volumes up to 6 mL/kg.

Dermal Administration

Materials are applied topically on the dorsal area of the trunk. The fur is removed with an electric clipper before dosing and as needed thereafter. Care should be taken to avoid abrading the skin and only animals with healthy, intact skin should be used. If the dosing material is a liquid, it can be applied diluted or undiluted. If the dosing material is a solid, it should be slightly moistened with saline, deionized water, or another suitable vehicle before application to ensure good contact with the skin. The dosing material is then applied uniformly over the exposure area (from approximately 5 cm^2 to an area estimated to constitute approximately 20% of the total body surface area). The exposure area can be left uncovered or it can be held in contact with the skin by a gauze dressing secured with tape, covered with cellophane wrap, and overwrapped with elastic tape. The animals should be fitted with flexible Elizabethan-type plastic collars to prevent them from removing the dosing material or coverings (Barnett 1958). At the end of the treatment period, the bindings and collar are removed and the exposure area can be washed with water or wiped clean with a towel moistened with water or a mild soap solution as thoroughly as possible without irritating the skin. The volume of material given is based on the size of the animal and the area of skin that is available for application.

Ocular Administration

Animals should be examined before the administration of the dose material and any that show prexisiting ocular defects, irritation, or corneal injury should not be used. If a solid or granular material is to be administered, it should be finely ground into a dust or powder. The material is placed in the conjunctival sac and the upper and lower lids are then gently held together for a second before releasing to prevent loss of the dose material. The amount given should not exceed 10 μL or the equivalent weight.

Intravenous Administration

Materials are usually administered intravenously into the marginal ear vein (along the posterior edge on the outside of the ear). The animal is restrained, the hair is gently plucked from the area of the vein, and a disinfectant is applied. If the vein is not readily apparent, the area can be rubbed with alcohol, or the vein can be occluded by gently pressing on the base of the ear or by placing a paper clip at the base of the ear. For IV injections, the needle should be sharp (prepackaged, sterile, disposable needles are the best) and should be the smallest size possible (1 in., 25 gauge or smaller). The needle should first be inserted into the skin beside and parallel to the blood vessel, and then inserted into the vein with the beveled edge up. The syringe should only contain the dosing solution and no air. If there are signs of engorgement or swelling at the injection site, indicating that the needle is outside the vein, reposition the needle. After the material has been injected,

withdraw the needle, and apply digital pressure to the puncture site for a short time to prevent bleeding.

Subcutaneous Administration

Subcutaneous injections are made under the skin of the neck or back, using a 1 in., 23- to 27-gauge needle. The skin is grasped with one hand and the material is injected into the tented skin. If large volumes are to be injected, the needle can be withdrawn slightly and redirected to spread the fluid over a wider area.

Intramuscular Administration

Intramuscular injections are made into the lateral aspects of the large muscles in the hindquarters (e.g., the gluteal or thigh muscles). Care must be taken to avoid hitting large blood vessels, nerves, and bone. First, the area of the injection site is immobilized, cleaned with disinfectant, and the needle is then inserted ½ in. to ¾ in. perpendicularly, depending on the muscle thickness. After the needle is inserted, the plunger of the syringe should be withdrawn slightly to confirm that no blood can be aspirated; that is, the needle has not inadvertently entered a blood vessel. If blood appears in the syringe, the needle should be carefully repositioned or withdrawn and reinserted. After the needle is placed properly, the fluid is injected slowly and the needle is withdrawn. The smallest gauge needle possible (1 in., 23–27 gauge) should be used for this procedure.

Intradermal Administration

Intradermal injections are given in the loin and flank areas where it is difficult for the rabbit to reach with its hind feet. The hair should first be removed from the site with clippers and depilatory cream. An antiseptic is then applied. The skin is stretched and the needle is inserted only into the dermis, forming a small bleb, and the injection is made using a 25-gauge needle attached to a syringe containing the dosing material. When the needle is removed, the site should be examined for seepage of the dosage material. If this occurs, the needle should be inserted further into the skin during the injection.

Intraperitoneal Administration

The rabbit is manually restrained with the hindquarters elevated and the needle (⅝ in., 23–25 gauge) is inserted bevel up into an area just lateral to the midline and just posterior to the area of the umbilicus at a steep angle toward the spine. The insertion is made with a quick thrust through the subcutaneous tissue and ventral abdominal muscles and abdominal wall, and into the peritoneal cavity. Using this procedure, up to 20 mL of fluid can be given in a single injection.

Vaginal Administration

The doe is placed in a restraining stock, and the tail is gently grasped between the thumb and forefinger while the rest of the hand is pressed firmly on the hip region of the animal. As the animal relaxes, the tail is pulled upward to expose the vaginal opening. A catheter (size 8 French, or a ball-tipped stainless steel 1½ in., 18-gauge needle) is gently inserted approximately 1 in. into the vagina, the dose is administered, and the catheter or needle is withdrawn. The animal is kept in the restraining stock for 1 to 2 min after the procedure is complete to ensure that the dose material is retained.

Collection Techniques

Blood Collection

Ear Vein

Blood is usually collected from the large, readily accessible marginal ear veins. To facilitate collection procedures, the animal is placed in a restraint box that allows access to the ears. A small amount of petroleum jelly is spread along the marginal vein. This causes the hair to lay flat in a natural direction toward the edge of the ear. A disinfectant such as 70% ethyl alcohol is applied and a paper clip can be applied as a tourniquet proximal to the intended bleeding site. Using a lamp as a heat source, the ear is warmed, which causes the vein to become engorged with blood. Slight digital irritation of the tip of the ear will also increase blood flow and facilitate blood collection. Although xylene is sometimes used for this purpose, it should not be used if the sample is to be used to determine white blood cell counts because of its severe inflammatory properties. While the ear is kept over the lamp, the vein is incised midway between the tourniquet and the tip of the ear, and blood is collected directly in appropriate tubes or containers. Alternatively, a needle attached to a syringe can be used and the blood drawn into the syringe by slow, steady manual pressure.

Auricular Artery

When larger blood samples are required, they can be collected from the centrally located auricular artery using a 20- or 23-gauge needle that is inserted into the artery toward the base of the ear.

Catheterization

Techniques for obtaining multiple blood samples from rabbits over short or extended periods of time, which are not traumatic, do not cause hematomas, and do not require anesthesia, invasive surgery, or sophisticated expertise involve implanting a flexible catheter into the auricular artery (Heim 1989; Smith et al. 1988). The site of the arterial puncture is depilated or the entire ear is shaved. A 2% nitroglycerin ointment can be applied over the artery to prevent arterial spasms, which can interfere with successful catheterization, and the area is stroked with the forefinger until the artery becomes engorged with blood. A 1-in., 22-gauge catheter is inserted into the artery approximately 3 cm from the tip of the ear with the needle at a 25° angle and the bevel up. The appearance of blood in the flash chamber confirms arterial entry. The catheter is then advanced into the arterial lumen until the hub meets the surface of the skin (the hub of the catheter should be below the tip of the ear) and an injection cap is attached to the hub of the catheter. Approximately 0.3 mL of heparinized (5 μ/mL) saline should be injected into the catheter to determine if the placement is correct. This is followed by rapidly infusing an additional 1 mL of heparinized saline and then aspirating blood until adequate blood flow is established. Tissue adhesive can be used to adhere the catheter hub to the skin surface. A piece of rolled gauze is placed inside the ear and then the ear and catheter hub are wrapped with adhesive tape, binding the ears together to prevent the rabbit from removing the injection cap and catheter. For longer sampling periods, gauze or bandaging tape should be used under the adhesive tape to prevent skin irritation.

For all these methods, blood flow from the puncture site is easily stopped by applying firm digital pressure at the site with a gauze pad. Alternatively, after the blood sample is collected, direct pressure can be applied to the incised area and a paper clip applied as a tourniquet to control bleeding until clotting takes place. The use of a surgical lubricant with the paper clip facilitates clotting.

Cardiac Puncture

Cardiac puncture can be used to collect larger quantities of blood or multiple blood samples from anesthetized or sedated rabbits. However, cardiac puncture is traumatic and can result in death. The rabbit should be anesthetized and placed in right lateral recumbency or dorsal recumbency on a restraining board. The heartbeat can be used to directly locate the heart, or the needle (1½ in., 18–21 gauge) is inserted at approximately a 30° angle immediately behind the xiphoid cartilage.

Posterior Vena Cava/Iliac Bifurcation

When large volumes of blood are needed at terminal sacrifice, and known enzyme alterations caused by cell trauma from other bleeding techniques are contraindicated, blood can be obtained from the posterior vena cava or iliac bifurcation of anesthetized animals.

The animal is placed on its back and a midline incision is made that exposes the abdominal viscera. The large intestine is moved laterally, away from the inguinal area, exposing the large abdominal vessels. The iliac vein is surgically isolated (cranial) just above the bifurcation of the internal iliac and femoral veins. If the posterior vena cava is to be used, it is isolated in a similar manner, just cranial to the common iliac vein and caudal to the renal vein. Because this is a terminal sampling procedure, a sterile surgical technique does not apply. Venipuncture is made using an appropriately sized flexible catheter and needle (the size depends on the size of the animal but will generally be 20–25 gauge). After the needle is inserted into the vein, the flexible catheter is slid forward past the tip of the needle, further into the vein, and the needle is withdrawn. Blood wells up in the hub of the catheter and is collected using the syringe. Any bleeding around the venipuncture site can be controlled by digital pressure with a piece of gauze or cotton. Alternatively, a ligature can be positioned around the vessel before venipuncture and then firmly tied after the needle is inserted.

To obtain a blood sample, the injection cap and catheter are flushed with heparinized saline, approximately 0.3 mL is then aspirated to clear the saline from the catheter, and the required amount of blood is withdrawn. Patency is maintained by flushing the catheter and injection cap with a higher concentration of heparinized (100 μ/mL) saline and then leaving them filled with the solution between sampling intervals.

This technique has been used successfully to collect as many as eight blood samples from a single rabbit within a 2-hr period (with sampling intervals as short as 5 min) and for infusion periods that have lasted as long as 7 days (patency was maintained by daily flushing with heparinized saline). It is particularly applicable for studies to establish the pharmacokinetic properties of new drugs and to observe the reaction of rabbits to drug administration. It can also be used to obtain large (greater than 30 mL) amounts of blood.

Urine Collection

The most common method used to collect urine is by placing the animals in stainless steel metabolic cages, where the urine and feces are separated by a cone-shaped device (the urine drains off the collecting walls into a tube and the feces drop into an inverted cone). Food and water are provided in such a way that the urine will not be contaminated. However, some hair and fecal contamination of the urine could occur.

If contamination of the sample with fecal and other material is not acceptable, urine samples can be obtained by urethral catheterization (practicable in males only) or by direct puncture of the bladder (cystocentesis).

Catheterization

For this procedure, the catheters and any lubricating gels and speculums must be sterilized before use. The animal is sedated or anesthetized, restrained in dorsal recumbency, and the penis is extruded. The catheter (commercially available cat urethral catheter) is introduced into the urethral opening and gently advanced into the urethra and then the bladder. If the catheter is left partially in its container, it can be held without becoming contaminated. When urine flow begins, a syringe can be attached to the catheter and used to aspirate the urine remaining in the bladder.

Cystocentesis

For urine collection directly from the bladder through the body wall, the animal should be sedated and the skin shaved in the midline in the inguinal region. The bladder, located by palpation in the posterior abdomen, is held firmly through the body wall and the needle (1 or 1½ in., 23 gauge) is introduced through the body wall in the midline at approximately a 45° angle. The needle should be inserted in the posterior section of the bladder so it will remain in the bladder lumen as the urine is withdrawn. The urine can be expelled through the needle into a collection tube or by attaching a syringe to the needle.

Special Procedure

Anesthesia

Because there is a great deal of interrabbit variability in sensitivity to anesthetics and because there is a narrow margin between anesthesia and death, a great deal of caution should be exercised in situations requiring anesthetization. In addition, the frequent presence of preexisting lung damage related to infection with *Pasturella multocida* could cause respiratory failure during a period of anesthesia. Recovery from anesthesia in rabbits is often slow, particularly following the use of barbiturates, and the prolonged inappetence that is often a postoperative complication can result in gastrointestinal disturbances.

For routine anesthesia of rabbits, Flecknell (1996) recommends fentanyl/fluanisone (Hypnorm; 0.3 mL/kg intramuscular) and midazolam or diazepam (2 mg/kg intramuscular, intravenous, or intraperitoneal). This drug combination provides good surgical anesthesia with excellent muscle relaxation for 20 to 40 min. It is recommended that fentanyl/fluanisone be administered first, followed 10 to 15 min later by midazolam or diazepam. Longer periods of anesthesia can be achieved by administering additional doses of Hypnorm (approximately 0.2 mg/kg intravenously every 30–40 minutes). See Flecknell (1996) for a complete discussion of the use of preanesthetic and anesthetic medication in rabbits.

The depth of anesthesia should be monitored by rate and depth of respiration and degree of jaw tension. Other indicators such as pedal reflexes, corneal reflex, and papillary size are unreliable.

Euthanasia

Euthanasia can be accomplished using an overdose of a barbiturate such as sodium pentobarbital or a commercial euthanasia solution such as T-61.

Reproduction Procedures

To determine the sex of adult rabbits, the skin is gently pressed back from the genital opening. In males, the penis will be everted; females have an elongated vulva with a short slitlike opening.

The mature male also has perineal, or inguinal, pouches lateral and anterior to the penis. In prepuberal rabbits, when pressure is applied against the genital orifice, the penis everts equally all the way around, whereas the vulva protrudes only laterally and ventrally; the posterior end that does not evert is attached near the anus.

Natural Mating

When breeding rabbits naturally, the doe must be taken to the male's cage. If the female is receptive, copulation will occur soon after introduction to the male. The buck mounts the doe and grasps the female's body with a foreleg on each flank. Intromission is usually accomplished after 8 to 12 rapid copulatory movements and ejaculation follows on the first intromission. Immediately after ejaculation, the male might fall backward or off the side (both hind feet are off the ground during ejaculation) and emit a cry.

Artificial Insemination

For artificial insemination procedures, semen is collected in an artificial vagina. The buck is introduced to a teaser doe. When the buck begins to mount, the vagina is positioned so that the buck can ejaculate into it. Semen samples show considerable variation between individuals for the same individual at different times. Therefore, the semen should be evaluated each time it is collected for amount (should have at least 0.5 mL) and color (if yellow or tan, it is probably contaminated with urine and should be discarded). To assess sperm viability, a small amount of semen is pipetted onto a glass slide and the sperm are observed using a 10∞ objective. Motility is graded as streaming (50% to 100% motility), waving, or shaking. Acceptable samples are streaming; waving or shaking samples should be discarded. The sample is then diluted 1:200, and a sperm count is done. The concentration of sperm should be at least of 40×10^6 sperm/mL. The semen preparation should be incubated at 37°C during the insemination procedure and must be used within 30 min of collection time.

Artificial insemination is done using methods similar to those for vaginal dosing. The doe is manually restrained, and the vaginal opening exposed. An insemination pipette (glass tubing approximately 18 cm in length with an inside diameter of 3 mm and an outside diameter of 4 mm) that is bent at a 45° angle approximately 4 cm from one end is warmed in a 0.9% saline maintained at 37°C. A rubber bulb is attached to the longer end of the pipette and approximately 0.25 mL of diluted semen is gently drawn into it. The short end of the pipette is gently inserted into the vagina with the tip directed toward the dorsal wall to avoid the urethral orifice. After the pipette has been inserted as far as the angle, it is rotated 180° so that the tip points toward the ventral wall and insertion is continued until slight resistance is felt. The bulb is gently squeezed to expel the semen while the pipette is slowly withdrawn. Ovulation is then induced by giving the doe an injection of 0.1 U/g of human chorionic gonadotropin into the marginal ear vein.

Study Designs

The rabbit is used in numerous study designs to evaluate toxicological responses to pesticides, drugs, and industrial chemicals. Depending on the type of material to be evaluated, these test are done according to the U.S. Food and Drug Administration (FDA), Environmental Protection Agency (EPA), Federal Hazardous Substances Act (FHSA), Department of Transportation (DOT), or the Organization for the Economic Cooperation and Development (OECD) guidelines and range from short-term acute studies to 90-day toxicity studies.

The rabbit is also the nonrodent species most frequently used to evaluate developmental toxicity. (In the 1960s, the drug thalidomide was tested and shown to be safe in rats, but caused

Table 6.5 Scale for Scoring Skin Reaction

Erythema	
0	None
1.0	Slight
2.0	Moderate (well-defined)
3.0	Severe (beet red)
Edema	
0	None
1.0	Slight (barely perceptible to well-defined by definite raising)
2.0	Moderate (raised approximately 1 mm)
3.0	Severe (raised more than 1 mm)
Atonia	
0	None
1.0	Slight (slight impairment of elasticity)
2.0	Moderate (slow return to normal)
3.0	Severe (raised more than 1 mm)
Desquamation	
0	None
1.0	Slight (slight scaling)
2.0	Moderate (scaling and flaking)
3.0	Severe (pronounced scaling and denuding)
Fissuring	
0	None
1.0	Slight (definite cracks in epidermis)
3.0	Moderate (cracks in epidermis)
3.0	Severe (cracks with bleeding)

severe birth defects in humans. When given to pregnant rabbits, this drug caused fetal malformations, and now it is required that chemicals must be tested for developmental toxicity in both rodents and nonrodent species.)

Ocular and dermal irritation studies and dermal toxicity studies are types of acute studies that are routinely done using rabbits. Table 6.5 presents a standard scoring chart for skin reactions. Typical designs for these studies and other specialized acute studies such as photoirritation and USP pyrogen tests are presented in tables 6.6 through 6.10.

Ocular Irritation

Eye, or ocular, irritation studies generally last 72 hr, but can be continued for up to 21 days if irritation persists. The guidelines that are followed indicate the number of animals tested and the number of test groups. The rabbits are given an examination to eliminate any animals with preexisting ocular defects, irritation, or corneal injury. The material is instilled in the conjunctival sac and the eye is examined at the specified intervals. If the pH of the test material evaluated is less than 3.0 or greater than 11.5, consideration should be given to not performing the test, as the material can be considered corrosive.

Dermal Irritation and Toxicity

Acute dermal irritation and toxicity studies last 14 days. The length of exposure is 24 hr and the numbers of animals and dose levels depend on the specific testing guidelines. Clinical observations and dermal irritation scores (table 6.5) are recorded daily. As with the ocular study, if the pH of the material is less than 3.0 or greater than 11.5, consideration should be given to not performing the test, because the test material can be considered corrosive.

Table 6.6 Typical Study Designs for Acute Primary Eye Irritation Studies with Rabbits

Low-volume dose (unwashed)	
Duration	72 hr
Dose level	10 Vtl or equivalent weight
Number of animals	6, either sex
Observations	Observe at 1, 24, 48, and 72 hr after instillation; if irritation persists, observe 96 hr, 7 days, 14 days, and 21 days after instillation or until irritation subsides (but not to continue past 21 days)
Termination	Euthanize and discard
Low-volume dose (unwashed and washed groups)	
Duration	72 hr
Dose level	10 Rl or equivalent wieght
Number of animals	9 rabbits; 6 unwashed and 3 washed
Dose administration	Washed at 4 sec after instillation (with 20 ml lukewarm water)
Observations	Observe at 1, 24, 48, and 72 hr; if irritation persists, observe 96 hr, 7 days, 14 days, and 21 days after instillation or until irritation subsides (but not to continue past 21 days)
Termination	Euthanize and discard
FHSA and EPA (unwashed)	
Duration	72 hr
Number of animals	6 rabbits
Observations	Observe at 1, 24, 48, and 72 hr after instillation; if irritation persists, observe 96 hr, 7 days, 14 days, and 21 days after instillation or until irritation subsides (but not to continue past 21 days)
Termination	Euthanize and discard
EPA, 1978 (unwashed and washed groups)	
Duration	7 days
Number of animals	9 rabbits; 6 unwashed and 3 washed
Observations	Observe at 1, 24, 48, 72, and 96 hr and 7 days after instillation; if irritation persists, observe 14 days and 21 days after instillation or until irritation subsides (but not to continue past 21 days)
Termination	Euthanize and discard
OECD (unwashed)	
Duration	72 hr
Number of animals	3 rabbits
Observations	Observe at 1, 24, 48, and 72 hr after instillation; if irritation persists, observe 96 hr, 7 days, 14 days, and 21 days after instillation or until irritation subsides (but not to continue past 21 days)
Termination	Euthanize and discard
OECD (washed)	
Duration	72 hr
Number of animals	6 rabbits; 3 washed for 5 min at 4 sec after instillation and 3 washed for 5 min at 30 sec after instillation
Observations	Observe at 1, 24, 48, and 72 hr after instillation; if irritation persists, observe 96 hr, 7 days, 14 days, and 21 days after instillation or until irritation subsides (but not to continue past 21 days)
Termination	Euthanize and discard

Dermal Toxicity

The rabbit is also used to evaluate the dermal toxicity of a test material. This study can be a short-term, 14-day study or can be up to 90 days in duration. The animals are clipped, treated, and wrapped like an acute dermal study, but the duration of treatment is daily, for 6 hr per day per week. Clinical observations, body weights, and food consumptions are recorded weekly and dermal irritation scores are recorded daily. The animals are necropsied and microscopic examinations are done. Animals are bled before the terminal sacrifice and hematology and clinical chemistry data are obtained. Specific tissue lists, clinical pathology test, and other specific requirements and tests for the 21-day and 90-day toxicity studies are listed in table 6.11.

Table 6.7 Typical Study Designs for Acute Primary Dermal Irritation Studies with Rabbits

DOT (corrosivity)	
Duration	48 hr
Dose level	0.5 g(ml)/rabbit; one intact site per rabbit; 4-hr exposure
Number of animals	6 rabbits
Observations	Observe at 4, 24, and 48 hr after dosing
Termination	Euthanize and discard
FHSA	
Duration	72 hr
Dose level	0.5 g(ml)/rabbit; one intact and one abraded site per rabbit
Number of animals	6 rabbits
Length of exposure	24 hr
Observations	Observe at 24 and 72 hr after dosing; if irritation persists, observe 96 hr, 7 days, and 14 days after dosing or until irritation subsides (but not to continue past 14 days)
Termination	Euthanize and discard
EPA	
Duration	72 hr
Dose level	0.5 g(ml)/rabbit; one intact site per rabbit
Number of animals	6 rabbits
Length of exposure	4 hr
Observations	Observe at 4, 24, 48, and 72 hr after dosing; if irritation persists, observe 96 hr, 7 days, and 14 days after dosing or until irritation subsides (but not to continue past 14 days)
Termination	Euthanize and discard

Teratogenicity

Rabbits are frequently used as the nonrodent species for teratogenicity studies (table 6.12). In this study design, the pregnant doe is treated during fetal organogenesis, days 7 to 19 of gestation. The day of breeding or artificial insemination is gestation day 0. The doe is euthanized prior to term (gestation day 29) and the uterine contents are examined for implantation sites, early and late resorption sites, and live or dead fetuses. The fetuses are weighed, sexed, and examined for external, soft-tissue, and skeletal development.

Parasites, Diseases, and Physical Anomalies

Because it is very difficult and expensive to obtain pathogen-free rabbits, the identification of common diseases and parasites is important to the investigator. In some instances, these conditions might cause an animal to be unsuitable for a study or might require euthanasia of a specific animal or an entire shipment of animals. This section includes a brief discussion of some of the more frequent conditions observed with rabbits, common clinical signs to aid in identification, and suggested treatments.

Mites

Ear Mites

Ear mange (otitis externa) is a condition caused by psoroptid mites, which are nonburrowing parasites that chew the epidermal layers of the skin and produce a tan or brown crusty exudate on the inner surface of the ear. Clinical signs are the rabbit shaking its head or scratching its ears. Although ear mites are nonpathogenic, they can spread from rabbit to rabbit, and, if left untreated, can result in a secondary bacterial infection. Treatment is simple: The ear canal should be cleansed and treated with a few drops of plain mineral oil or mineral oil with a miticide added. A high standard of hygiene should be maintained (Kraus 1974).

Table 6.8 Typical Study Designs for Acute Dermal Toxicity Studies with Rabbits

OECD	
Duration	72 hr
Dose level	0.5 g(ml)/rabbit; one intact site per rabbit
Number of animals	3 rabbits
Length of exposure	4 hr
Observations	Observe at 4, 24, 48, and 72 hr after dosing; if irritation persists, observe 96 hr, 7 days, and 14 days after dosing or until irritation subsides (but not to continue past 14 days)
Termination	Euthanize and discard
FHSA limit test	
Duration	14 days
Dose level	2 g/kg
Number of animals	5 males and 5 females
Skin preparation	Half abraded, half nonabraded
Length of exposure	24 hr
Observations	Clinical observations daily Dermal irritation scored daily (table 6.5)
Necropsy	Necropsy all rabbits found dead
Termination	Euthanize and discard after 14 days
FHSA estimation of lethal dose	
Duration	14 days
Dose levels	3 dose levels; determined by limit test
Number of animals	5 males and 5 females/dose level
Skin preparation	Half abraded, half nonabraded
Length of exposure	24 hr
Observations	Clinical observations daily Dermal irritation scored daily (table 6.5)
Necropsy	Necropsy all rabbits found dead
Termination	Euthanize and discard after 14 days
EPA/OECD limit test	
Duration	14 days
Dose level	2 g/kg
Number of animals	5 male and 5 females
Length of exposure	24 hr
Observations	Clinical observations daily Dermal irritation scored daily (table 6.5)
Necropsy	Necropsy all rabbits found dead
Termination	Euthanize and discard after 14 days
EPA/OECD estimation of lethal dose	
Duration	14 days
Dose levels	3 dose levels; determined by on limit test
Number of animals	5 males and 5 females/dose level
Length of exposure	24 hr
Observations	Clinical observations daily Dermal irritation scored daily (table 6.5)
Necropsy	Necropsy all rabbits found dead
Termination	Euthanize and discard after 14 days

Table 6.9 Typical Study Designs for Special Dermal Studies with Rabbits

Photoirritation/toxicity	
Duration	72 hr
Dose level	0.5 g(ml)/rabbit; two intact sites per rabbit
Number of animals	6 rabbits
Length of exposure	2-hr occluded exposure 10 J/cm^2 UVA to one site
Observations	Observe at 24 and 72 hr
Termination	Euthanize and discard

Table 6.10 Typical Study Designs for Biological Safety Tests with Rabbits

Pyrogen test	
Duration	3 hr
Dose level	1 dose level; single IV injection
Number of animals	3 or 8 rabbits
Observations	Record rectal temperatures at 1, 2, and 3 hr after injection

Fur Mites

There are two types of fur mites that are commonly seen in laboratory rabbits, the cheyletid and sarcoptid mites. The cheyletid fur mite is seen mainly on the dorsal trunk in the scapular area. Often there are no clinical signs except for partial alopecia and a grayish white skin surface; generally there is no scratching by the rabbit. This mite is nonburrowing and ingests the keratin layer of the epidermis. Treatment for this mite consists of the application of a topical gel acaricide (Holmes 1984).

Mange Mites

Another mite that can be seen in groups of laboratory rabbits is the sarcoptid mange mite. Unlike the noninvasive cheyletid fur mite, the sarcoptid mite tunnels through the skin, ingests epithelial cells, and might suck lymph. Clinical signs are rubbing and alopecia and a whitish yellow crust. The rubbing can lead to skin lesions and secondary bacterial infections. This condition is contagious and can become severe, leading to emaciation and death (Holmes 1984). The recommended treatment is elimination of affected animals and clean, sanitary conditions.

Protozoan Infections

Coccidiosis

One of the most important diseases in rabbits is coccidiosis, which is caused by protozoan organisms of the genus *Eimeria*. There are two major types of coccidiosis that are detrimental to rabbits; one affects the liver and the other affects the intestine. Transmission is by ingestion of sporulated oocysts that are in the feces (Harkness 1987).

Encephalitozoonosis

Encephalitozoonosis is a chronic disease seen in rabbits and is caused by the protozoan *Encephalitozoon cuniculi*. It is usually latent and actually prevalent in many colonies (estimates range from 15% to 78% affected). However, it is extremely difficult to diagnose because clinical signs are only occasional neurological signs such as convulsions, tremors, and torticollis (Harkness 1987). Transmission is thought to be via infectious urine. Pathologically, lesions are noted in the kidney and the brain. There is no known treatment (Holmes 1984). The disease is important because it can interfere with and complicate the interpretation of experimental data.

Hepatic Coccidiosis

Eimeria stiedae is the organism that affects the liver. Clinical signs such as diarrhea and weight loss are often seen only in young animals; adults usually show no physical change. Pathologically this organism affects the bile duct epithelium and causes an enlarged or irregularly shaped liver (Pakes 1974).

Table 6.11 Typical Study Design for Dermal Toxicity Studies with Rabbits

21-day toxicity	
Duration	21 days
Dose levels	Four groups of 10 males and 10 females each; dose ml/kg based on most recently recorded weight
Number and age of animals	80 (40 males and 40 females) rabbits 10 to 12 weeks of age
Area of exposure	Approximately 10% of total body surface
Length of exposure	6 hr/day, 5 days per week
Dose administration	Applied uniformly over exposure area
Body weights	Weekly and at sacrifice
Food consumption	Monitor quantitatively or qualitatively, depending on specific guidelines
Observations	At least twice daily for signs of moribundity, mortality, toxicity, and abnormal behavior or poor health
Dermal irritation	Scored daily, before each application of test material and on the day of necropsy Score using scale presented in table 6.5
Clinical pathology	Before terminal sacrifice
Hematology	Red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet count, white blood cell count, differential blood cell count, blood cell morphology, reticulocyte count smear
Clinical chemistry	Glucose, urea nitrogen, creatinine, total protein, albumin, globulin, total bilirubin, cholesterol, aspartate, amino transferase, alanine aminotransferase, y glutamyl transferase, calcium, inorganic phosphorus, sodium, potassium, calcium
Moribund sacrifices/deaths	Gross necropsy, save tissues
Scheduled sacrifice	Gross necropsy, record organ weights, save tissues
Tissues weighed	Brain, kidneys, liver, ovaries, or testes
Tissues saved	Skin (treated and untreated); target organs; lesions
Microscopic examination	Tissues saved from control and high-dose rabbits and macroscopic lesions from all rabbits
90-day toxicity	
Duration	90 days
Dose levels	Four groups of 10 males and 10 females each; additional rabbits (10/sex) can be added to the control and high-dose groups and used as recovery animals; dose ml/kg based on most recently recorded weight
Number and age of animals	80 (40 males and 40 females) rabbits 10 to 12 weeks of age
Age of exposure	Approximately 10% of total body surface
Length of exposure	6 hr/day, 5 days per week
Dose administration	Applied uniformly over exposure area
Body weights	Weekly and at sacrifice
Food consumption	Monitor quantitatively or qualitatively, depending on specific guidelines
Observations	At least twice daily for signs of moribundity, mortality, toxicity, and abnormal behavior or poor health
Dermal irritation	Scored daily, before each application of test material and on the day of necropsy Score using scale presented in table 6.5
Ophthalmic examination	Before initiation of treatment and before terminal necropsy
Clinical pathology	Before terminal sacrifice
Hematology	Red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular, hemoglobin concentration, platelet count, white blood cell count, differential blood cell count, blood cell morphology, reticulocyte count smear
Clinical chemistry	Glucose, urea nitrogen, creatinine, total protein, albumin, globulin, total bilirubin, cholesterol, aspartate, aminotransferase, Y glutamyl transferase, calcium, inorganic phosphorus, sodium, potassium, calcium
Moribund sacrifices/deaths	Gross necropsy, save tissues
Scheduled sacrifice	Gross necropsy, record organ weights, save tissues
Tissues weighed	Brain, kidneys, liver, ovaries, or testes

Table 6.11 Typical Study Design for Dermal Toxicity Studies with Rabbits (continued)

Tissues saved	Adrenals, aorta, bone marrow smear, brains, cecum, colon, duodenum, epididymides, esophagus, eyes, femur and bone marrow, gallbladder, heart, ileum, jejunum, kidneys, lacrimal gland, liver, lungs, mammary gland, mesenteric lymph node, muscle, ovaries or testes, pancreas, pituitary, prostate, rectum, sciatic nerve, seminal vesicles, skin (both treated and untreated), spinal cord, spleen, sternum and bone marrow, stomach, submandibular salivary glands, thymus, thyroid with parathyroid, trachea, urinary bladder, uterus, and lesions
Microscopic examination	Tissues saved from control and high-dose rabbits and macroscopic lesions from all rabbits

Intestinal Coccidiosis

Eimeria irresidua, E. magna, E. media, and E. perforans are the organisms responsible for intestinal coccidiosis. Clinical signs vary depending on the extent of infection and the age of the animal. In cases of mild infections, there are no clinical signs; severe infections result in weight loss, diarrhea, dehydration, and secondary bacterial infections (Holmes 1984). Prevention and control of coccidiosis is by strict sanitization practices and elimination of infected animals.

Table 6.12 Typical Study Designs for Rabbit Range-Finding and Definitive Teratology Studies

Range-find	
Duration	Days 0—29 of gestation
Dose levels	5 groups/5 inseminated females/group; dose ml/kg based on daily weight, most recently recorded weight, or day 7 weight
Number of animals	25 young adult females 5 to 6 months of age
Length of exposure	Days 7–19 of gestation
Body weights	Days 0, 7, 10, 13, 16, 20, 24, and 29 of gestation
Observations	At least twice daily for signs of moribundity, mortality, toxicity, and abnormal behavior or poor health
Moribund sacrifices/ deaths/abortions	Necropsy, examine uterus for implantation sites, resorption sites, and fetuses, if possible
C-section	Day 29 of gestation, gross necropsy, remove and weigh uterus then examine for corpora lutea, implantation sites, resorption sites, live and dead fetuses Options: Expose uterine contents and examine fetuses for external malformations; or expose uterine contents, remove fetuses, examine for external development, and weigh
Definitive	
Duration	Days 0–29 of gestation
Dose levels	Four groups/16 inseminated females/group (must have 12 pregnant does per group; dose ml/kg based on daily weight, most recently recorded weight, or day 7 weight
Number of animals	64 young adult females 5 to 6 months of age
Length of exposure	Days 7–19 of gestation
Body weights	Days 0, 7, 10, 13, 16, 20, 24, and 29 of gestation
Food consumption	Monitor quantitatively or qualitatively, depending on specific guidelines
Observations	At least twice daily for signs of moribundity, mortality, toxicity, and abnormal behavior or poor health
Moribund sacrifices/ deaths/abortions	Gross necropsy, examine uterus for implantation sites, resorption sites, and fetuses, if possible
C-section	Day 29 of gestation, gross necropsy, remove and weigh uterus then examine for corpora lutea, implantation sites, resorption sites, live and dead fetuses Expose uterine contents, remove fetuses
Fetal development	Examine for external development, and weigh. Euthanize fetuses, examine internally (modification of Staples technique), and sex. Midcoronal slice of head and brain, examine brain; remove eyes and examine; eviscerate fetuses and fix in alcohol Macerate fetuses with potassium hydroxide, stain skeletons with calcium-positive alizarine red s, and evaluate skeletal structures for development.

Bacterial Infections

Pasteurella Multocida

Pasteurella multocida is a frequent cause of bacterial disease in rabbits. It causes a multitude of clinical diseases such as snuffles, pneumonia, otitis media, abscesses and conjunctivitis, metritis, or pyometra, and orchitis. Many rabbits carry the organism without any clinical signs of infection until they are stressed as a result of experimental procedures.

Snuffles. This disease is also referred to as rhinitis or sinusitis. It occurs when *P. multocida* is found in nasal cavities (Flatt 1974). It is quite common for rabbits to have this infection without any clinical signs. When stressed, sneezing, coughing, and a nasal discharge might develop. The disease can persist as snuffles or it might progress to other clinical forms.

Pneumonia. *P. multocida* can cause pneumonia in young rabbits 4 to 6 weeks of age. It can progress to cause consolidation in the lungs or pleuritis, or in the case of acute pneumonia, might result in death without any clinical signs (Holmes 1984).

Septicemia. Septicemia has often been associated with snuffles and pneumonia. Clinical signs for septicemia are not observed because, once infected, the animal dies quickly (Holmes 1984).

Otitis. *P. multocida* can spread from the nasal cavities to the inner ear and cause torticollis or otitis interna (Holmes 1984). This should not be confused with otitis externa, which is caused by mites. Rather, it is an infection of the inner ear that is often characterized by a head tilt or wry neck, and can be so severe that the head is upside down.

Metritus and Orchitis. Another condition caused by *P. multocida* is metritis, or pyometra, in the doe and orchitis or epididymitis in the buck. Genital infections occur more in the doe, although they also are observed in young rabbits and adult bucks. Clinical signs of the acute or subacute infection in the doe are seldom seen except for a vaginal discharge. The condition can cause a reduction in fertility either by a failure of the doe to conceive or the buck might have enlarged testes (Flatt 1974).

Abscesses and Conjunctivitis. These conditions are frequently seen in rabbits that have been exposed to *P. multocida* (Flatt 1974). The abscess swellings contain a thick, tan or white creamy exudate and might be surrounded by a fibrous capsule. Conjunctivitis, possibly caused by the bacteria entering the conjunctival sac via the nasolacrimal duct, can occur in both young and mature rabbits. Clinical signs of conjunctivitis are reddened conjunctivae, moderately swollen or closed eyelids, and a mucus exudate (Flatt 1974).

P. multocida is easily transmitted from rabbit to rabbit. It can be spread from dam to young by the respiratory route. It can have venereal transmission when genital infections are present. Because rabbits are infected with the disease without any clinical signs, it can be transmitted when new aminals are introduced into an established colony. The bacteria is sensitive to a number of antibiotics such as chlortetracycline, oxytetracycline, penicillin, ampicillin, and cholramphenicol (Holmes 1984). However, rarely do these treatments provide a complete cure. More often, remission occurs and infection is reestablished when treatment is ceased or when the rabbit is stressed. The best cure is elimination of the disease by euthanasia of affected animals and stringent sanitation practices. Also, it is recommended that investigators purchase rabbits from commercial colonies that provide rabbits that were cesarian derived and maintained in isolation (Holmes 1984).

Tyzzer's Disease

Bacillus piliformis causes a bacterial disease commonly referred to as Tyzzer's disease. There is not a lot of information available on Tyzzer's disease; however, it is thought to be widespread. It is often diagnosed as mucoid enteritis because of the watery diarrhea, stained hindquarters, and dehydration that are seen clinically and very few animals survive (Flatt 1974). This disease generally affects young rabbits, 10 to 12 weeks of age, but can occur in adults and preweanlings. It is transmitted through ingestion of feces of infected animals. Then, if the rabbit is stressed because of overcrowding, heat, or experimentation, the organisms multiply. Treatment of infected animals rarely results in complete elimination of the bacteria. The best remedy is to eliminate infected animals and apply good sanitation practices (Holmes 1984).

Staphylococcal Infections

There are few data concerning the incidences of staphylococcal infections; however, *Staphylococcosis aureus* is the most commonly identified pathogen. Clinical identification of the disease is difficult and is dependent on the site and duration of infection. These infections can range from dermatitis and slight subcutaneous swellings to septicemia and death (Flatt 1974). Staphylococcal infections can be treated with systemic antibiotics, but they are best controlled by maintaining animals in a clean, sanitary environment.

One of the more common staphylococcal infections is mastitis, or "blue breast." It is most prevalent in does that are heavy milk producers, or that have sustained an injury and are maintained in unsanitary conditions Clinical signs are fever, decreased appetite, and inflamed mammary glands. These infections are not limited to lactating does and might also be seen in pseudopregnant does that are maintained in unsanitary conditions (Holmes 1984). The disease is contagious and infected does should be isolated.

Escherichia Coli

Clinical signs of *Escherichia coli* infections are similar to other bacterial infections: diarrhea and death. This type of infection is thought to be a result of changes in the intestinal flora due to changes in environmental conditions. Although this organism is the predominant bacteria cultured, it is also thought to be a secondary infection rather than the primary cause of death (Flatt 1974).

Treponematosis

Treponematosis, or rabbit syphilis, is characterized by inflammation of the face, genitalia, or regional lymph nodes. It is caused by a bacterium, *Treponema cuniculi.* Although it is not commonly seen clinically, the disease is often observed serologically. Treponematosis has been confused with hutch burn, and the lesion might look like that seen with ear mites or sarcoptic mange. The lesions are primarily seen on the external genitalia, but they are also found on the chin, lips, nose, and eyelids. Treponematosis is transmitted by direct contact and is easily treated by isolation of infected individuals and penicillin (Flatt 1974).

Mucoid Enteropathy

Mucoid enteropathy is probably one of the most common diseases affecting rabbits and at the same time is the least understood. It has been called bloat, enteritis, mucoid enteritis, mucoid diarrhea, and scours. It is most prevalent among young rabbits, 7 to 10 weeks of age, and is

associated with the change in nutrition and feed of the suckling rabbit and the weanling. It is not certain if mucoid enteropathy is a disease in itself or just occurs in conjunction with other diseases. It is one of the most common causes of death in young rabbits. Clinical signs are diarrhea, depressed food consumption, hypothermia, bloated abdomen, depression, hunched posture, and the perineal region stained with yellow- to brown-colored fecal material. Macroscopic examinations frequently show stomach and sections of the intestines distended with gas or a watery fluid, gelatinous contents in the colon, and noninflammatory lesions in the intestinal mucosa. Microscopic examinations of the intestinal lesions support the noninflammatory condition and indicate an increase in the production of goblet cells (Flatt et al. 1974). The most productive treatment of mucoid enteropathy is management by prevention, good sanitation, reduced stress, and elimination of animals with clinical signs. Maintaining the animals on restricted feed rations is sometimes effective, or if that is not possible, restricting feed for the first few days postarrival and then gradually increasing to ad libitum. Also, feeding a high-fiber diet has been shown to be effective in reducing the incidence of mucoid enteropathy.

Ulcerative Pododermatitis

Ulcerative pododermatitis is often seen among groups of older rabbits that have been maintained in wire cages for long periods of time. It is a pressure necrosis of the skin and is considered the result of heavy body weight on wire cage floors. It has mistakenly been called sore hocks, but this is an inaccurate term, because the affected area is the entire metatarsal region and is not restricted to the hocks (Flatt et al. 1974). The lesions vary in size and are well defined, ulcerated areas in the skin that are covered by a crusty scab. Secondary bacterial infections and abscesses might occur in the adjacent dermis (Holmes 1984). The affected animal might appear clinically healthy, or it might show a weight loss, hunched appearance, and shifting of weight. The lesion can be treated and the reoccurrence reduced by housing the animal in solid-bottom cages with soft bedding material.

Fractures

The rabbit skeleton is very fragile and is only about 8% of its body weight (compared to a cat skeleton, which is 13% of its body weight; Harkness and Wagner 1983). Because the long bones and lumbar spine are surrounded by powerful muscle masses, the rabbit is particularly susceptible to fractures of the legs and back. Thus, although not a disease, the identification and diagnosis of the severity of a traumatic vertebral fracture is important. It is a posterior paralysis resulting from a vertebral fracture and damage to the spinal cord (Flatt et al. 1974). The onset is sudden, and often the fracture is the result of a struggle during restraint or improper support of the hindquarters when handled. Diagnosis is by clinical examination or radiography. Depending on the location of the lesion and the severity of the fracture, clinical signs are motor paralysis, loss of control of the anal sphincter and urinary bladder, and edema at the site. After a few weeks, function might return and the rabbit might not show any obvious signs of a previous injury (Flatt et al. 1974). However, if the injury is serious enough, it might be necessary to euthanize the animal.

Moist Dermatitis

Moist dermatitis, also known as sore dewlap or hutch burn, is the result of constant wetting of the fur. It is a chronic, progressive disease and there can be various degrees of bacterial involvement. It can be caused from drooling because of malocclusion; drinking from open pans; cold, damp contact bedding; or continuous wetting of the fur because of experimental design (Holmes 1984).

Treatment is by the elimination or reduction of the causes, such as using water bottles and sipper tubes or automatic watering systems instead of open pans, good sanitation, and assuring that the fur is kept clean and dry.

Trichobezoar

Hair that has been ingested can accumulate in the stomach in the form of a ball, or trichobezoar. It generally does not cause any problems and is only noted at necropsy unless the mass becomes very large in size, in which case obstruction can occur. If without apparent reason, an animal suddenly stops eating and drinking, a hairball might be the cause. Hairballs can be detected by palpitation or radiography. Successful treatment consists of giving mineral oil by a stomach tube if the hairball is not too large.

Buphthalmia

Congenital or infantile glaucoma, buphthalmia, is a relatively common disease in laboratory rabbits. It is an increase in the size of the anterior chamber of the eye and can progress to cloudiness in the cornea, flattening of the cornea, and increased prominence of the eyeball. The condition progressively worsens over time and there is no treatment (Lindsey and Fox 1974).

Heat Prostration

Rabbits are very sensitive to heat and husbandry practices should assure controlled temperatures. Clinical signs of heat prostration include rapid respiration, cyanosis, and prostration. Does near kindling and young rabbits in nesting boxes are particularly prone to temperature extremes. Hyperthermia can also cause abortion and a reduction of male fertility.

Malocclusion

Another clinical finding that is not necessarily life threatening but could have an adverse effect on an investigation is malocclusion. Rabbit teeth grow continuously and malocclusions occur when the incisors do not properly occlude and, therefore, are not worn away. Causes could either be a genetic shortening of the upper jaw or the result of an injury. This condition can be easily managed by clipping the teeth on a regular basis.

PATHOLOGY

The previous section presented some of the most common diseases, with their treatments, that might be encountered in laboratory rabbits during toxicology studies. This section on pathology provides additional information on these diseases, as well as spontaneous gross and microscopic changes seen in the common diseases. Some less common conditions of laboratory rabbits are also discussed. The occurrence of spontaneous diseases must be considered when interpreting the outcome of toxicology experiments (Baker 1998 2003). During some experimental studies, changes will be observed that are incidental and have no effect on the results. In other studies, the experiments will exacerbate the severity or incidence of common findings (Dahlgren 1992). The material is presented by organ system with a brief review of diseases primarily affecting that system, their gross and microscopic anatomy, and some of the associated spontaneous pathology.

Integumentary System

Skin

The rabbit body is covered with an abundant, fine, curly undercoat and stiffer guard hairs. The skin has a well-differentiated superficial fascia composed of elastic and dense collagen. Numerous blood vessels lie immediately under the dermis. These features facilitate subcutaneous injections into folds of skin over the back, shoulder, and neck regions. The ears are highly vascular and have prominent marginal veins that can be used for venipuncture. The neck has a large fold of skin called the dewlap that is predisposed to moisture accumulation and moist dermatitis (see later).

Dermal irritation studies and acute dermal toxicity studies are routinely done using rabbits (Anderson and Henck 1994). In these studies, test materials are applied to the skin. Afterward, depending on the experimental protocol, the sites of application are observed at defined time periods for possible adverse effects. Frequent gross observations range from erythema to ulcer formation. The severity of these lesions depends on the duration and frequency of application in addition to the chemical properties of the material involved. Microscopically, these lesions range from mild hyperemia and edema to extensive ulceration. Rabbits were more sensitive than human subjects when exposed to similar amounts of isopropylmyristate (Campbell and Bruce 1981). Griffith and Buehler (1981) compared the responses of skin from rabbits to those from guinea pigs and humans to primary irritants. Rabbit skin reacted with greater severities than guinea pigs or human. Guinea pig reactions were more predictive of the human response to primary irritants than those of rabbits.

Alopecia, the loss or thinning of the hair, is most often observed on the head or back. It can be the result of ectoparasite infestations involving a variety of mites, mycotic infections such as dermatomycosis or ringworm, and hair chewing or "barbering" by cagemates. Alopecia is more common in old rabbits. Alopecia was associated with ribavirin administration in rabbits (Gillett et al. 1990).

Otitis externa is a manifestation of the ear mite, *Psoroptes cuniculi,* the most common and costliest ectoparasite in domestic rabbits (Suckow and Douglas 1997). These are obligate, nonburrowing mites that chew the epidermal layers of the skin and produce marked inflammation. Affected rabbits are often observed shaking their heads or scratching their ears. In heavily parasitized ears, foul-smelling branlike tan or brown crusty exudates might fill the inner surface of the external ear. The ear is often thickened and edematous.

Alopecia and dermatitis of the face, nose, lips, and external genitalia have been associated with *Sarcoptes scabei, S. cuniculi,* and *Notoedres cati.* Unlike the noninvasive fur mites, these mites tunnel through the skin, ingest epithelial cells, and might suck lymph. Early skin changes are alopecia with a whitish yellow crust. Common clinical signs of sarcoptic infestations are pruritis including intense rubbing and scratching that can lead to severe skin lesions and secondary bacterial infections, followed by anemia, leucopenia, self-mutilation, general debility, emaciation, and death (Percy and Barthold 2001). Notoedres infestations are less common and usually mild (Hofing and Kraus 1994).

Fur mites, *Cheyletiella parasitovorax,* can be present with little clinical evidence or a mild alopecia with a grayish white skin surface that affects the back especially over the shoulders. These mites are nonburrowing and ingest the keratin layer of the epidermis. Areas of variable degrees of hair loss, hyperemia, scaliness, and crusts are present. Microscopically, these mites cause a mild subchronic dermatitis with hyperkeratosis, congestion, and inflammatory cell infiltrations. These inflammatory cells might include polymorphonuclear neutrophils, mononuclear cell phagocytes, lymphocytes, plasma cells, and small numbers of eosinophils (Foxx and Ewing 1969). This alopecia is accompanied a pruritis that is only evident after careful observation. Generally there is no scratching by affected rabbits. Another fur mite, *Listrophorus gibbus,* has been reported sporadically in domestic rabbits. This mite might be more common than currently realized because it causes no clinical signs of its presence. The preferred site of attachment is the underside of the tail and with

little movement in the hair (Hofing and Straus 1994). See the preceding Toxicology section for treatment of ectoparasites.

Morgan et al. (1985) described treatment-related skin lesions of epidermal necrosis, hyperkeratosis, and inflammation during skin irritation tests that became infected by dermal mucormycosis. *Rhizopus sp.* were identified in the tissues. The development of fungal dissemination might require more time than the usual 7- to 21-day studies.

Dermal mycotic infections such as dermatomycosis or ringworm are uncommon. They might be subclinical or characterized by raised, circumscribed, erythematous areas with crusted surfaces and hair loss. Lesions usually occur on the head, face, and ears with less frequent secondary spread to the forepaws. They can be very pruritic with affected rabbits scratching vigorously at lesions. The most common cause is *Trichophyton mentagrophytes.* Less commonly *Microsporum canis* is involved. Microscopically, characteristic changes include hyperkeratosis, epidermal hyperplasia, and folliculitis with mononuclear and polymorphonuclear cell infiltration. Fungal arthrospores in infected hair shafts are demonstrated by methenamine silver and PAS-stains.

Hair chewing or barbering is occasionally observed in young, group-housed rabbits. It occurs on the face and back as a patchy alopecia with no evidence of a concurrent dermatitis. Contributing factors of boredom and low-roughage diets have been implicated. Abrasions with hair loss are common as a result of fighting among group-housed rabbits that have reached sexual maturity. The lesions after fighting include lacerations around the external genitalia. Aggressive males might injure both bucks and does, thereby resulting in skin abrasions and amputation of the tips of the ears.

Hair pulling can result in patchy thinning or hair loss in adult rabbits especially in nest-building females as well as boredom, or seasonal and idiopathic moulting.

Exfoliative dermatosis and sebaceous adenitis have been reported, primarily in older adult pet rabbits. It is characterized as a nonpruritic scaling dermatosis with patchy to coalescing areas of alopecia. Microscopic changes include hyperkeratosis, follicular interface dermatitis, interface folliculitis, reduction in the numbers of sebaceous glands with destruction and lymphocytic infiltration, and perifollicular to diffuse dermal fibrosis (White et al. 2000).

Ulcerative dermatitis or pododermatitis (sore hocks) is often seen among groups of older rabbits that have been maintained in wire cages for long periods of time. The condition is commonly termed sore hocks, but this is inaccurate, because the affected area is the entire metatarsal region and is not restricted to the hocks. Affected animals might appear clinically healthy, or might show a weight loss, hunched appearance, shifting of body weight, or reluctance to move. Lesions occur on the plantar aspect of the foot, adjacent to the metatarsal bones. They are caused by pressure necrosis from heavy body weight on wire cage floors. Lesions of various sizes consist of well-defined, ulcerated areas in the skin covered by a crusty scab. Secondary bacterial infections and abscesses might occur in the adjacent dermis. Microscopically, it consists typically of a circumscribed, ulcerated area covered by granulation tissue and necrotic debris with purulent exudate on the surface. The condition most commonly affects heavy, mature adults. Additional factors such as poor sanitation, trauma from wire-bottom cages with rough edges, and hereditary predisposition might contribute to the occurrence of the disease. *Staphylococcus aureus* is frequently isolated from these lesions (Percy and Barthold 2001). Incidence can be reduced by housing animals in solid-bottom cages with soft bedding material.

Moist dermatitis, also known as sore dewlap or hutch burn, is the result of constant wetting of the fur. It might be caused by panting associated with high environmental temperatures; drooling because of malocclusion; drinking from open pans; cold, damp contact bedding; or continuous wetting of the fur because of experimental design. The dermatitis that results is a chronic, progressive disease with various degrees of bacterial involvement. Inflammation in the subcutaneous tissues can progress to suppuration and abscesses with ulceration of the overlying skin. The most common bacterial agent is *Fusobacterium necrophorum* that causes necrobacillosis (Bergdall and Dysko 1994). Another organism sometimes associated with moist dermatitis is *Pseudomonas aeruginosa*

that can result in discoloration of the fur, blue fur disease (Richardson 2000). Prevention consists of elimination or reduction of the causes to assure that the fur is kept clean and dry.

In addition to pododermatitis, dermatitis at other sites can be caused by bacterial infections. *Staphylococcus aureus* is the most commonly identified pathogen. These infections vary in duration and range from a localized mild dermatitis with slight subcutaneous swellings to abscesses, septicemia, and death (Percy and Barthold 2001). These subcutaneous abscesses usually contain a thick, tan or white creamy exudate and might be surrounded by a fibrous capsule. *Corynebacterium pyogenes* and *Pasteurella multocida* also can cause suppurative and ulcerative skin lesions in rabbits. Streptococci have been reported to cause septicemic infections in young rabbits. Skin lesions affecting the genitalia, perineal region, nose, mouth, and eyelids can result from infections caused by *Treponema cuniculi.* These lesions can be raised, crusted, or ulcerated.

Multifocal aggregates of lymphocytes can be found surrounding hair follicles or in the deep dermal areas of untreated, healthy rabbits. The stimulus for their development is unknown.

In addition to the skin conditions previously described, inflammation can also result from lacerations and abrasions caused by sharp objects in the environment (e.g., nails and wire), as well as the teeth and claws of other rabbits when fighting. Painful skin problems can change the temperament of affected rabbits for the worse (Stein and Walshaw 1996).

Viral infections of rabbits can cause neoplasia involving the skin and connective tissues. These viruses are not a problem in toxicology studies. For details about these virus infections, see DiGiacomo and Mare (1994).

Skin tumors other than viral fibromas or fibromatosis, myxomatosis, and papillomas include lipoma, squamous cell carcinoma, trichoepithelioma, basal cell tumors, and melanomas (Weisbroth 1994).

Mammary Gland

Does have four or five pairs of mammary glands that extend in broad bands from the throat to the groin (Richardson 2000). The milk is high in fat and protein (Harkness and Wagner 1989). Inflammation of the mammary glands, mastitis or "blue breast," is sometimes observed in recently kindled does. It is most prevalent in does that are heavy milk producers. Teat traumatic injuries including biting of the nipples and unsanitary conditions are additional predisposing factors. Mastitis is not limited to lactating does and can also be seen in pseudopregnant does that are maintained in unsanitary conditions (Richardson 2000). In severe cases, the doe and young rabbits could die. Clinical signs typical of inflammation include fever, decreased appetite, abnormal milk, and infected, swollen, firm mammary glands. The overlying skin has a red to dark blue discoloration. On section, the affected gland might contain material having various appearances, ranging from watery fluids to thick purulent exudates. Abscesses can form, containing a thick, tan or white creamy exudate and surrounded by a fibrous capsule. *Staphylococcus, Pasteurella,* and *Streptococcus spp.* are the most common causative agents.

Cystic mammary gland hyperplasia is associated with a condition termed cystic mastitis. This lesion occurs in one or more glands of nonbreeding does over the age of 3 years and appears to be a preneoplastic change. Often it is associated with uterine hyperplasia and uterine adenocarcinomas. Clinical signs are a nonpainful swelling of the affected gland and a bluish skin color with no evidence of inflammation. The nipple might have a brown serosanguinous discharge. Masses or fluid-filled cysts are sometimes palpable within the mammary tissues (Richardson 2000). A biopsy can be useful in distinguishing between cystic hyperplasia and neoplasia.

Mammary gland dysplasia has been associated with pituitary adenomas and uterine adenocarcinomas. Benign and malignant tumors of the mammary gland are less common than uterine adenocarcinomas and most often occur in multiparous females that are 3 to 4 years of age. Papillomas and adenocarcinomas have been observed, especially in some families of breeding rabbits (Weisbroth 1994). Animals with mammary adenocarcinomas frequently also have uterine adenocarcinomas.

Musculoskeletal System

Rabbits must be picked up and held correctly to prevent both animal and human injuries. Because they are very shy animals and are easily frightened, they often struggle and try to escape. If a rabbit is picked up incorrectly or not held securely, it will kick violently with its hind legs, jump, or thrash (Suckow and Douglas 1997). The rabbit skeleton is fragile, almost birdlike, and comprises only 7% to 8% of the total body weight, whereas the skeletal muscle constitutes more than 50% of the body weight (Percy and Barthold 2001). Because the long bones and lumbar spine are surrounded by powerful muscle masses, the rabbit is particularly susceptible to fractures of the legs and back (Harkness and Wagner 1989). Traumatic lumbar vertebral fracture, usually in the lumbosacral region at L6 and L7, results in posterior paresis or paralysis following damage to the spinal cord. The onset is sudden, often after thrashing during restraint, or handling without proper support of the hindquarters. Depending on the location and severity of the fracture, the results are partial or complete motor paralysis with loss of control of the urinary bladder and anal sphincter. After a few weeks of intensive nursing care, function might return and the affected rabbit might show no obvious signs of a previous injury (Richardson 2000). However, if the injury is serious enough, it could be necessary to euthanize the animal. Young rabbits can fracture legs if there are holes in wire cages large enough to trap their feet. Geriatic rabbits can develop paresis and paralysis as a result of vertebral degeneration in association with aging. Vertebral spondylosis is common (Richardson 2000).

Deficiencies in vitamin E can result in muscle damage. Affected rabbits have muscle soreness and stiffness. At necropsy, gross findings consist of pale mineralized streaks in skeletal and cardiac muscle. Common gross sites are the diaphragm, paravertebral regions, and the hind limbs. Microscopic findings are hyaline degeneration and necrosis of affected myofibers with mineralization of the sarcoplasm and histiocytic or granulomatous inflammation. Later lesions have collapse of the sarcolemmal sheaths and interstitial fibrosis (Percy and Barthold 2001).

Rabbits, especially adults, are very sensitive to vitamin D toxicity. This toxicity results in calcification of many organs and tissues. Increased resorption of calcium from bone leads to osteodystrophy with osteoid dysplasia and osteosclerosis. Microscopically, the bone lesions are characterized by excessive production and deposition of a highly cellular, abnormal basophilic osteoid with many active osteoblasts. These changes are observed on the periosteal and endosteal surfaces, medullary trabeculae, and haversian systems (Percy and Barthold 2001).

Young rabbits with "splay leg" keep one or more legs spread and lack the ability to adduct the affected limbs. They cannot come to a standing position. These rabbits can have a variety of congenital abnormalities including inherited syringomelia, hypoplasia pelvis, femoral luxation, and distal foreleg curvature. Greene (1965) investigated several hereditary disorders in rabbits, including oxycephaly, brachydactylia, dwarfism, cretinism, dwarf-cretin complexes, and hydrocephalus. He also studied the role of heredity in the toxemia of pregnancy and neoplasia. Large doses of vitamin A given to pregnant rabbits can produce a high incidence of gross congenital malformations (Shenefelt 1972). Inherited diseases and developmental abnormalities are described by Palmer 1976 and Lindsey and Fox 1994).

The mandible of rabbits is the most frequent site for inflammation of the bone, osteomyelitis, and rare bone neoplasms, osteosarcomas (Stein and Walshaw 1996). An osteoma (Stedham 1976), an osteochondroma, and an osteosarcoma involving the appendicular skeleton (Weisbroth 1994) have been reported.

Digestive System

Rabbits are herbivores with a large and relatively complex digestive tract that fills a capacious abdominal cavity. Sometimes, they have been referred to as pseudoruminants (Arrington 1978). The stomach typically contains approximately 15% of the ingesta present in the digestive tract.

The small intestine is relatively short for a mammal, having approximately 12% of the total volume of the GI tract. Rabbits are hindgut fermenters. Based on the body weight, the rabbit has the largest cecum of any living mammal. The cecum typically holds about 40% of the ingesta present in the digestive tract. Fine particulate materials are selectively channeled into the cecum during the passage through the large intestine, and larger particulate material composed of undigestable fiber is usually directed into the colon and passed as dry fecal pellets. At night, rabbits practice coprophagy; that is, they consume small, mucous-coated, soft, moist fecal pellets, cecotrophs, high in B vitamins and protein produced during normal cecal fermentation. Lymphoid tissues are abundant in the intestinal tract of rabbits. Lymphoid (Peyer's) patches are large and prominent in the small intestine; however, most of the lymphoid tissues are found in the large intestine. The gut-associated lymphoid tissue (GALT) represents more than 50% of the total mass of lymphoid tissue in the body, which might account for the relatively small spleen seen in rabbits. A common incidental finding in rabbits is the presence of large histiocytes filled with particulate debris in the follicular centers of GALT.

Not only is it important to understand how different parts of the digestive tract function in the rabbit, but also the importance of a proper diet. A balanced diet with adequate amounts of plant fiber is necessary to ensure normal gut motility and thereby prevent or control several diseases of the digestive system (Richardson 2000).

Normally, the stomach will effectively sterilize the food consumed with its pH of 1 to 2. Nutrients are absorbed in the small intestine from the fluid ingesta exiting the stomach. In the large intestine, as noted earlier, the indigestible fiber is processed into hard fecal pellets and the smaller particles are fermented in the cecum before being processed into cecotrophs. When conditions for movement of ingesta are disrupted, disease can follow. The transit time for food in the rabbit digestive tract is normally short. Diets high in indigestible fiber, especially lignocellulose, stimulate the rapid movement of ingesta through the GI tract. Diets low in fiber lead to reduced gut motility and gastric stasis with increased incidences of impaction. Diets with high sugar and starch contents exceed the small intestine's ability to absorb them and their passage into the cecum where proliferation of microorganisms can result in enterotoxemia. Anorexia and other illnesses can cause the pH of the stomach to rise, reducing the effectiveness of the stomach to sterilize the contents. Also, a compact ball of concentrated dry food in the stomach can prevent effective sterilization, allowing microorganisms to pass further down and cause adverse effects (Richardson 2000). If the ball of food becomes large enough, impaction will result.

A high-fiber, low-protein, low-carbohydrate diet is more natural for the rabbit. The digestive system of the rabbit works best when provided ample plant fiber such as diets containing good-quality hay with decreased amounts of pelleted diets. Also, offering leafy greens is advisable. Changes in the diet such as new vegetables should be done gradually. Starch and fat should be minimal. Proper diets will help prevent many digestive tract problems. Rabbits need to be able to exercise daily.

Teeth

The dental formula for a rabbit's 26 to 28 teeth is 2 (2/1 incisors, 0/0 canines, 3/2 premolars, and 2–3/3 molars). Rabbits have a set of small incisors called peg teeth directly behind the front incisors. These small incisors are thought to protect the upper gum during chewing (Richardson 2000). The upper incisors fit anteriorly to the bottom incisors. Folds of skin between the incisors and premolars form an antechamber with limited visibility and access to the rest of the oral cavity (Cruise and Brewer 1994).

Malocclusion is not life threatening but could have an adverse effect on a research study. All rabbit teeth have open roots and therefore grow continuously. Incisor teeth of rabbits can grow 10 to 12 cm a year (Harkness and Wagner 1989). Malocclusions occur when the incisors do not properly occlude and, therefore, are not worn away. The most common cause is an inherited shortening of the upper jaw, mandibular prognathia, or the result of fractured or missing teeth

following head injuries. Affected rabbits have weight loss, difficulty in chewing their food, and excessive drooling, ptyalism (Suckow and Douglas 1997). This condition can be easily managed by clipping the teeth on a regular basis.

Ulceration of the mouth is common. The first signs are salivation and anorexia. The cause is malocclusion of the cheek teeth. The lower molars cause ulcerations on the tongue, whereas the upper molars generally grow laterally and cause buccal ulcerations of the cheek (Richardson 2000).

Tongue

The tongue is large and covered by spine-like structures called filiform papillae that have no taste buds. The base of the tongue has a pair of large circumvallate papillae on the dorsal surface that are occasionally mistaken for papillomatous growths. Also, rabbits have rows of well-developed foliate papillae on the caudal, lateral border, and fungiform papillae on the rostal border of the tongue. The circumvallate, foliate, and fungiform papillae are all shown to have taste buds (McLaughlin and Chiasson 1979).

Oral papillomatosis (warts) is a rare virus disease in rabbits. It results in white lesions on the underside of the tongue that can become ulcerated. The lesions are limited and usually regress after several weeks (Richardson 2000).

Salivary Glands

Four pairs of salivary glands are present: the parotid, submaxillary, sublingual, and zygomatic. The largest are the whitish-brown parotids. They extend below and in front of the base of the ears. The submaxillary are oval glands at the angle of the mandible. Sublingual are small, flattened glands adjacent to the mandible and the submaxillary glands. The zygomatic or infraorbital glands lie in the anteroventral angles of the orbit, just below the lacrimal glands.

Stomach

The stomach has cardiac, fundic, and pyloric portions. The cardiac portion is thin walled and relatively immobile, with numerous small glands. The exocrine secretory region of the highly glandular fundus is the major source of acid for the pH of 1 to 2 in the stomach. It has gastric pits lined with parietal (oxyntic) cells that secrete acid and intrinsic factor and peptic (chief) cells that secrete pepsinogen. The pyloric region has thick layers of smooth muscle. Submucosal areas often contain heterophils and lymphocytes. Grossly, the mucosa often appears to have red foci that are not apparent microscopically. These areas are assumed to be areas of hyperemia that bleach out during fixation. Because of its thin wall, the stomach must be handled with care. It is easily ruptured during necropsy. Also, the stomach undergoes autolysis rapidly after death and might spontaneously rupture before the necropsy can begin (Feldman and Seely 1988).

Fastidious grooming habits predispose rabbits to ingestion and subsequent accumulation of hair, leading to the formation of hairballs (trichobezoars) in the stomach or pylorus. The rabbit is unable to vomit because of a well-developed cardia sphincter, so these accumulations cannot be removed by that route. Hairballs are common in the stomach, especially in older adult animals that lick or pull their own fur. Reduced gastric motility from low-fiber diets, anorexia, a lack of exercise, and long-haired breeds are predisposing factors for the development of hairballs (Richardson 2000). They generally do not cause any problems and are only noted incidentally at necropsy. In a few rabbits, the hair mass becomes large enough to obstruct the flow of food resulting in anorexia, weight loss, and decreased fecal output. If the obstruction is prolonged, death could result. Gastric rupture with peritonitis has been observed (Suckow and Douglas 1997). Hairballs are less common in the duodenum.

Small Intestines

The small intestines consist of a relatively long duodenum with a short jejunum and ileum. Brunner's glands are distributed throughout the length of the duodenum. The common bile duct from the liver and gallbladder opens into the duodenum near its origin at the pylorus and the pancreatic duct opens into the duodenum near the origin of the jejunum. The wall of the jejunum is thinner and appears less vascularized than the duodenum. Lymphoid aggregates (Peyer's patches) are large and prominent throughout the jejunum and ileum. A rounded enlargement, the sacculus rotundus, is formed where the ileum ends at the ileocecal valve.

Marked plasma cell infiltrations in the intestinal tract have been described as intestinal plasmacytosis in rabbits used in research studies. Older rabbits were particularly at risk, especially those used for antibody production or cholesterol studies. The lesions were frequently only observed during microscopic examinations of the tissues. Severely affected animals had grossly thickened intestinal mucosa. Microscopically, lesions were most prominent in the small intestine and cecum with less involvement of the lymphoid tissues of the sacculus rotundus or cecal appendix. Variable degrees of mucosal erosion, dilatation of lacteals, and blunting of the overlying villi occurred in the small intestine. Marked infiltrations of well-differentiated plasma cells with uniform sizes and shapes were observed in affected tissues. When severe, infiltrations of dense aggregates in the lamina propria completely replaced the normal architecture. These infiltrates were usually confined to the intestinal tract; however, in a few animals, plasma cells were increased in the spleen and mesenteric lymph nodes.

Intussusceptions are uncommon, but usually occur in young rabbits with enteritis. Anorexia and a cessation of droppings are clinical signs in affected rabbits.

Large Intestines

The large intestines consist of a spiral cecum, a sacculated colon, and the rectum. At the ileocecal junction adjacent to the sacculus rotundus, the cecum has a thickened round patch of lymphoid tissue called the cecal tonsil. The cecum is very large with thin and smooth walls. The inner surface is greatly increased by a long spiral fold or valve. The cecum terminates in the vermiform or cecal appendix, a thick-walled narrow blind tube with abundant lymphoid tissues. The grossly thickened walls of the sacculus rotundus, cecal tonsil, and vermiform appendix are due to aggregates of organized lymphoid tissue and macrophages in the lamina propria and submucosa. The colon can be divided into three major segments: the ascending, transverse, and descending colons. These segments are characterized by numerous pouches or haustra formed between bands of tissue termed taeniae. In the ascending colon, the taeniae form three rows of haustra. The haustra gradually decrease to a single row in the descending colon. The ascending colon is a long and coiled tube that ascends from the ileocecal junction to the liver. Because the ascending colon is within the pelvic cavity, this location can be used to collect accurate sections of the colon for microscopic examination. The transverse colon is a short segment between the ascending and descending colon. Along the greater curvature of the junction of the transverse and descending colons is a slightly curved spindle-shaped structure, the fusus coli, which is unique to lagomorphs. The fusus coli is an area 5 cm to 8 cm in length of thickened circular muscle and mucosa that has numerous ganglion cell aggregates. The mucosa here is four to five times thicker than in the remainder of the descending colon. The fusus coli serves as a pacemaker regulating the movement of ingesta into the descending colon and transporting more fluid chyme back into the cecum (Cruise and Brewer 1994). The long descending colon terminates in the short, straight rectum and anus. Lymphoid patches can be found throughout the submucosa of the cecum and colon. Sections of the rectum often contain submucosal lymphocytes that do not form aggregates or foci.

In the cecum, bacteria ferment small pieces of cellulose to produce B vitamins and volatile fatty acids that are contained in cecotrophs. The predominant organisms are *Bacteroides spp.* with

smaller numbers of *Clostridium sp., E. coli,* and *Streptococcus faecalis*. Saccharomycetaceae yeasts are considered to be part of the normal flora and nonpathogenic. The *Bacteroides* are gram-negative cellulolytic anaerobes. The cecotrophs have a mucilaginous coating that forms a capsule to survive passage through the stomach's acidity after ingestion. Nutrients from the cecotrophs are absorbed in the small intestine (Richardson 2000). When excessive fermentable carbohydrates are fed, the cecal contents become more acid. The amount of normal flora is decreased and the *Clostridium sp.* proliferate. The result is enteritis. As the fiber content of the diet goes down, numbers of *Clostridium sp.* and *E. coli* increase. Reduced intestine motility is another result of a low-fiber diet. Because the stomach and cecum empty very slowly, the rabbit produces fewer and fewer fecal pellets. The rabbit will stop eating. This might be followed by impaction from a mass of ingesta. Feeding of diets that are high in protein, low in fiber, and high in carbohydrates or sugar will cause rabbits to produce more cecotrophs than are needed with accumulation of excess cecotrophs on the fur. Other causes for the accumulation of excess cecotrophs include rabbits that are overfed who lose the urge for coprophagy, large rabbits in hutches so small that they do not have room for coprophagy, and rabbits that are very overweight, have excessive dewlap, incisor or molar malocclusion, or arthritis and spinal pain preventing coprophagy (Richardson 2000).

Diets low in fiber result in reduced motility and the build-up of food material in the cecum. This excess in food material can result in a cecal impaction. Fermentation of the excess food material can lead to tympany. Further, the presence of excessive amounts of sugars or starch can result in an altered pH of the cecum contents with proliferation of *Clostridium spiriformes* and a fatal enterotoxemia.

Young rabbits experience extensive stress during shipment and on arrival at a testing or research facility, including variations in diet and temperature, as well as unusual noises and unfamiliar handling. This stress can predispose these young animals to infections by agents in the new environment and agents that might have been carried from the production facility.

Enteritis complex has been used to encompass the multifactorial group of enteric diseases of rabbits, especially those 5 to 12 weeks of age. The most common clinical sign is diarrhea. It is associated with many causes including dietary changes; bacterial, protozoal, and viral infections; antibiotic use; or times of stress. A combination of several causative factors are often involved. The higher stomach pH (3–7) in rabbits with diarrhea allows bacteria to pass into the large intestine. Several causative factors have been shown to be associated with enteritis including the stress of weaning, changes in diet, insufficient fiber, carbohydrate overload with subsequent alterations in gut flora and intestinal pH changes, crowding, and transport. Infectious agents are now recognized to have an important role in causing enteritis. These agents include rotaviruses, coronaviruses, *Clostridia, Escherichia coli, Lawsonia, Salmonella, Vibrio,* and coccidia. Mucoid enteropathy does not appear to be caused by a specific infectious agent, but might be associated with bacterial toxins, although the pathogenesis is unclear. A condition known as antibiotic toxicity occurs when certain antibiotic agents alter the normal intestinal flora, allowing an overgrowth by enterotoxic *Clostridia*. Enteric diseases vary greatly in their outcome. Some are transient with little effect on the health of the rabbit, whereas others are often fatal. Some specific disease entities are discussed later and in more detail in Percy and Barthold (2001) and Manning et al. (1994).

Enteritis in newborn or suckling rabbits can be precipitated by hypothermia and maternal neglect. The most common bacterial organisms associated with enteritis in newborn or suckling rabbits are enteropathogenic strains of *Escherichia coli* and *Staphylococcus spp.* (Percy and Barthold 2001). The clinical signs are usually a yellow, watery diarrhea that stains the perineum and abdomen. Within a litter, the morbidity and mortality can reach 100%. At necropsy, the gross findings are milk in the stomach, whereas the remaining digestive tract is filled with watery contents. Isolation of *E. coli* is significant in rabbits under 2 weeks of age because it is not normally present in the flora at this age. Many of these young rabbits die as a result of septicemic infections.

Weanling rabbits of 4 to 8 weeks of age are more susceptible to enteric diseases than adults. Up to 21 days, protective antimicrobial fatty acids found in the stomach of newborn and suckling

rabbits control the digestive tract microflora. After 21 days, these protective fatty acids decrease and the large intestine acquires its bacterial flora. At the time of these changes in the digestive tract, these rabbits become more susceptible to enteritis (Richardson 2000). Other causative factors include the stress of weaning, changes in diet, insufficient fiber, carbohydrate overload with subsequent alterations in gut flora and intestinal pH changes, crowding, and transport. The clinical signs in these young rabbits are profuse diarrhea, dehydration, and a rough hair coat. Mortality can reach 100%. Lesions occur in the cecum that resemble those of *Clostridium spiroforme* enterotoxemia. The predominant findings are hemorrhage and edema of the cecum with watery or mucoid blood-tinged contents.

Several species of *Clostridium* have been implicated in enteritis and enterotoxemia in rabbits including *C. perfringens, C. difficile,* and *C. spiroforme. C. piliforme,* the cause of Tyzzer's disease, is discussed separately. These organisms are gram-positive bacilli that reside in the large intestine and grow under anaerobic conditions. After significant changes in the diet, pathogenic strains are able to produce powerful enterotoxins or cytotoxins that can result in severe and often fatal enteric disease. Early studies demonstrated the type E "iota" toxin in fatal cases of enterotoxemia that was believed to have been produced by *C. perfringens.* Other studies have demonstrated the type E iota toxin can be produced by *C. spiroforme*. Fatal colitis and enterotoxemia have been associated with overgrowth of *C. difficile* following prolonged treatment with antibiotics against gram-positive organisms such as procaine penicillin, cephalexin, erythromycin, ampicillin, amoxicillin, clindamycin, lincomycin, tylosin, and metronidazole. The toxic effects of these antibiotics vary from animal to animal. *C. difficile* infection was observed in specific pathogen-free rabbits in the absence of antibiotic treatment. The organism was found in the small intestine and cecum. It was able to produce toxins A and B (Percy and Barthold 2001).

Enterotoxemia generally occurs within the first 24 to 96 hr after shipment during the quarantine period and is rarely, if ever, diagnosed in animals after they have been removed from the quarantine regimen. Clinically, these animals appear to be somewhat depressed, inactive, anorexic, and often have a greenish brown discoloration on the perineal area. Diarrhea is moderate to severe. These animals are generally dehydrated, but death often occurs before dehydration is apparent. Affected animals often have a high death rate.

Gross findings depend on the course of the disease. In acute illness, the carcass is usually in good condition with perineal staining of the fur. In subacute to chronic cases, carcasses are usually thin and dehydrated. There is extensive staining of the perineum, belly, and rear legs with watery green to tarry brown feces. Straw-colored fluid might be present in the peritoneal cavity. Extensive ecchymotic hemorrhages are usually present in the serosal of the cecum, sometimes with the distal ileum and proximal colon affected. Ecchymoses can also occur in the epicardium and thymus. The large intestine, especially the cecum, is frequently dilated with watery to mucoid, green to dark brown contents with gas formation. The affected large intestine can be markedly thickened as a result of edema. The mucosa findings can vary from hemorrhage to ulceration and fibrinous exudates. Microscopic changes in the cecum are those of a necrotizing typhlitis with irregular denuding of the mucosa, ulceration, fibrinous exudation, and leukocytic infiltration, predominantly heterophils. The mucosal epithelial cells, enterocytes, can be swollen, vacuolated, flattened, denuded, or undergoing replacement proliferation. There can be relative sparing of the crypt bases and the lamina propria. The mucosa and submucosa are usually congested, with frequent hemorrhages and thrombi. Large numbers of gram-positive bacilli can often be demonstrated adhered to the mucosal surface. In animals that survive the earlier necrotic changes, the mucosa undergoes repair characterized by epithelial hyperplasia. At this stage, the clinical signs are those of malabsorption with continued diarrhea (Percy and Barthold 2001).

Another major source of enteritis in commercial rabbitries and occasionally in research facilities is colibacillosis, caused by enteropathogenic strains of *Escherichia coli.* Colibacillosis results in an acute, sometimes explosive diarrheal disease that often produces a high mortality rate young rabbits in the 10- to 20-week age group. *E. coli* is normally absent or present in small numbers in

the digestive tract of suckling and weanling rabbits. The absence or small numbers are attributed to the low pH of the stomach. Therefore, the stomach and small intestine are usually relatively free of these bacteria. Colibacillosis is an infrequent problem in laboratories that provide good care and adequate diet. Clinical disease is associated with rapid proliferation of *E. coli* and a shift from the normal predominantly gram-positive in the large intestine to a gram-negative bacterial flora. This rapid growth can be associated with several factors, including abrupt changes in diet or management, experimental studies with compounds such as antibiotics that can affect the bacterial flora of the intestinal tract, intestinal coccidiosis with its accompaning mucosal damage and resorption of endotoxins, and a change in cecal pH. The normal pH of the large intestine exerts an antibacterial effect in rabbits. Strains of *E. coli* isolated during outbreaks of this disease are classified as enteropathic. These strains cause intestinal disease, do not produce enterotoxins, and are not considered enteroinvasive. Isolates vary widely in pathogenicity as well as their response to antibiotics and improved sanitation. The ability of these isolates of *E. coli* to attach to mucosal epithelial cells of the small or large intestine is closely related to the age of the rabbits from which they were isolated (Percy and Barthold 2001). Although *E. coli* is often the predominant bacteria isolated, it should be noted that this organism can also be secondary to the primary cause of death.

Gross findings are dehyration and a perineal region stained with watery yellow to brown feces. The small intestine usually appears normal. Frequently, the cecum and colon are distended with watery yellow to gray-brown contents. Other findings are edema with mucosal and subserosal ecchymotic hemorrhages in the cecum or colon, edematous mesenteric lymph nodes, and prominent lymphoid tissues in Peyer's patches and the sacculus rotundus. Microscopic changes in suckling rabbits with coliform enteritis are characterized by large numbers of coccobacilli attached to the mucosal epithelial cells in both the large and small intestine with infiltration of the lamina propria by heterophils. Microscopic lesions in weanlings are usually more extensive with blunting of the villi in the ileum, edema of the lamina propria, and leukocytic infiltration, predominantly by heterophils. Epithelial cells at the tips of the villi in the small intestine and at the tips of the large intestine crypts are swollen and have attached bacterial colonies. Also, the cecum and colon frequently have detachment and ulceration of the epithelium at the tips of the cecal folds.

Colibacillosis occurs less frequently in rabbits at any age past weaning. The disease can range from a mild diarrhea and weight loss to a severe diarrhea with mortalities up to 50%. The diarrhea can lead to intussusception or rectal prolapse. After recovery, affected rabbits might remain undersized. Gross examinations can reveal "paintbrush" hemorrhages on the cecum and colon serosal.

Another form of bacterial enteritis characterized by a sudden outbreak of profuse watery diarrhea, stained hindquarters, and dehydration is a component of Tyzzer's disease caused by *Clostridium piliformis,* previously named *Bacillus piliformis.* This disease clinically resembles mucoid enteritis, has a short course of illness with mortality rates of 10% to 50%. It usually affects young rabbits, 10 to 12 weeks of age, but can occur in adults and preweanlings as well. It is transmitted through ingestion of feces of infected animals. The onset of illness is often associated with stress such as overcrowding, excessive heat, or experimental procedures that allow the organisms to multiply (Percy and Barthold 2001). Gross findings are usually extensive ecchymotic hemorrhages, occasionally with fibrinous exudate on serosal surfaces in the cecum and colon. Frequently the liver has disseminated pale foci. Less frequently the myocardium has pale, linear streaks, particularly near the apex of the left ventricle. Animals that survive the initial disease have thin carcasses and usually identifiable circumferential regions of fibrosis and stenosis in the terminal ileum or large intestine. Microscopic changes are consistently present in the intestinal tract, usually in the liver, and infrequently in the myocardium. The intestinal tract has focal to segmental necrosis of the mucosa of the cecum with variable involvement of the distal ileum and proximal colon. Large numbers of bacteria are often present on the damaged mucosal surface. The affected intestine wall has extensive edema, muscle necrosis, and leukocytic infiltrates, predominantly heterophils. Liver lesions are characterized by periportal foci of coagulation or caseation necrosis, often with a conspicuous scarcity of peripheral inflammatory cells. Long

filamentous intracytoplasmic organisms in characteristic bundles stained by the Warthin-Starry silver or Giemsa methods can often be found in or near the areas of hepatic necrosis. Tyzzer's bacilli are not present in lesions examined after the acute phase of the disease. Heart lesions consist of focal or linear areas of coagulation necrosis with a minimal inflammatory response (Percy and Barthold 2001).

Mucoid enteritis or enteropathy is one of the most common diseases affecting weaned young rabbits 7 to 14 weeks of age, a vulnerable time when the cecal microflora is still becoming established. It does not appear to be caused by a specific infectious agent but is associated with changes in the diet that decrease the cecal pH and disrupt the normal cecal flora. A possible role for bacterial toxins has been suggested. Inadequate dietary fiber with cecal and colonic impactions, and use of antibiotics such as clindamycin and lincomycin appear to contribute to this disease. Mucoid enteritis rarely occurs in rabbits that are fed a high-fiber diet with limited amounts of grains, proteins, and fats. Enteritis associated with extensive mucus production in older rabbits is usually a part of the enteritis complex and has a lower mortality rate. Affected rabbits produce abundant gelatinous or mucoid feces. Other clinical signs include mucoid diarrhea, depressed food consumption, depression, a bloated abdomen, grinding of the teeth, hypothermia, weight loss, polydipsia, hunched posture, and the perineal region stained with yellow to brown-colored fecal material. Mortalities are very common in these young rabbits. Up to 60% of affected rabbits might have an accompanying pneumonia. Surviving rabbits grow very slowly. Gross findings include a stomach and sections of the intestines filled with gas or a watery fluid, excessive production of mucus in the cecum, gelatinous contents in the colon, and cecal or colon impaction without evidence of mucosal inflammation. A characteristic microscopic finding is marked noninflammatory goblet cell hyperplasia in the small and large intestine. This finding is consistent with the clinical observations (Percy and Barthold 2001).

Salmonellosis is an uncommon enteric disease in rabbits that is usually associated with contaminated food or water. The most frequent types isolated are *Salmonella typhimurium* and *S. enteritidis.* Clinical signs include septicemia, depression, pyrexia, and rapid death, often accompanied by diarrhea. Pregnant does might abort. Gross findings of septicemia are diffuse petechial hemorrhages and vascular congestion of various organs. Does can develop metritis. Necrotic pale foci are found on the liver and spleen. Lymph nodes are edematous (Percy and Barthold 2001).

Infections by *Lawsonia intracellularis,* previously named *Campylobacter*-like organism, can result in proliferative and histiocytic lesions in the small intestines of rabbits as well as a number of species including rats, hamsters, and guinea pigs. This is an obligate intracellular bacterium that can reach large numbers within the enterocytes of the small intestine. Infections are common in some rabbit colonies. Lesions are often mild and found as incidental findings in affected rabbits. Clinical signs vary with the stage of infection and the age of the affected rabbit. Acute infections can result in diarrhea and death in suckling, weanling, and young adult rabbits. Gross findings in the acute disease can consist of semifluid mucinous contents in the colon and rectum. In more chronic infections, there might be thick opaque loops of small intestine. In these animals, the mucosal surface of the small intestine has a markedly thickened and rugose appearance (Percy and Barthold 2001). Microscopic findings in the mucosa vary from acute inflammation with erosions and suppuration to proliferation with multifocal to diffuse hyperplasia of enterocytes lining crypts and villi associated with a prominent histiocytic infiltration accompanied by occasional multinucleated giant cells in the lamina propria. Special stains reveal typical clusters of small bacteria in the apical cytoplasm of enterocytes (Percy and Barthold 2001).

Other bacteria that have been associated with enteritis and diarrhea include *Vibrio sp.* and *Pseudomonas sp.* infections from contamination of drinking water.

Coccidiosis is the most common parasitic disease in rabbits. Twelve species of protozoan organisms are known to infect the rabbit, all in the genus *Eimeria.* The disease has two forms, one affecting the liver and the other affecting the intestine. Transmission is by ingestion of sporulated

oocysts that are in the feces. Cecotrophs do not contain sporulated oocysts because cecotrophy does not allow time for sporulation. Therefore, cecotrophs are not infective (Richardson 2000).

Liver infections are caused by a single species, *Eimeria stiedae.* Clinical signs such as diarrhea and weight loss are often seen only in young animals; adults might show no physical change. This species colonizes the bile duct epithelium and causes an enlarged or irregularly shaped liver (Pakes and Gerrity 1994). Affected livers can result in distended or pendulous abdomens. Grossly numerous yellow or white spots might be present on and in the liver. Heavily infected rabbits can develop signs of bile duct obstruction with liver enlargement, ascites, jaundice, diarrhea, and anorexia, and result in death (Richardson 2000).

Intestinal coccidiosis can result from infections by several species, including *Eimeria irresidua, E. magna, E. media,* and *E. perforans.* Although all of these species colonize intestines, the site and pathogenicity depend on the specific species. *E. magna* has high infectivity for the small intestine, whereas *E. perforans* has low infectivity for the small intestine. *E. media* has moderate infectivity for both the small and large intestine (Richardson 2000). Mixed infections with more than one species are common. Clinical signs vary depending on the extent of infection and the age of the animal. Young rabbits are most commonly affected with diarrhea, weight loss, and anorexia. The diarrhea frequently is characterized by blood and mucus. Severe diarrhea can result in intussusception. In mild infections, no clinical signs might be evident. Severe infections can result in dehydration and death due to secondary bacterial infections (Richardson 2000).

At least two viruses are known to cause enteritis in suckling and weanling rabbits. Both rotavirus and coronavirus have been isolated, and both affect rabbits between 3 and 12 weeks of age. Infections with rotavirus alone are often subclinical and can be endemic in some rabbit populations. In young rabbits under stress and exposed to other secondary agents, both rotavirus and coronavirus infections can result in severe clinical signs, including anorexia, diarrhea, dehydration, and death. Adult rabbits can have subclinical coronavirus infections. The gross findings for both diseases are similar with intestines distended and congested and petechial hemorrhages in the small and large intestines. Microscopically, both viruses cause villous atrophy of the jejunum and ileum. Identification of which virus is involved requires virus isolation procedures (Richardson 2000). A highly contagious entercolitis has been reported in suckling, weanling, and lactating rabbits in Europe that appears to be caused by an as yet unidentified virus. Clinical signs are characterized by anorexia, moderate watery diarrhea, reduced GI motility that progresses to ileus, and death in 2 to 3 days. Gross findings are abundant fluid in the small intestine with gas and mucus in the colon (Richardson 2000).

The nematode *Passalurus ambiguus,* a pinworm, is a common parasite of domestic rabbits. Adult worms are very small and found in the large intestine, primarily in the cecum and colon. Juvenile stages are found in the mucosa of the small intestine and cecum. Infestations of small to moderate numbers are considered nonpathogenic with no clinical signs. Heavy infestations are reported to cause impaired weight gain, poor breeding performance, and occasionally death. Tapeworms are rarely seen in laboratory rabbits. More detailed information on parasites of rabbits is available in Pakes and Gerrity (1994), Hofing and Kraus (1994), and Percy and Barthold (2001).

Liver

The liver has two major lobes and several smaller ones. A deep median fissure at the falciform ligament divides the liver into a large right and smaller left lobe. The right lobe is further divided into a right medial lobe adjacent to the gallbladder, the quadrate lobe, and the small caudate lobe often found in the groove for the vena cava. The primary nonspecific microscopic changes consist of lymphocyte infiltration in the periportal area and varying degrees of hepatocytic cytoplasmic vacuolation or fatty change. The incidence of lymphoid infiltration varies with age and is more common in females. The severity is generally minimal to mild. Vacuolation of hepatocytes is nonspecific but appears to be more common in females (Wells et al. 1988), and is greatly affected

by ad libitum feeding of standard diets and fasting prior to blood collection (Weisbroth et al. 1990). Extreme fatty vacuolation of hepatocytes and hepatic necrosis can develop in pregnant, pseudopregnant, and postparturient females resulting in a condition known as pregnancy toxemia. A similar toxemia occurs in obese male rabbits. Although the cause of this disease appears complex, there are some consistent findings. The affected animals are obese and quit eating. Lactation might be an additional causative factor. The rabbits show signs of depression and anorexia, might abort or even convulse, and die with few clinical signs of illness. The urine is clear. Urinalysis demonstrates evidence of protein and ketones. Gross findings are an empty stomach, obesity, a pale yellow liver and kidneys, possible dead fetuses, and uterine hemorrhage. Hill et al. (1988) have described a spontaneous storage-like disease characterized by hepatocytes with expanded, foamy cytoplasm and distinct cell borders. These lesions occurred singularly or as multifocal aggregates. Necrosis of individual or small groups of hepatocytes with no zonal distribution is not uncommon. It is assumed that the etiology is circulatory in origin.

Cytoplasmic vacuolation of hepatocytes associated with glycogen accumulation is a variable finding seen microscopically in rabbits fed commercial diets (Percy and Barthold 2001).

In rabbits, torsion of the caudate process generally results in a fatal subcapsular hepatic rupture several centimeters long with hemorrhage into the peritoneal cavity. The gross lesion resembles a hemorrhagic tumor. Microscopic examination reveals the lesion is an infarct (Ruebner et al. 1965).

A systemic viral disease has been reported that results in hepatic necrosis and hemorrhages in the lungs, kidneys, and other tissues. (See the cardiovascular system for more details.)

Bile duct dilation with striking hyperplasia of the epithelium can be a feature of hepatic coccidiosis. Milder coccidial infections that heal can resemble biliary cirrhosis (Ruebner et al. 1965). Bile duct tumors are relatively common spontaneous neoplasms in rabbits (Weisbroth 1994).

Gallbladder

The gallbladder is located in a rounded cavity in the medial surface of the right medial lobe of the liver and contains a dark greenish fluid. At necropsy, the gallbladder often has ecchymotic serosal hemorrhages. These hemorrhages rarely extend the full thickness of the wall. Mucosal hyperplasia with frond-like papillary projections has been observed in animals that were being fed 40% to 50% of a normal libitum diet. It is assumed that this hyperplasia is in response to cholestasis or gallbladder distention.

Pancreas

The pancreas is a diffused irregular mass of tissues located in the mesentery of the small intestine. It often contains foci of lymphocyte infiltration within acinar areas with no apparent acinar necrosis. Individual acinar necrosis or granular depletion of the cytoplasm can be found in animals that have undergone prior fasting for 12 to 16 hr. The islets are microscopically similar to other species. New Zealand White rabbits have been reported to develop a disease resembling maturity-onset diabetes mellitus.

Respiratory System

Rabbits are very sensitive to heat. Clinical signs of heat prostration include rapid respiration, cyanosis, and prostration. Does near kindling and young rabbits in nesting boxes are particularly prone to temperature extremes. Open mouth breathing is an indication of severe respiratory distress and carries a guarded prognosis (Richardson 2000). Normally rabbits are obligate nose breathers. Twitching of the nostrils is normal behavior in active rabbits; however, it might be absent in resting or sick animals. Nasal and ocular discharge, anorexia, labored breathing, abscesses, reduced reproductive ability, head tilt, and circling are usually associated with *Pasteurella multocida* infections.

Some breeds of rabbits, such as the Flemish Giants, appear to be more susceptible to *P. multocida* infections than others.

Nasal Cavity

The nasal cavity presents no unusual anatomical structures. The respiratory surface might contain multifocal areas of mucoid material. The submucosa can contain individual or small aggregates of lymphocytes but no lymphoid follicles have been recognized.

Snuffles is a rhinitis or sinusitis characterized clinically by a mucous or mucopurulent nasal discharge that stimulates the animal to rub the nares with the medial surface of the front limbs and paws. The resulting wetness of the paws might be visible even though the nasal area exudation is dry. Sneezing, coughing, and respiratory "snuffling" are common symptoms. The gross and microscopic lesions are usually limited to the nasal cavity. Purulent exudate is present on the surface of the cavity or turbinates with heterophils (neutrophils) and edema present within the submucosa or on the mucosal surface. Gram-negative rod-shaped bacteria can be found within the exudate with the aid of special stains. Usually *P. multocida* is isolated from the exudates. The incidence varies from 20% to 80%, but with the establishment of breeding colonies free of *P. multocida* the current incidence could well be below 10%. Rabbits commonly are infected with this organism without any clinical signs. After being stressed, these rabbits can begin sneezing and coughing, followed by a nasal discharge. The snuffles can persist, spread to the middle ears, or progress to pneumonia and septicemia.

Trachea and Lung

The trachea is an unpaired tubular air passageway that extends from the larynx to where it divides into primary bronchi. It is supported by segmented incomplete cartilage rings and lined by respiratory epithelium with histologic features similar to those of rats and mice. The lungs are divided into six primary lobes with both the left and right lungs having apical, cardiac, and diaphragmatic lobes. The left apical lobe is very small. The right diaphragmatic lobe is further divided into a large lateral and a smaller accessory lobe (Wingerd 1985). There are peribronchiolar lymphoid foci that tend to increase in size with age. There are no respiratory bronchioles in the rabbit.

Several of the barbital-based anesthetic agents cause grossly apparent petechial hemorrhages on the surface of the lungs that disappear with fixation. Macrophage-like cells with foamy-appearing cytoplasm can be found within the alveolar spaces of untreated, control rabbits. These cells can occur in lungs that have no other evidence of inflammation.

Enzootic pneumonia is an acute fibrinous pneumonia. Extension to the pleural surface, pleuritis, pericardium, and pericarditis are common secondary complications. Although some animals might have clinical signs of pneumonia or septicemia, others die acutely without any clinical signs (Percy and Barthold 2001). Often the only signs of pneumonia are anorexia and depression. This disease is caused by *P. multocida.* Typically, it occurs in young rabbits 4 to 6 weeks of age, and might follow an outbreak of snuffles. Mortality rates can reach 40% of affected rabbits. The gross lesions are typical of acute or peracute fibrinous pneumonia with anteroventral consolidation of the lungs, pleuritis, and pericarditis. Other findings include lung congestion, petechial and ecchymotic hemorrhages, and peritonitis. Microscopically, the bronchioles and alveoli are filled with heterophils, fibrin, and necrotic debris (Dahlgren 1992).

Even though it is possible to obtain rabbits that are *Pasteurella* free, pasteurellosis continues to be a significant bacterial disease in rabbits. Infections by *Pasteurella multocida* can be manifested in several forms such as snuffles, enzootic pneumonia, otitis media, genital infections (metritis, or pyometra and orchitis), abscesses, conjunctivitis, and septicemia. Many rabbits carry this organism without any clinical signs of infection. *P. multocida* is easily transmitted from rabbit to rabbit and can spread from an infected dam to her offspring by the respiratory route shortly after birth. Venereal

transmission can occur when genital infections are present. Because it is difficult to recognize infections in carrier animals, an asymptomatic infected rabbit might be introduced with subsequent spread throughout an established colony. These asymptomatic rabbits usually carry the organisms in the nasal cavity. It is believed that stress such as experimental procedures can compromise the rabbit's ability to resist these bacteria, allowing them to multiply rapidly and spread to other sites as well as other animals. The development of the specific clinical disease might depend on the route of infection or extent of dissemination. Other bacterial agents that can cause respiratory disease in rabbits include *Staphylococcus aureus* and *Pseudomonas aeruginosa* infections. *Bordetella bronchioseptica* is considered to be a part of the normal bacterial flora in rabbits and in most situations is nonpathogenic. A coronavirus has been identified that causes pleural effusions in rabbits. Clinical signs are fever, anorexia, weight loss, hind limb weakness, and difficult breathing (Richardson 2000). Gross findings are pulmonary edema and pleural effusions. Microscopic changes in fatal infections were characterized by lymphoid depletion of the spleen follicles, focal degenerative changes in the thymus and lymph nodes, proliferative changes in glomerular tufts, and uveitis. Animals that survive the acute disease have myocardial degeneration and necrosis, focal hepatic necrosis, and proliferative changes in the spleen, lymph nodes, interstitial pulmonary tissue, and glomeruli (DiGiacomo and Mare 1994). Pulmonary aspergillosis has been observed in apparently healthy young rabbits (Matsui et al. 1985).

Metastatic uterine adenocarcinomas are the most frequent neoplastic lesions in the lungs. Clinical signs of affected rabbits include weight loss, dyspnea, and lethargy (Richardson 2000). Primary neoplasms in the lung are very rare (Weisbroth 1994).

Endocrine System

Pituitary Gland

The pituitary often contains multiple endothelium-lined spaces at the junction between the pars distalis and intermedia. True cystic spaces or hyperplasia are not common in this species. An eosinophil adenoma has been reported in the anterior pituitary (Weisbroth 1994).

Adrenal Gland

The adrenal glands are paired organs located anterior and medial to the kidneys. They appear flattened against the dorsal body wall. The left adrenal is adjacent to origin of the superior mesenteric artery and the right adrenal lies adjacent to the postcava. Adrenal glands have the usual cortex areas with a distinct medulla seen in other species. The cortex often contains vacuolated cytoplasmic areas in cells that compromise the zona fascicularis. Accessory cortical nodules are found beneath the cortical capsule or embedded within the adipose tissue adjacent to the capsule. Greene (1965) reported that localized areas of adrenal cortical hyperplasia resembling adenomas occurred in his rabbits associated with the coming of spring. These hyperplastic lesions disappeared as the seasons changed. The adrenal medulla can contain areas of hyperplasia involving small groups of cells. Although these foci might suggest preneoplasia, there is no reported evidence that pheochromocytomas develop from these hyperplastic foci.

Two adrenal cortical carcinomas have been reported in rabbits (Weisbroth 1994).

Thyroid and Parathyroid Glands

The bilobed thyroid gland is located in the usual lateral aspect of the trachea, distal to the cricoid cartilage. Microscopically, the parathyroid is often embedded with the body of the thyroid. The thyroid contains follicles of various sizes that contain colloid. Typical C cells are difficult to identify by standard light microscopy. Thyroids of adult animals often contain lymphoid aggregates

within the interstitial areas. Ectopic thymic tissue can be identified within the capsule of the thyroid. The parathyroid presents no unusual changes with advancing age.

Urinary System

Kidney

The overall kidney structure is similar to that of the rat. It is bean shaped. On longitudinal section, a single papilla has extensive evaginations of the pelvic fornices. The number of glomeruli increases after birth. About 60 ectopic glomeruli are present in each kidney of adult rabbits. Aging glomerular changes begin to be observed at 1 year of age. These changes consist of mesangial proliferation with a multifocal distribution. Progression to a more diffuse distribution and extension into the capillary loops is the end stage of this mesangial proliferation. The capillary wall appears to thicken with age, but the exact nature of this thickening has not been well documented. Numerous experimental studies exploring the histological and ultrastructural changes in immune complex glomerulonephritis have shown a deposition of immune complexes at varying locations along the capillary wall. It is not known if such immune complex disease(s) occur in the rabbit in a natural setting. It is not unusual to find obsolete glomeruli or glomeruli that contain peripheral capillary loops that are collapsed or adherent to each other. There are no other changes and clinical chemistry and urinalysis values do not suggest renal disease. Perhaps ultrastructural changes occur, but such reports have not been forthcoming. Amyloid and hyaline material containing lipid has been found within capillary configurations. These deposits have been found in animals fed high-lipid diets and used in cholesterol-related studies.

Observations of evidence of kidney disease are fairly common during necropsies of old rabbits. Although renal failure is rare, a history of polydipsia, polyuria, or urine scald in a depressed, thin, aged rabbit should suggest advanced renal disease. Kidney damage can be caused by infections by *Encephalitozoon cuniculi, Staphylococcus aureus,* and *P. multocida*.

Tubular and interstitial changes consist of the deposition of pigments or lipids and lymphoid and macrophage infiltration. Lipid droplets are often found in the cytoplasm of the convoluted tubular epithelium in animals that have been fasted prior to sacrifice. Other pigments have been found in the convoluted or collecting tubules and usually represent products associated with red cell destruction or hepatic failure. Focal or multifocal areas of interstitial mononuclear cell infiltration are common findings. Usually, there is no evidence of tubular damage or evidence of specific infectious agents. However, leptospirosis has been suggested as a possible etiological agent, but there has been no documented case in the recent English literature. The cause of these mononuclear infiltrates remains undetermined.

Embryonal nephromas are relatively common spontaneous neoplasms in rabbits (Weisbroth 1994). Experimentally nephroblastomas can be produced in large numbers by exposure to N-ethylnitrosourea (Haenichen and Stavrou 1979; Hard 1986; Hard and Fox 1983, 1984).

Urinary Bladder

Rabbit urine is normally cloudy due to the large amounts of crystals. The normal color can range from yellow to red or red brown. The pH is typically alkaline. The urinary bladder in mature rabbits contains alkaline urine having abundant quantities of dull yellow to brown calcium carbonate and triple phosphate crystals (ammonium, magnesium, and calcium carbonate monohydrate). In rabbits, the calcium is absorbed in proportion to the amount in the diet. Excess calcium and magnesium are excreted primarily in the urine. Occasionally, the urine of normal rabbits has a dark red to orange color. This color is thought to be due to dietary porphyrins. Pigmented urine has also been associated with elevated levels of urobilin.

Adult pregnant females can develop urinary retention due to the position of the uterus. Occasionally microscopic examination of the urinary bladder will reveal acute suppurative inflammation of the mucosa (cystitis). Frequently, this cystitis will extend into the ureter (ureteritis) and renal pelvis (pyelitis). The pyelitis can lead to pyelonephritis and pyonephritis.

The blood in the urine, hematuria, is observed in animals with uterine adenocarcinomas, uterine polyps, episodic bleeding from endometrial venous aneurysms, cystitis, urinary bladder polyps, pyelonephritis, and renal pelvic hemorrhage (Percy and Barthold 2001).

Genital System

Inguinal canals that connect the abdominal cavity to the inguinal pouches do not close in the rabbit. The inguinal pouches are blind structures located lateral to the genitalia in both sexes. These pouches often accumulate white to brown secretions produced by scent glands in their walls. The male secondary sex glands (i.e., seminal vesicles, vesicular glands, prostate, and bulbourethral glands) are not separable except in general terms.

Does reach puberty at 4 to 9 months of age and their breeding life span is 3 to 4 years. The placenta is hemochorial, in which maternal blood flows into sinus-like spaces where the transfer of nutritients to fetal circulation occurs. Passive immunity is transferred to the young via the yolk sac prior to birth rather than by colostrum or the placenta.

As noted earlier, rabbits are very sensitive to heat. Does near kindling and young rabbits in nesting boxes are particularly prone to temperature extremes. Hyperthermia can also cause abortion and a reduction of male fertility.

The rabbit is also the nonrodent species most frequently used to evaluate developmental toxicity. In the 1960s, the drug thalidomide was tested and shown to be safe in rats, but caused severe birth defects in humans. When given to pregnant rabbits, this drug caused fetal malformations, and now many governmental agencies require that chemicals be tested for developmental toxicity in both rodent and nonrodent species (Anderson and Henck 1994). Vitamin A imbalances can result in hydrocephalus, infertility, abortions, resorption, and increased neonatal mortalities (Richardson 2000). Vitamin E deficiencies can cause infertility, muscular dystrophy, prenatal mortalities, and seminiferous tubal degeneration (Cheeke 1994). Vitamin A deficiencies are a cause of hydrocephalus in rabbits (Newberne 1973). Large doses of vitamin A given to pregnant rabbits can produce a high incidence of gross congenital malformations (Shenefelt 1972).

Skin lesions of rabbit syphilis, treponematosis, primarily affect the external genitalia and perineal region, but also are found on the chin, nose, lips or mouth, eyelids, and regional lymph nodes. These lesions can be raised, crusted, or ulcerated. They result from bacterial infections caused by *Treponema cuniculi*. This is a rabbit venereal disease that spreads among adult rabbits during breeding and is transmitted to young rabbits at the time of parturition or lactation. Clinical disease is not common. Transient infertility can occur in both sexes. Infertility has been attributed to metritis or retained placentas in females and to preputial inflammation in males. More often infected animals are detected by serological testing. Treponematosis has been confused with hutch burn. Also, the skin lesions can resemble those of ear mites or sarcoptic mange.

Genital infections are commonly associated with pasteurellosis and salmonellosis causing metritis and pyometra in females, and orchitis and epididymitis in males. These infections occur more frequently in does, but they also are observed in young rabbits and adult bucks. They often result in a reduction in fertility (Dahlgren 1992).

Pregnancy toxemia is a problem in obese rabbits and can occur in males and nonpregnant does.

Ovary and Oviducts

The ovaries are slightly elongated, small structures that are frequently obscured by a mass of fat, which should be carefully removed for examination. Microscopically, the ovaries are similar in appear-

ance to other species. An enlarged portion of the oviduct forms a funnel-shaped structure, the ostium tubae that partially surrounds the ovary. The other end of the oviduct opens into a horn of the uterus.

Ovarian abscesses commonly caused by *P. multocida* are often found during gross and microscopic examinations at the end of reproductive or teratology studies. These abscesses usually contain a thick, tan or white creamy exudate and might be surrounded by a fibrous capsule.

It is not unusual to find oviductal "cysts" on one or both oviducts. Microscopic examinations of these cysts suggest ductal origin. According to Arey (1965), the cranial portion of the mesonephric duct persists as the duct of the epoophoron and its tip becomes the cystic vesicular appendage. These cysts could be termed mesonephric or Wolffian duct cysts.

Ovarian tumors are rarely reported in older female rabbits (Weisbroth 1994). Affected rabbits might have a history of decreased reproduction rate or failure to conceive.

Uterus and Vagina

The doe has two separate uterine horns with two separate cervices that unite to form the vagina. The uterine horn has a characteristic thick muscular wall. The thickened muscular tissue between the uterus and the vagina is termed the cervix. The urethra lies along the ventral surface of the vagina and joins with the vagina to form a common tube, the urogenital sinus or vaginal vestibule. The external margins of the urogenital sinus form the vulva.

The clitoris is the female homolog of the male penis and lies along the ventral surface of the urogenital sinus. The glans clitoris projects into the urogenital aperture.

Uterine infections, metritis and pyometra, are commonly caused by *P. multocida*. A vaginal discharge is usually the only clinical sign of acute or subacute metritis and pyometra in the doe. Reduced fertility as a result of does that fail to conceive is often observed. The lesions are usually found during gross and microscopic examinations at the end of reproductive or teratology studies.

Endometrial venous aneurysms can result in persistent vulvar or urogenital bleeding. At necropsy, the endometrium has multiple blood-filled, thin-walled dilated veins and blood clots in the lumen of the uterus. These veins apparently rupture and bleed periodically into the uterus lumen resulting in a bloody discharge or hematuria. They appear to be congenital defects seen in multiparous does (Percy and Barthold 2001).

Bloody vulvar discharge with anorexia, weight loss, and reproductive failure has been observed in animals with uterine hyperplasia and adenocarcinomas. Labored breathing can be a sign of metastasis to the lungs by adenocarcinomas. Cystic mammary glands frequently accompany these uterine changes. Uterine adenocarcinomas are common spontaneous, highly invasive neoplasms that occur in does 2 years of age or older (Baba and von Haam 1972). The incidence rate of uterine hyperplasia and adenocarcinomas in female rabbits has been reported as high as 80% in does over 5 years of age. The incidence appears to be influenced by breed (Greene 1965). The highest rate is observed in Dutch rabbits, with moderately high incidences in the New Zealand White and Californian rabbits (Stein and Walshaw 1996).

Leiomyomas and leiomyosarcomas have been reported sporadically in female rabbits (Weisbroth 1994).

Scrotum

The scrotum of the rabbit is hidden beneath the fur cranial to the penis and ventral to the pelvis. This location is unique for placental mammals. The scrotum consists of a sac of skin, connective tissue, and muscle that encloses the testes.

Testes

The testes are paired, elongated, whitish organs with a marbled appearance that are enclosed within a thick sheath, the tunica albuginea. They descend at 12 to 14 weeks (Richardson 2000).

Spontaneous testicular findings in adult rabbits include perivascular mononuclear cell infiltrations. In some rabbits, there is evidence of vascular damage and inflammation (periarteritis). It is not uncommon to have a few peripheral seminiferous tubules in a section of the testes appear to be aspermatogenic or necrotic. This is most likely a fixation and handling artifact (Dahlgren 1992).

The most frequent testicular neoplasms are interstitial cell tumors. They occur predominantly in 5- to 7-year-old bucks used for breeding. These tumors cause enlargement of the testes with areas of necrosis. Seminomas have been rarely reported (Weisbroth 1994).

Epididymis

The epididymis is a highly vascularized and highly convoluted tubule that is a continuation of the seminiferous tubules from the testes. Interstitial perivascular mononuclear cell infiltrations are common observations in the epididymis, as well as the seminal vesicle, vesicular gland, and prostate.

Inflammation of the testes, orchitis, and the epididymis, epididymitis, in the male can be caused by *P. multocida*. The lesions are usually found during gross and microscopic examinations at the end of reproductive or teratology studies. These infections can cause a reduction in fertility by preventing conception of the does (Dahlgren 1992). *P. multocida* can be transferred from rabbit to rabbit by males with genital infections. Clinical signs of orchitis, and epididymitis in the buck, can include enlarged testes or development of abscesses with purulent drainage. These abscesses usually contain a thick, tan or white creamy exudate and might be surrounded by a fibrous capsule.

Seminal Vesicle

The seminal vesicle is an elongated sac originating from the base of the bladder and lying along its dorsal side. Spontaneous vesicular epithelial squamous metaplasia, hyperplasia, and keratinized nodules were described by Zwicker et al. (1985).

Vesicular Gland

The vesicular gland, also termed the coagulating gland, is a weakly bilobed tubuloalveolar gland adjacent to the prostate and separated by a thin connective tissue septum with smooth muscles.

Prostate Gland

The prostate is a glandular thickening of the dorsal wall of the basal portion of the seminal vesicle. Spontaneous prostate epithelial squamous metaplasia, hyperplasia, and keratinized nodules were described by Zwicker et al. (1985).

Bulbourethral Gland

The bulbourethral gland is a small structure lying on the dorsal surface of the urethra behind the prostate gland.

Penis

The penis consists of a pair of heavy muscles, the crura, a body formed by the corpora cavernosa, and the end, the pars libera. The pars libera is protected by a fold of skin, the prepuce.

Cardiovascular System

The heart is similar to other species except that it is relatively small and the right atrioventricular valve is bicuspid rather than tricuspid. The right chambers of the heart are relatively thin walled,

and a frequent postmortem finding is a quantity of clotted blood in the right ventricle, with no evidence of postmortem contraction (Percy and Barthold 2001). Veins of rabbits are delicate, thin walled, and tear easily (Harkness and Wagner 1989).

Pale streaks running parallel to the long axis of the ventricle are frequent gross findings in obese females and in females late in gestation (26–30 days). These streaks are on the epicardial surface and can be confused with pericardial fat unless the pericardial sac is removed. Microscopically, the streaks have areas of mineralized fibers or a loss of fiber staining but no evidence of inflammation. The etiology is unknown.

Spontaneous interstitial myocarditis consisting of irregularly distributed foci of mononuclear inflammatory cells has been frequently reported in apparently healthy adult rabbits. In other reports, small lymphocytic infiltrates with no evidence of myocardial damage or other inflammatory reactions were observed in a very large percentage of clinically healthy and grossly normal rabbits (Lehr 1965).

Cardiomyopathy has been observed in rabbits. A possible cause is the rabbit coronavirus. This cardiomyopathy is associated with pleural effusions. Clinical signs are those of congestive heart failure, including increased respiratory rate and effort with generalized muscle weakness. Gross findings are pleural effusion, a dilated right ventricle, and pulmonary edema.

A systemic viral disease has been reported in Europe and China. The cause is considered to be a calicivirus that causes a hemorrhagic disease. The course of the disease is acute with high morbidity and mortality. When observed, clinical signs are depression, anorexia, fever, and incoordination that can progress quickly to death. Pathologic findings are hepatic necrosis and hemorrhages in the lungs, kidneys, and other tissues.

Both arteriosclerosis and atherosclerosis occur in rabbits. Arteriosclerosis is common in several breeds. Spontaneous arteriosclerosis occurs in the aorta of rabbits, especially in the arch (Lehr 1965). Vitamin D, calcium, and phosphorus imbalances, especially hypervitaminosis D, have been associated with arteriosclerosis in rabbits. Clinical signs have included anorexia, dehydration, and weight loss. One affected rabbit had seizures. Gross and microscopic examinations reveal mineralization at the aortic arch and in the thoracic aorta. Atherosclerosis occurs as a result of hereditary hypercholesterolemia in the Watanabe rabbit. Rabbits of this breed develop severe atherosclerosis and are used as a research model for studying this disease (Jayo et al. 1994; Kraus et al. 1984).

Also, rabbits provide a useful model for the study of Purkinje fibers in the heart (Brewer and Cruise (1994).

When a ketamine–zylazine combination or the alpha agonist detomidine was administered to rabbits, multifocal myocardial degeneration with interstitial fibrosis was observed. The cause is attributed to ischemia secondary to vasoconstriction and a reduction in coronary blood flow. The myocardium of rabbits has limited collateral circulation. Early lesions have degeneration of myofibers with mononuclear and polymorphonuclear cell infiltrations. Late lesions have loss of myofibers and marked interstitial fibrosis (Percy and Barthold 2001).

Hematopoietic and Lymphatic System

The rabbit has been an important experimental animal in the study of immunology and infectious agents. They were very valuable in the development of a cure for diphtheria (Arrington 1978). Rabbits are an important source for the production of polyclonal antibodies (Stills 1994). A strain of rabbits deficient in the sixth component of complement inherit this defect as an autosomal recessive trait (Kraus et al. 1984).

When transported from the breeding rabbitry to a testing or research facility, young rabbits experience extensive stress during shipment and on arrival including variations in temperature, unusual noise, and unfamiliar handling. Toth and January (1990) have shown the effects of shipment on various hematological and physiological parameters affecting adult male New Zealand White rabbits.

Systemic disease involving the blood and multiple organs, especially those of the lymphoid tissues, include *Pasteurella multocida, Staphylococcus aureus, Salmonella typhimurium, Salmonella enteritidis,* and *Toxoplasma gondii.* Septicemia has been often associated with snuffles and pneumonia caused by *P. multocida.* Affected animals die peracutely with no evidence of clinical disease and few if any gross or microscopic changes of the infection. The diagnosis is determined by isolation of the organism (Richardson 2000). Other organisms that might result in septicemia are *Staphylococcus aureus* and *Bordetella bronchiseptica.*

Hypercalcemia is a frequent complication of neoplastic disease. The transplantable VX-2 carcinoma of rabbits provides a model for studying mechanisms of osseous-mediated hypercalcemia in the absence of skeletal metastases (Young et al. 1978).

Blood and Bone Marrow

Neutrophils and lymphocytes are present in blood smears in approximately equal numbers (30%–70%). Basophils occur more commonly than in most mammals (2%–7%; Harkness and Wagner 1989).

The rabbit neutrophil contains eosinophilic granules and is commonly referred to as the heterophil or pseudoeosinophil. In suppurative lesions, it is often mistaken for the similarly appearing eosinophil, although the heterophil is distinguished by smaller, lighter stained granules. Hyposegmented neutrophils can occasionally be observed in blood smears. This is known as the Pelger-Huet anomaly. This anomaly is inherited as a partial dominant trait in rabbits.

Clinical biochemistry and hematology values are presented by several sources with emphasis on normal or untreated rabbits. Detailed information is available in McLaughlin and Fish (1994). Other sources are Fox and Laird (1999), and Loeb and Quimby (1999).

Spleen

The spleen is a flat, elongated, dark red structure on the left dorsolateral surface of the greater curvature of the stomach. The microscopic anatomy is similar to that of mice and rats.

Thymus

The thymus persists into adult life so it is usually large in adult rabbits. It lies somewhat ventral to the heart, and extends forward into the thoracic inlet. The microscopic anatomy is similar to that of mice and rats. Thymomas occur infrequently in mature rabbits.

Lymph Nodes

Lymph nodes are bean-shaped structures spread throughout the body connected to a system of the lymph vessels. Nodes beneath the skin and between muscles are bilateral. These include the cervical, axillary, brachial, inguinal, and sciatic nodes. Most visceral lymph nodes are not bilateral but a few are associated with paired organs. These include the mediastinal, pancreatic, renal, mesenteric, and lumbar. The microscopic anatomy is similar to that of mice and rats. The lymph nodes have a fibrous outer capsule that extends trabeculae into the node and subdivides it. A subcapsular sinus communicates with the lymphatic system. Blood vessels and nerves enter and efferent lymph vessels and veins drain from the hilus. The node consists of a peripheral cortex, paracortex, and medulla. The cortex consists largely of lymphoid follicles including germinal centers. The paracortex is lymphoid tissue between the follicles of the outer cortex and the medulla. The medulla consists of sinuses and cords of lymphoid cells.

Lymphosarcoma is the most common tumor of juvenile and young adult rabbits. There might be a genetic predisposition in this species. Clinical signs include lethargy, anorexia, weight loss,

and pale mucous membranes. Most affected rabbits have the visceral form involving the liver, spleen, kidney, mesenteric lymph nodes, stomach, adrenal glands, lungs, and bone marrow.

Nervous System

Torticollis, or twisting of the neck, is occasionally seen in rabbits. Affected rabbits might also have inappetence and ataxia. The usual cause is infections of the middle or inner ears by *P. multocida*. Other causes are encephalitozoonosis, encephalitis, or mechanical injuries of nervous tissues by migration of aberrant parasites, such as *Baylisascaris procyonis.*

Baylisascaris procyonis is a common parasite of the raccoon. When rabbits consume hay or bedding contaminated with raccoon feces containing infective *B. procyonis* eggs, larvae are released in the intestine. Because these larvae are in an unnatural host, they aggressively migrate through the lungs and various other organs, especially the central nervous system, causing devastating cerebrospinal damage. Clinical signs of this damage include torticollis, ataxia, circling, opisthotonus, and recumbancy with fatal results. Gross findings often include multiple, small circumscribed and raised white nodules in the subepicardial and subendocardial surfaces of the heart and the serosa of the liver. Microscopic examinations of these nodules reveal focal granulomatous inflammation with infiltrations of mononuclear cells and heterophils. Parasite remnants are usually present. Lesions in the central nervous system consist of malacia and astrogliosis where the larvae have migrated. These areas have Gitter cells and a variety of inflammatory cells including lymphocytes, macrophages, eosinophils, and heterophils. Frequently, nematode larvae can be identified in the adjacent tissues (Percy and Barthold 2001). Visceral larval migrants can also result from infections by *B. columnaris,* a skunk ascarid, and *Toxocara canis,* a dog ascarid, if rabbits are exposed to contaminated feed and bedding.

Encephalitozoonosis is an infection of rabbits caused by the protozoan, *Encephalitozoon cuniculi*. This protozoan usually causes latent infections with no clinical signs of a central nervous system disease. However, tremors, ataxia, paresis, head tilt, and convulsions have been reported in affected animals. Usually the disease is discovered when granulomas are observed in brain sections, most often the cerebrum, from animals that displayed no clinical signs. This protozoan also infects renal tubular epithelial cells (Flatt and Jackson 1970; Shaddock and Pakes 1971). Vertical transmission through infective urine is strongly suspected.

Focal meningeal aggregates of lymphocytes are often found in routine sections of the brain and the spinal cord of clinically healthy rabbits. Similar aggregates have been observed in the optic nerve as it leaves the globe.

Otitis media infections occasionally can extend from the middle ear to the ventral surface of the brain, and especially to the area of the cerebellum.

Epilepsy has been reported in certain breeds of rabbits with white fur and blue eyes (Okerman 1994). Vitamin A deficiency is uncommon, but can result in hydrocephalus. Vitamin A deficiency in the rabbit provides an animal model for production of hydrocephalus (Newberne 1973). Hypervitaminosis A can result in numerous malformations including anencephaly and spina bifida (Shenefelt 1972). Methylmercury poisoning in the rabbit provides an animal model of this disease that causes lesions of the central nervous system, especially in the cerebellum, similar to those in man (Koller 1978).

Special Senses System

Ear

Ears of rabbits are large, highly vascularized organs that serve for heat regulation as well as in sound gathering. They are fragile and sensitive to trauma (Harkness and Wagner 1989).

Otitis externa is a manifestation of the ear mite, *Psoroptes cuniculi.* (See the section on skin for more information.) These mites can produce marked inflammation. Affected rabbits are often observed shaking their heads or scratching their ears. The inner surface of the external ear might be filled by foul-smelling, branlike tan or brown crusty exudates. The ear is often thickened and edematous.

Otitis media is inflammation of the inner ear that is clinically characterized by ataxia, head tilt, wry neck, or torticollis. Usually, it is the result of an infection caused by *P. multocida* that has spread from the nasal cavities. Other bacterial organisms that have been implicated are *Staphylococcus aureus* and *Bordetella bronchiseptica.* Many rabbits with otitis media are asymptomatic. Lesions consist of inflammatory exudate in the tympanic cavities. Occasionally, the infection can extend to the ventral surface of the brain.

Eye

A very wide field of vision reaches 190° for each eyeball. Wide papillary dilation results in a light sensitivity approximately eight times greater than that of humans (Harkness and Wagner 1989).

Ocular irritation studies are routinely performed using rabbits (Anderson and Henck 1994; Kraus et al. 1984). Although the eye is used in many toxicological studies, only gross or clinical observations are frequently recorded (Dahlgren 1992). Microscopic changes often involve the conjunctiva and cornea and consist of multifocal areas of cellular infiltrates and necrosis of the superficial and deep corneal epithelium. Lymphoid infiltration is more common in the conjunctiva. The cellular infiltrates are an admixture of heterophils and macrophages. Corneal erosions can progress to ulceration, the final stage of many eye lesions. Sections of the globe might reveal anterior or posterior synechiae. The filtration angle is often difficult to visualize with H&E-stained section; however, the presence of inflammatory cells in the ciliary body or the base of the iris might contribute to the misconception of increased ocular tension. Although the choroid lacks pigment, it is not unusual to find foci of melanin-like pigment that appears to extend into the layer of rods and cones of the retina. This pigment does not appear to be the result of inflammation.

Toxic effects of 6-aminonicotinamide were observed in eyes of rabbits given intraperitoneally cumulative doses from 13 to 49 mg/rabbit. The principal lesions were diffuse, bilateral cytoplasmic vacuolation of the iridial and ciliary epithelium in male and female albino and pigmented rabbits (Render and Carlton 1991).

Conjunctivitis, possibly caused by the bacteria entering the conjunctival sac via the nasolacrimal duct, frequently occurs in both young and mature rabbits. Clinical signs of conjunctivitis are reddened conjunctivae, moderately swollen or closed eyelids, and a serous, mucus, or purulent discharge (Flatt 1974). If chronic, the discharge can cause a loss of hair on the face below the medial canthus. The inflammation might be primary or secondary as part of acute or chronic respiratory disease. The organism most frequently isolated is *P. multocida.* This organism can be present in grossly normal eyes. Subsequent ocular irritation studies can be compromised by development of the inflammatory response to this organism.

Entropion, either from congenital defects or inflammation, can damage the conjunctiva and the cornea. Affected rabbits have blepharospasm, conjunctivitis, epiphora, and corneal ulceration, depending on the severity of the entropion.

Buphthalmia is a condition in which the globe of the eye is enlarged and distended. An autosomal recessive inherited defect impairs the drainage of aqueous humor from the anterior orbital chamber, primarily in New Zealand White rabbits. The disease can be bilateral or unilateral with various times of onset. The increased intraocular pressure causes changes in the shape and size of the eye, resulting in clinical enlargement and corneal opacity. The cornea has a bluish or cloudy appearance (Lindsey and Fox 1994). Conjunctivitis often results from trauma and drying of these large eyes (Suckow and Douglas 1997).

Rabbits serve as useful models for ophthalmology and vision research (Peiffer et al. 1994).

Corneal dystrophy, cataracts, and intraocular tumors are reported infrequently in the rabbit. A malignant melanoma of the eye that infiltrated the choroids, iris, and ciliary body has been described (Weisbroth 1994).

The rabbit has a well-developed nictitating membrane, the third eyelid, a semilunar fold at the anterior angle of the eye. During sleep, the third eyelid moves across the cornea.

Harderian Gland

The Harderian gland is a large bilobed structure located behind the third eyelid. Swelling and protrusion of the third eyelid is associated with the Harderian gland. Affected animals have unilateral or bilateral large red masses protruding from the third eyelid at the medial canthus of the eye. Possible causes of this protrusion are inflammation of the Harderian gland (Richardson 2000) or an abnormal laxity of the connective tissue attaching the Harderian gland to the bony orbit (Percy and Barthold 2001).

Nutrition and Metabolic Diseases

Malnutrition is uncommon in rabbits. Rabbits, especially adults, are very sensitive to vitamin D toxicity. This toxicity results in calcification of many organs and tissues. Relatively uncommon nutritional problems include vitamin D, calcium, and phosphorus imbalances (atherosclerosis); vitamin A (hydrocephalus, prenatal death) and vitamin E (muscular dystrophy, prenatal mortality, seminiferous tubal degeneration) deficiencies; and some specific mineral or amino acid deficiencies (Cheeke 1994). Vitamin A deficiencies are a cause of hydrocephalus in rabbits (Newberne 1973). Large doses of vitamin A given to pregnant rabbits can produce a high incidence of gross congenital malformations (Shenefelt 1972).

Bacterial, Mycotic and Viral Diseases

For detailed descriptions of bacterial diseases and the causative microorganisms, see Flatt (1974), Gleiser (1974), and DeLong and Manning (1994). Mycotic infections occur but are less frequent in rabbits (see Bergdall and Dysko 1994). Viral diseases and the causative agents are described in detail by DiGiacomo and Mare (1994). Use of rabbits as models in infectious disease research is presented by Fox et al. (1994). Health considerations can involve common disease agents (Broderson and Gluckstein, 1994). The impact of bacterial, mycotic, and viral infections on research studies is discussed by Baker (1998, 2003).

Parasitic (Protozoan and Metazoan) Diseases

For detailed descriptions of protozoal diseases and the causative organisms, see Pakes and Gerrity (1994). Arthropod and helminth parasites are described by Hofing and Kraus (1994). The impact of parasites on research studies is discussed by Baker (2003).

Neoplastic Diseases

Weisbroth (1974, 1994) reviewed the literature very thoroughly for neoplasms reported in rabbits, with emphasis on naturally occurring or spontaneous neoplasms, unrelated to treatment. Although the overall incidence of neoplasia is reported to be 2% to 3%, this incidence is biased by the high percentage of rabbits at necropsy at ages too young to be in cancer-prone age groups. A normal healthy rabbit has a life span of approximately 6 to 8 years; however, a few can live up to 15 years (Harkness and Wagner 1989). Rabbits, as do other species, demonstrate that age is a predominant factor in determining neoplastic incidence. In a report of 849 female rabbits dying of various causes from a

colony where animals were permitted to die of natural causes, 16.7% had uterine adenocarcinomas (Greene 1965). The average age at death was greater than 4 years. In the affected rabbits, uterine adenocarcinomas had a 4.2% incidence rate in females 2 to 3 years of age and 79.1% in females 5 to 6 years of age. In a smaller study, the incidence of neoplastic disease was seven times higher after the second year of life. An incidence of approximately 8% was the same for males and females over 2 years of age. Uterine adenocarcinomas in female rabbits between 1 and 2 years of age resulted in an overall tumor incidence that was six times higher in females than males (Weisbroth 1994). If the life span of the rabbit is considered to be 7 years, the ratio of the rabbit to the human life span would be approximately 1:12. A rabbit of 6 years would be roughly equivalent to 75 years in man, over 90% of the human life span (Peckham 1980). Some tumors in rabbits have shown a clear genetic predisposition. Other tumors are associated with oncogenic viruses. A few transplantable rabbit tumors are available for research studies. In addition to uterine adenocarcinomas, a wide variety of other neoplasms have been reported. These tumors affect most organ systems, including the uterus, vagina, mammary glands, kidney, urinary bladder, liver, skin, bone, mesenchymal tissues, lymphoid tissues, testes, stomach, intestines, lung, nervous system, and endocrine glands (Weisbroth 1994). The most commonly occurring spontaneous tumors, in order of frequency, are uterine adenocarcinomas, lymphosarcomas, embryonal nephroma, and bile duct adenoma of the liver. Lymphosarcomas occur most commonly in young rabbits. Rabbits used in "typical" toxicological or teratological studies rarely have spontaneous neoplasms because of the limited duration of these studies.

Conclusions

The rabbit provides useful animal models for toxicology and a broad variety of experimental diseases and research studies in several organ systems. Advantages of the rabbit include that it is hardy, small but large enough to easily observe irritation and evaluate fetuses, relatively clean, inexpensive, and easily housed and handled. It also requires moderate amounts of test materials, has a short gestation period, and is sensitive to teratogens. Rabbits are good antibody producers and blood is relatively easy to collect. The scientific literature provides ample background historical and reference information for investigators. The rabbit is generally the nonrodent species of choice for studies that evaluate potential adverse effects on reproduction, organ development, and risks for teratologic effects. Other examples of studies routinely using rabbits include ocular, dermal and mucous membrane irritation, dermal toxicity, toxicokinetic studies, pyrogen testing, intracutaneous implants, neurotoxicity, nephrotoxicity, and immunotoxicity (Anderson and Henck 1994). Investigators using rabbits must be aware of a few potential major disadvantages. The microflora of the GI tract is easily disrupted, which can lead to diarrhea and poor health. The diets fed and nutrition management are very important. Also, the rabbit is highly susceptible to exposure to certain types of antibiotics. The impact of bacterial, mycotic, viral, and parasitic agents on research studies is discussed by Baker (1998, 2003). For additional information on subjects related to the use of the rabbit in research studies, the reader is referred to the comprehensive publications edited by Weisbroth et al. (1974), Melby and Altman (1974, 1976), Hillyer and Quesenberry (1997), and Manning et al. (1994). Some specific subjects are covered in the following references. Anatomy is described by Cruise and Brewer (1994) and McLaughlin and Chiasson (1979) and details are illustrated in an atlas by Popesko et al. (1992). Dissection instructions are given by Wells (1968), Wingerd (1985), and McLaughlin and Chiasson (1979). Necropsy of the rabbit is described by Bivin (1994) and in greater detail by Feldman and Seely (1988). Physiology is discussed by Kaplan (1962), Kozma et al. (1974), Kaplan and Timmons (1979), and Brewer and Cruise (1994). Clinical chemistry and hematology values are presented in detail by McLaughlin and Fish (1994), Fox and Laird (1999), and Loeb and Quimby (1999). General husbandry and disease management, including clinical descriptions of common and uncommon diseases with possible treatments, are presented by Sanford (1979), Williams (1976, 1979), Whitney (1979), Holmes

(1984), Adams (1987), Harkness and Wagner (1989), Stein and Walshaw (1996), Suckow and Douglas (1997), and Richardson (2000). Pathology findings are presented in Benirschke et al. (1978) and described in detail by Percy and Barthold (2001). Weisbroth (1974, 1994) has completed a comprehensive review of neoplastic findings in the rabbit. Other references on neoplasia in rabbits include Squire et al. (1978) and Stedham (1976).

METABOLISM

Hepatic microsomal CYP-450 (cytochrome P-450) activity in the rabbit has been studied and compared with that in other laboratory animals such as the mouse, rat, hamster, and guinea pig (Chhabra et al. 1974; Davies et al. 1969; Litterst et al. 1976; Litterst et al. 1975), as have the levels and activities of its substrates. The cytochrome P-450 content (1.05–1.09 nmol/mg) and the activity of NADP:cytochrome c reductase (130–150 nmol/min/mg) in the rabbit were similar to those in the other species examined (table 6.13). Although CYP-450 reductase activity is much lower in the rabbit (3.0–3.4 nmol/min/mg) than in the mouse and rat, the full range of CYP-450 isoenzyme activities is present (see table 6.14).

Table 6.13 Summary of Hepatic Xenobiotic Drug Metabolizing Enzymes in the Rabbit

Enzyme	Concentration or Activity
Cytochrome P-450 (nmol/mg protein)	1.1[a], 0.779[b], 1.05 for male and 1.09 for female[c], 0.720[d], 0.67[c], 0.81[f,g]
Cytochrome b_5 (nmol/mg protein)	0.3[e], 0.84[b]
NADPH: cytochrome c reductase (nmol/mg protein)	185 [a], 152[g], 130 for male and 150 for female[c]
NADPH: cytochrome P-450 reductase (nmol/mg protein)	3.0 for male and 3.4 for female[c]
Hydroxylase (nmol/min/mg)	
Aniline hydroxylase	0.65 [a], 0.542[b], 0.6[g], 0.72[h]
Benzo(a)pyrese hydroxylase	0.061[d], 0.11[i]
Biphenyl 4-hydroxylase	3.9 [a], 1.7[g], 1.1[f], 2.0[i]
Styrene oxide hydroxylase	5.6[j], 5.8–6.4[k], 4.2[l], 2.3[m], 10.1[n]
O-dealkylase (nmol/min/mg)	
Ethoxycoumarin O-dealkylase	2.3[f]
P-nitroanisole O-dealkylase	5.4[f]
N-demethylase (nmol/min/mg)	
Aminopyrene N-demethylase	15.0[b], 9.8[g], 8.0[h]
Ethylmorphine N-demethylase	4.0 [a], 4.0 for male and 4.3 for female[c], 2.0–2.2[o]
N-hydroxylase (nmol/min/mg)	
Dibenzylamine N-hydroxylase	2.9[p]
2-acetylaminoflourene N-hydroxylase	0.2[f], 0.1[i]
Glutathion S-transferase (nmol/min/mg)	
1-chloro-2,4-dinitrobenzene	4091[n] (cytosol), 81[n] (microsomes), 156[q] microsomes
Hexachloro-1,3-butadiene	0.14[n] (microsomes)
Ethacrynic acid	5.81[r]
Styrene oxide	30.5[j], 21.3–26.9[k], 36[l]
Protein estimates (mg/g)	
Microsomal	24.1[g], 13.8–22.8[k], 18.1[d]
Cytosolic	72.7–82.0[k], 86.5[d]

[a] Chhabra et al. (1974)
[b] Oppelt et al. (1970)
[c] Davies et al. (1969)
[d] Gregus et al. (1983)
[e] Souhaili-el Amri et al. (1986)
[f] Atlas et al. (1975)
[g] Thorgeirsson et al. (1979)
[h] Kato (1979)
[i] Thorgeirsson et al. (1979)
[j] James et al. (1976)
[k] James et al. (1977)
[l] Pacifici et al. (1981)
[m] Oesch (1973)
[n] Oesch and Wolf (1989)
[o] Nerland and Mannering (1978)
[p] Beckett and Gibson (1975)
[q] Morgenstern et al. (1984)
[r] Gregus et al. (1985)

Table 6.14 CYP-450 Isoenzyme Activities in the New Zealand White Rabbit

Activity	CYP-450 Isozyme
7-Ethoxyresorufin *O*-dealkylation	1A1/2[a]
7-Methoxyresorufin *O*-dealkylation	1A2[a]
Caffeine 3-demethylation	
Benzphetamine *N*-demethylation	2B4/5[b,c]
7-Benzoxyresorufin *O*-dealkylation	2B4/5[b]
7-Pentoxyresorufin *O*-dealkylation	2B4/5[b]
Coumarin 7-hydroxylation	2A10/11[d,e]
7-Ethoxy-4-trifluoromethylcoumarin deethylation	2B4[f]
Ethoxycoumarin *O*-dealkylation	2B4/5, 2C3[b,g]
Tolbutamide methyl-hydroxylation	3A6[h]
Chlorzoxazone 6-hydroxylation	
4-Nitrophenol hydroxylation	2E1/2[i]
N-Nitrosodimethylamine *N*-demethylation	2E1/2[i]
Androstenedione 15α-hydroxylation	2B5[b,g]
Androstenedione 16α/β-hydroxylation	2B4/5[b,g]
Dextromethorphan *O*-demethylation	
Dextromethorphan *N*-demethylation	
Testosterone → Androstenedione*	2A10/11[d]
Testosterone 2α-hydroxylation	
Testosterone 2β-hydroxylation	
Testosterone 6β-hydroxylation	2C3, 3A6[a]
Testosterone 7α-hydroxylation	
Testosterone 15α-hydroxylation	
Testosterone 15β-hydroxylation	
Testosterone 16α-hydroxylation	2B4, 2C3[a]
Testosterone 16β-hydroxylation	2B
Lauric acid 11-hydroxylation	4A1, 4B1[j]
Lauric acid 12-hydroxylation	4A1, 4B1[j]

[a] Donato et al. (1999)
[b] Grimm et al. (1994)
[c] Adali et al. (1996)
[d] Ding et al. (1994)
[e] Pearce et al. (1992)
[f] Roberts et al. (1997)
[g] Ryan et al. (1993)
[h] Veronese et al. (1990)
[i] Ding et al. (1990)
[j] Makowska et al. (1992)

Souhaili-el Amri et al. (1986) conducted a similar study in six laboratory animal species and humans. Although the values for the CYP-450 concentrations were somewhat lower in this study compared with those in the previous studies, the value in the rabbit (0.67 nmol/mg) was generally similar to those in the other animals, which is consistent with earlier findings. The CYP-450 content in the rabbit was somewhat higher than the value in humans (0.31 ± 0.09 nmol/mg).

Studies of rabbit CYP-450 using SDS-acrylamide gel electrophoresis have demonstrated at least 15 separable forms from liver microsomes other than P-450. The PB-induced form, P-450 LM_2, and BNF/3-MC-induced form, P-450 LM_4, are the most active, although all of the P-450 isozymes displayed enzymatic activities. P-450 LM_2 was most effective in the metabolism of benzphetamine, whereas P-450 LM_4 was specific for acetanilide hydroxylation. However, they displayed comparable activity toward p-nitrophenetole, p-nitroanisole, and 7-ethoxycoumarin. The isozyme LM_3, which is similar to cytochrome P-450_p in the rat, was also found in the rabbit treated with macrolide antibiotics such as erythromycin and triacetyloleandomycin (Bertault-Peres et al. 1987; Fabre et al. 1988). This isozyme specifically mediates metabolism of cyclosporin. The isozymes LM5 and LM6 exclusively mediate the metabolism of 2-aminolfluorene and 7-ethocyresorufin, respectively.

The distribution pattern of mixed function oxidases for xenobiotics between smooth and rough microsomal membrane is known to be highly species dependent. In the rabbit, concentrations of all components of the mixed function oxidase system are four to five times higher in the smooth

endoplasmic reticulum than in the rough endoplasmic reticulum of the hepatic microsomal fractions (Gram et al. 1971), whereas some animals (e.g., the rat and mouse) showed fairly even distribution between the smooth and rough membranes.

As reported for other species, the drug-metabolizing enzymes in newborn rabbits are not fully developed. For example, phenobarbital was more toxic to newborn than to adult rabbits and produced longer loss of the righting reflex in newborn rabbits (Weatherall 1960).

The content of CYP-450 and other electron transport chain components are highest in the rabbit liver among the organs examined, being approximately 2.5- to 5.0-fold higher than those in the lung and kidney (Oppelt et al. 1970; Uehleke 1969). However, comparison of mono-oxygenase activity toward various substrates undergoing the same route of metabolism shows considerable variation in the ratio of lung to liver.

Distribution of mono-oxygenase activities among the tissues is dependent on the substrates employed. For example, when benzypyrene hydroxylase activity was examined in the small intestinal mucosa preparations of the rabbit, mouse, cat, guinea pig, and rat, the rabbit had medium benzpyrene hydroxylase activity in the small intestine, although the hydroxylase activity in the liver microsomes was least active in the rabbit (Hietanen and Vainio 1973). The UDP-glucuronyl transferase measured using p-nitrophenol as a substrate was highest in the small intestinal mucosa of the rabbit, although the transferase activity of the rabbit liver was intermediate and similar to the activity of rat and mouse liver.

Neither the concentrations of CY P-450 nor the activities of CY P-450 reductase and NADPH:cytochrome c reductase were similar between the male and the female rabbit (Davies et al. 1969), nor were the N-demethylation of ethylmorphine and aminopyrine or hydroxylation of phenobarbital (Davies et al. 1969; Testa and Jenner 1976). An exception is found in rabbit lung metabolic response to ozone, which was found to be more sensitive in females (Delaunois et al. 1999).

Strain differences in drug-metabolizing ability could account for varying biological responses to such compounds as hexobarbital, adrenal cortex hormones, and 2-naphthylamine. In contrast to two- to threefold variation in drug-metabolizing ability seen in the mouse and rat strains, up to 20-fold variations were observed between various rabbit strains. California rabbits, wild cottontails, and jack rabbits exhibited the most striking difference, each being fairly deficient in some enzymes. For example, the *in vitro* hepatic microsomal metabolism rate of hexobarbital and amphetamine was 19 and 8 nmol/mg protein/hr, respectively, in the cottontail rabbit, whereas the metabolism rate of hexobarbital in the New Zealand rabbit was 254 nmol/mg/hr and the metabolism rate of amphetamine in the Dutch rabbit was 154 nmol/mg/hr (Cram et al. 1965).

The capacity for aromatic hydroxylation in the rabbit appears to be medium to high in general as illustrated with aniline, biphenyl, and coumarin. After IV administration of aniline, about 50% of the dose was excreted as p-aminophenol (Parke 1960). Whereas 4-hydroxylation of biphenyl was observed in the rabbit as in humans, 2-hydroxylation was not observed. Biphenyl 2-hydroxylation was limited to a few species such as the mouse and hamster. When the *in vitro* metabolism of N-benzyl-4-substituted anilines were studied using the rabbit, mouse, hamster, and guinea pig, ring hydroxylation was the major pathway in the rabbit and guinea pig, whereas in the mouse and hamster, N-debenzylation was the major pathway (Gorrod and Gooderham 1987). The rat used both pathways to an equal extent. Coumarin 7-hydroxylase was present in rabbit hepatic preparations but absent in the rat and mouse preparations (Kulkarni and Hodgson 1980).

In contrast to the preceding examples, the aromatic hydroxylation activity in the rabbit was low with benzpyrene, amphetamine, and ethyl biscournacetate. The hepatic benzpyrene hydroxylase activity of the rabbit was two- to fivefold lower compared with that of the rat and mouse (Gregus et al. 1983; Hietanen and Vainio 1973) and also somewhat lower than that in humans with a variation of about sixfold (Pelkonen et al. 1975). The range of aryl hydrocarbon hydroxylase activity in humans was approximately 20 to 320 pmol/g liver/min with no sex difference. In the rabbit, ethyl biscournacetate, an anticoagulant drug, was exclusively deesterified and no aryl hydroxylation was observed, whereas in humans and dogs, aryl hydroxylation of the drug was observed.

In the metabolism of amphetamines, aromatic hydroxylation was minor and deamination was the major pathway in the rabbit (Caldwell 1976, 1981). Only small amounts were excreted as the unchanged drugs. In humans, deamination and unchanged drug excretion were equally important. In addition, substantial amounts of aromatic hydroxylated metabolites were excreted. N-dealkylation of amphetamines was a minor pathway in humans as observed in the rabbit (Brodie 1962). Green et al. (1986) have studied amphetamine metabolism using isolated hepatocyte suspension from the rabbit, rat, dog, squirrel, monkey, and human livers. As observed in the *in vivo* studies, rabbit hepatocytes metabolized amphetamine almost exclusively to the products of the oxidative deamination pathway, whereas rat hepatocytes primarily metabolized by aromatic hydroxylation. Metabolism of the drug by the hepatocytes from three other species (dog, monkey, and human) was mixed but oxidative deamination was somewhat more active than aromatic hydroxylation.

N-dealkylating activity in the rabbit was highly variable, ranging from low to high depending on the substrate. For example, N-demethylation of benzphetamine by the rabbit was about twofold higher than that in the rat and mouse, about fourfold higher than that in the dog (Gregus et al. 1983), and more than tenfold higher than that in humans (Souhaili-el Amri et al. 1986). The maximum velocity (V_{max}) of N-dealkylase activity for aminopyrine in the rabbit was slightly lower compared with the mouse and male rat (SD strain), similar to that of the monkey and female rat, and about twofold higher than that of humans (Souhaili-el Amri et al. 1986). N-demethylase activity of ethylmorphine in the rabbit was less than half that of the mouse and male rat, similar to that in the dog and female rat, and about twofold higher than that in humans (Gregus et al. 1983; Souhaili-el Amri et al. 1986). In contrast, N-dealkylation of benzylaniline in the rabbit was minor and ring hydroxylation was the major pathway. Similar results were also observed with tolbutamide, an antidiabetic drug. Tolbutamide is metabolized via methyl hydroxylation and N-dealkylation pathways. In rabbits as well as in humans, monkeys, and rats, the methyl hydroxylation was the major pathway (Thomas and Ikeda 1966; Tagg et al. 1967; Gee and Green 1984). In dogs, N-dealkylation was the major pathway (Remmer et al. 1964).

N-hydroxylase activity is predominantly mediated via the polycyclic hydrocarbon-inducible cytochrome PI-450 (Felton et al. 1976) and is of great importance for toxicity evaluation. For example, acetaminophen-induced hepatic injury was found to be related to the rates of N-hydroxylation of the drug by the hepatic microsomes (Davis et al. 1974). In the rabbit, N-hydroxylation activity was medium to high as illustrated with 4-aminobiphenyl and 2-acetylaminofluorene (2AAF). The major metabolic pathway of 4-aminobiphenyl was N-hydroxylation in the rabbit, mouse, guinea pig, and hamster (McMahon et al. 1980). Interestingly, the N-hydroxylase activity in the rabbit was not induced by methylcholanthrene or Aroclor 1254, a potent cytochrome PI-450 inducer, although it was induced by phenobarbital. N-hydroxylation of 4-aminophenyl in the rat, mouse, and guinea pig was enhanced more than fivefold by Aroclor 1254.

The N-hydroxylase activity of 2-acetylaminofluorene in rabbit liver microsomes was higher than that in the mouse, rat, and guinea pig (Lotlikar et al. 1967). The N-hydroxy metabolite was practically undetectable in the guinea pig, which explains resistance of the guinea pig to hepatoma induction by 2-acetylaminofluorene. When *in vitro* metabolism of dibenzylamine was studied using hepatic microsomes of the rabbit, rat, mouse, hamster, guinea pig, chick, and cat, N-oxidase activity in rabbit microsomes was highest (86.9 nmol/mg/30 min) and approximately five and seven times greater than the activity of mouse and rat microsomes (Beckett and Gibson 1975). *In vitro* metabolism of N-benzyl-4-substituted anilines were studied using rabbit, rat, mouse, hamster, and guinea pig liver homogenates (Gorrod and Gooderham 1987). In contrast to the findings with dibenzylamine, the rabbit had the lowest N-oxidase activity, which was followed by the rat, mouse, guinea pig, and hamster in increasing order. In the rabbit, ring hydroxylation was the major pathway.

Hepatic microsomal levels of epoxide hydrolase activity in rabbits have been compared with those of humans and several commonly used animal models, including the mouse, rat, guinea pig, and rhesus monkey (Oesch 1973, 1980). With benzo(a)pyrene 1, 1-oxide as a substrate, the hydrolase activity in the rabbit was similar to that in the rat but approximately half that in humans and guinea

pigs. The monkey had the highest activity and the mouse had the least activity. When the activity of epoxide hydrolase was measured with styrene oxide as a substrate, the enzyme activity in rabbit liver (4.2–10.1 nmol/min/mg) was similar to that in rat liver as observed with benzo(a)pyrene 1, 1-oxide but three- to sixfold lower than that in human liver (Gregus et al. 1983; Oesch and Wolf 1989; Pacifici et al. 1981). As observed with other enzyme activities, the styrene oxide hydrolase activity in the rabbit kidney and lung (1.5 and 0.4 nmol/min/mg) was lower compared with that in the liver.

The N-acetyltransferase enzyme system is important in understanding the toxicity induced by arylamines. This enzyme system can be viewed as a component of activation pathways with respect to arylamine hepatocarcinogenesis and as a component of detoxification pathways with respect to arylamine bladder carcinogenesis, whereas N-hydroxylating enzyme systems can be viewed as components of activation pathways with respect to both arylamine bladder carcinogenesis and arylacetamide hepatocarcinogenesis. The rabbit is known as the best acetylator of aromatic amines and sulfonamide with low arylacetamide deacetylase activity. For example, consistently high levels of N-acetyl transferase activity were observed in the rabbit toward p-aminobenzoic acid, isoniazid, sulfamethazine, 2-aminofluorene, and O-naphthalylamine, whereas the rat and mouse showed markedly different activity depending on substrate (Gregus et al. 1983). When acetylase activity was studied with sulfanilamide (Williams 1967) and various mono- and dimethoxy-6-sulfanilamidopyrimidines (Bridges et al. 1969) in various species (e.g., rabbit, human, monkey, and rat) the major metabolites in the rabbit urine were N^4-acetyl derivatives except in the case of the 2,5-dimethoxy compound, which was excreted largely unchanged. The rat also favored the formation of N^4-acetyl derivatives, but overall excreted more unchanged drug than the rabbit. Comparable results were apparent in humans with a lesser degree of N^4-acetylation compared with the rabbit. Rabbits display a genetic polymorphism with respect to acetylation, as do humans. This leads to speculation that the rabbit might be a predictive animal model for population-based acetylation of aromatic amines (Calabrese 1988).

Glowinski et al. (1978) compared rates of acetylation in both fast and slow acetylator phenotypes in rabbits and humans for seven compounds: sulfamethazine, p-aminobenzoic acid, and five arylamine carcinogens (α-naphthylamine, 8-naphthylamine, benzidine, 2-aminofluorene, and methylene-bis-2-chloroaniline). In general, the fast acetylator rabbits displayed a 10- to 50-fold greater rate of activity than the fast acetylator humans. For all compounds except p-aminobenzoic acid, the acetylation activity for the fast acetylator rabbits was 90- to 580-fold higher than that of the slow acetylator rabbits, whereas in humans the activity in fast acetylators was approximately four- to 13-fold higher. Therefore, for some compounds, the levels of acetylation activity in the slow acetylator rabbits were in the range of the fast or slow acetylator humans. For example, the slow acetylator rabbits were very similar to the slow acetylator humans for 2-aminofluorene (0.013 vs. 0.021 μmol/mg/hr) and benzidine (0.016 vs. 0.019 μmol/mg/hr), but more closely comparable to the fast acetylator human for β-naphthylamine (0.28 vs. 0.23 μmol/ mg/hr).

Many studies have been conducted to determine UDP-glucuronyltransferase activity in the rabbit. However, the enzyme activity appears to be highly substrate specific and no general conclusion could be drawn. For example, the transferase activity in the rabbit was high toward 1-naphthol, p-nitrophenol, estrone, morphine, and chloramphenicol when compared to the activity in other species such as the mouse, rat, guinea pig, and cat (Gregus et al. 1983). The transferase activity was medium or low toward phenolphthalein, diethylstilbestrol, testosterone, digitoxigenin, valproic acid, and bilirubin. In the rabbit, the concentration of hepatic UDP-glucuronic acid, which is required in the glucoronidation reaction as a glucuronic acid donor, was about half the values of the rat and guinea pig and similar to that of the dog. Therefore, UDP-glucuronic acid did not appear to be a limiting factor for glucuronidation metabolic pathway.

Emudianughe et al. (1978; Emudianughe et al. 1987) studied glucuronidation and other conjugation reactions of radiolabeled naphthylacetic acids. Following 1-naphthylacetic acid administration, the majority of urinary radioactivity (88%) was accounted for by a glucuronide conjugate,

whereas only a small amount (6%) of glycine conjugate was present in the rabbit. However, with 2-naphthylacetic acid the majority of urinary radioactivity was accounted for by amino acid conjugates (glycine, glutamine, and taurine), whereas the glucuronide conjugate represented about 24% of the urinary radioactivity. This was explained by the steric hindrance of 1-naphthylacetic acid for activation in amino acid conjugation (Caldwell 1981). The amino acid conjugations require that the carboxylic group of the xenobiotic acid should be readily accessible for activation to the essential acyl CoA intermediate.

Interestingly, solubilized rabbit liver microsomes had relatively high UDP-glucuronyl transferase activity toward estrone and G-estradiol but not testosterone (Falany et al. 1983). Glucuronidation observed at the 3-OH position of β-estradiol is 20-fold greater than at the 17-OH position. In contrast, solubilized liver microsomes from female rats possessed approximately fourfold more activity toward testosterone (17-OH) than estrone (3-OH). Rat liver microsomes formed 2.5-fold more β-estradiol 17-glucuronide than 3-glucuronide. The highly substrate- and species-specific UDP-glucuronyltransferase appears to be due to, at least in part, multiple forms of the enzyme.

Sisenwine et al. (1982) studied *in vivo* and *in vitro* stereoselective effects in the glucuronidation of oxazepam in various species. They reported that conjugation of S-(+)-oxazepam was favored in rabbits as well as in humans, dogs, rats, and miniature swine. In rhesus monkeys, conjugation of R-(-) isomer was favored. In contrast to oxazepam, glucuronidation of propranolol was stereoselective for (R)-propranolol in the rabbit and for (S)-propranolol in humans and dogs (Von Bahr et al. 1982; Yost et al. 1981). These workers also found that glucuronic acid conjugation of 4-hydroxypropranolol, a metabolite of propranolol, was not stereoselective. After administration of racemic 2-arylpropionic acid, the formation of the glucuronide was enantioselective for the S(-) isomer in the rat and mouse but showed no stereoselectivity in the rabbit (Fournel and Caldwell 1986). These findings as a whole demonstrated complex stereoselectivity of UDP-glucuronyl transferases among different species and broad generalization could not be made for the suitability of animal models for evaluating stereoselectivity of the enzyme.

Hydrolysis of glucuronide conjugates is carried out by the lysosomal enzyme β-glucuronidase, which is present in most tissues, particulary liver, kidney, spleen, intestinal tract, and endocrine and reproductive organs. The level of β-glucuronidase in multiple tissues has been studied in a variety of animal species as well as in humans. When β-glucuronidase activity was measured with phenolphthalein glucuronide, the enzyme activity in the rabbit liver (5,000 μg/g liver/hr) was about 1.7-fold higher than that of human liver, similar to that of guinea pig and hamster liver, and three- to sixfold lower than that of rat liver (Calabrese 1988). In contrast to liver enzyme activity, the enzyme activity in the kidney was lower in the rabbit (300 μg/g liver/hr) than in humans (2,000 μg/g liver/hr).

GI tract levels of β-glucuronidase activity could markedly affect the response to some carcinogenic agents. Many carcinogenic agents such as benzo(a)pyrene are conjugated with glucuronic acid and excreted via the bile. Low 6-glucuronidase activity in the small intestine is expected to reduce enterohepatic circulation, thereby reducing the residence time of the carcinogens in the body. Rabbits are estimated to have approximately 120- and 50-fold higher β-glucuronidase activity in the proximal and distal small intestine, respectively, compared with humans (Calabrese 1988). Mice and rats are estimated to have approximately 60,000- and 15,000-fold, respectively, higher β-glucuronidase activity in the proximal small intestine than humans. Such findings suggest that the rabbit model might offer a closer approximation to the human even though the rabbit far exceeded the human values.

Glutathione transferase activity in rabbit liver was highly variable depending on the substrate. For example, glutathione transferase activity toward 1-chloro-2,4-dinitrobenzene was approximately five- and twofold higher in the rabbit than in the rat and mouse, respectively (Gregus et al. 1983; Igarashi et al. 1986). However, toward 1,2-dichloro-4-nitrobenzene, the enzyme activity in the rabbit was less than one-sixth of the activity in the rat and mouse. Gregus et al. (1985) studied glutathione transferase activities in the 60-day-old rabbit and other commonly used laboratory animals, such

as rat, mouse, and guinea pig, toward seven substrates. The rabbit was the least active in the conjugation of six substrates (3,4-dichloronitro-benzene, sulfobromophthalein, p-nitrobenzyl chloride, ethacrynic acid trans-4-phenyl-3-butene-2-one, and 1,2-epoxy-3-(p-nitrophenoxy propane) out of seven, yet it was the most active in the conjugation of 1-chloro-3,4-dinitrobenzene. The transferase activity toward styrene oxide in rabbit liver (36 nmol/min/mg) was two- and fourfold lower than that in rat and mouse liver, but about 1.9 and 1.4 times higher than that in dog and human liver (Pacifici et al. 1981). This high variation of the enzyme activity appears to be in part due to different substrate specificities and subunit composition of the enzymes among the different species (Igarashi et al. 1986). The glutathione transferase activities toward styrene oxide in the rabbit kidney and lung (18.0 and 6.5 nmol/min/mg, respectively) were lower than that in the liver (Pacifici et al. 1981).

Oesch and Wolf (1989) determined glutathione transferase activity in liver microsomes and cytosol from various species, including rabbits and humans. The glutathione transferase activity toward the microsomal specific compound, hexachloro-1,3-butadiene in rabbit liver microsomes (0.14 nmol/min/mg) was about one-tenth of that in human liver The transferase activity toward the cytosol enzyme-specific compound, 1-chloro-2,4-dinitrobenzene, in rabbit liver cytosol fractions (4,091 nmol/min/mg) was more than twofold higher than that in human liver cytosol fractions.

When perinatal development of glutathione S-transferase and epoxide hydrolase was studied in rabbit liver and extrahepatic organs, activity of glutathione S-transferases toward 1,2,dichloro-4-nitrobenzene and styrene oxide was different in liver, intestine, lung, and kidney (James et al. 1977). In the liver, which has higher activity compared with the other organs, the transferase activity toward both substrates was within the adult range at 70 days after birth. The transferase activity toward styrene oxide in fetal rabbit liver was 1 to 2 nmol/mg/min. This activity increased sharply and reached about 50% of the adult value between 1 and 6 days after birth. However, the age development of glutathione transferase activities in rabbits was highly substrate specific (Gregus et al. 1985). For example, the glutathione transferase activity in rabbit liver for ethacrynic acid increased only 1.8-fold from the neonatal value during the first 120 days after birth, whereas the enzyme activity toward 1,2-epoxy-3-(p-nitrophenoxy)-propane increased about 18-fold.

Depletion of glutathione in the liver was found to be directly correlated with acetaminophen hepatotoxicity (Green et al. 1984). After treatment of acetaminophen, hepatocytes of acetaminophen-resistant species such as the rabbit retained higher amounts of glutathione, produced no detectable covalent adducts, and metabolized more extensively to polar metabolites compared to hepatocytes from other species. In contrast, hepatocytes of acetaminophen-susceptible species such as the hamster were depleted of glutathione more rapidly, produced more covalent adducts of acetaminophen, and formed polar metabolites at a slower rate than the rabbit hepatocytes.

Although information on S-methyl reactions in the rabbit is limited, a membrane-bound enzymatic activity has been found in rabbit liver microsomes that catalyzes the transmethylation from S-adenosylmethionine to a series of C1-C3 alkane thiol (Holloway et al. 1979). Methane and ethane thiols are known to be endogenous toxins, which might play an important role in the pathogenesis of hepatic coma, and methylation could provide an important pathway for metabolic detoxification through neutralization of the highly reactive sulfhydryl group.

The rabbit appears to have low to moderate sulfate conjugation capability. After administration of dopamine about 1% of the dose was excreted as sulfate conjugate in the 0 to 72-hr rabbit urine, whereas about 61%, 5.3%, and 7.0% of the dose was excreted as sulfate conjugates in the dog, rat, and mouse urine, respectively, over the same time periods. Following administration of phenacetin, the sulfate conjugate was a minor metabolite (4%–9%) and N-acetyl-p-aminophenylglucuronide was the major metabolite in the rabbit, whereas in humans glucuronide and sulfate conjugates were about equally important (36%–42% and 23%–31%, respectively; Smith and Timbrell 1974). However, with phenol, the percentage of the total sulfate conjugate excreted in rabbit urine was 54% of the administered dose (Capel et al. 1972). This value is comparable to those in the rat (55%) and mouse (51%) but somewhat lower than that in humans (78%).

The formation of arylsulfoconjugates is known to be rate limited by the endogenous sulfate ion, and thus the extent of conjugation is dose dependent. Liver concentrations of adenosine 3'-phosphate-5'-phosphosulfate (PAPS), which is required for sulfation reactions as the sulfate donor, was 32.7 nmol/g of tissue in the rabbit. This concentration was about half the value in the rat, similar to that in the hamster and mouse, but about twice as high as that in the dog (16.1–17.3 nmol/g). The concentrations of PAPS in the rabbit kidney, lung, and intestine were approximately one-third of the value in the rabbit liver.

The rabbit utilizes the glycine conjugation pathway in the metabolism of many arylacetic acid compounds, such as phenylacetic acid, indoleacetic acid, and phenylcysteine. In contrast, humans did not form any substantial amount of glycine conjugate with these compounds. Interestingly, 1-naphthylacetic acid was conjugated only with glycine in addition to glucuronic acid in the rabbit, whereas 2-naphthylacetic acid formed conjugated metabolites with glycine, glutamine, and taurine as well as glucuronic acid (Emudianughe et al. 1978; Emudianughe et al. 1979).

The drug-metabolizing enzymes in the rabbit are generally induced or inhibited with the known enzyme inducers or inhibitors in the other species, respectively. However, the effects of some inducers and inhibitors have been reported to differ in rabbits from those in the other species. When differential inductive effects in the rabbit liver were studied after treatment with phenobarbital, G-naphtho-flavone (BNF), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and 3-methylcholanthrene (3-MC), metabolism of 2AAF to the mutagenic metabolite N-hydroxy 2AAF was increased from seven- to 13-fold after treatment with 3-MC, BNF, or TCDD (Atlas et al. 1975). The N-hydroxylation of 2AAF increased twofold with phenobarbital in the rabbit but not in the rat, mouse, hamster, and guinea pig. A more detailed study revealed that in the rabbit, a significant portion of the microsomal metabolism of 2AAF to the mutagenic metabolite was initiated by deacetylation to aminofluorene followed by N-hydroxylation, which was catalyzed by phenobarbital-inducible cytochrome P-450 LM_5 as well as by 3-MC-inducible cytochrome P-450 LM_4.

Acetanilide hydroxylation in the rabbit was induced from 2.4- to 3.9-fold with BNF, TCDD, 3-MC, and phenobartital (Atlas et al. 1975). However, arythydrocarbon hydroxylase (AHH) activity toward benzo(a)pyrene was increased only by phenobarbital but not by any of the other inducers in this study. Furthermore, Aroclor 1254 and methylcholanthrene, which were potent inducers of N-hydroxylation of 4-aminophenyl in the rat and guinea pig, were not inducers in the rabbit (McMahon et al. 1980). Interestingly, TCDD stimulated the demethylase activity for p-nitroanisole in the rabbit after 3 days of treatment, but the effect was lost after 6 days.

Cytochrome P-450 LM_5, which is found in rabbit liver and extrahepatic tissues, does not appear to be similar in all properties to any cytochrome P-450 isozymes isolated from other species. Although phenobarbital induces the synthesis of both isozyme LM_5 and LM_2 in rabbit liver, the relationship between these isozymes is not comparable to that between P-450_b and P-450_e, highly related isozymes that are induced by phenobarbital in rat liver.

Vanderslice et al. (1987) examined the presence of homologues of rabbit cytochrome P-450 LM_5 in pulmonary and hepatic preparations from the rat, mouse, hamster, guinea pig, and monkey. Homologues of isozyme LM_5 were detected in pulmonary preparations from all five species. However, only hepatic preparations from the hamster, in addition to those from the rabbit, contained detectable levels of this isozyme. Although LM_5 isozyme in rabbit liver was induced by phenobarbital, treatment of other animals with phenobarbital did not increase the hepatic or pulmonary content of isozyme LM_5 homologues or the amount of 2-aminofluorene metabolism that was inhibited by antibodies to isozyme LM_5. Subchronic treatment of phenobarbital induced renal cortical microsomal mono-oxygenase and aryl hydrocarbon hydroxylase and cytochrome P-450 content in rabbits but did not induce these renal enzyme systems in the rat (Kuo et al. 1982).

In addition to cytochrome LM_5 isozyme, rabbit pulmonary cytochrome P-450 comprises two other isozymes, LM_2 and LM_6. In the untreated rabbit lung, cytochrome LM_2 and LM_5 isozymes are present in approximately equal proportions. However, the isozyme LM_6 has been identified only after treatment with TCDD. In the liver, the isozymes LM_2 and LM_5 make up a small fraction of

the total complement, which comprises at least nine forms. Phenobarbital, an inducer of forms 2 and 5 in liver, has no inductive effect in the lung. Induction of form 4 by aromatic hydrocarbons occurs in the liver but not in the lung, except perhaps in the neonate. Pulmonary concentrations of isozyme 2 were decreased to trace levels by the administration of Aroclor 1260 (Serabjit-Singh et al. 1983; Ueng and Alvares 1981; Ueng et al. 1980). The pulmonary content of isozyme 6 was also decreased twofold by treatment with phenobarbital but increased five- and tenfold by administration of Aroclor 1260 and TCDD, respectively.

It has been reported that metabolism of stereoisomers was selectively induced by pretreatment with different inducing agents. For example, when the stereoselective glucuronidation of oxazepam was studied using rabbit liver microsomes with a series of inducing agents, the ratio of diastereomeric products produced varied dramatically relative to noninduced animals (Yost and Finley 1985). The R/S enantiomer ratio for noninduced rabbits was 0.76, but this was reversed to 1.41 after treatment with β-naphthaflavone. These data clearly suggest that various forms of glucuronyltransferase with different stereoselectivities were present in rabbit liver.

α-Naphthoflavone has been reported to be a potent inhibitor of reconstituted rabbit liver P-450 IA2 (LM_4) biphenyl 4-hydroxylation (IC_{50} = 27 nM) and 7-ethoxy-resorufin-O-demethylation (IC_{50} = 10 nM; Johnson et al. 1979). In contrast, P-450 IIB4-dependent activity (rabbit LM_2) was only inhibited to a minor extent at an α-naphthoflavone concentration of 240 nM. Thorgeirsson et al. (1979) also studied the *in vitro* effect of α-naphthoflavone on four hepatic mono-oxygenase activities (aryl hydrocarbon hydroxylase, 2-acetylaminofluorene N-hydroxylase, biphenyl 2-hydroxylase, and biphenyl 4-hydroxylase) before and after methylcholanthrene treatment of the rabbit and other laboratory animals (mice, rat, hamster, and guinea pig). *In vitro* addition of α-naphthoflavone selectively inhibited 2AAF N-hydroxylase and biphenyl 4-hydroxylase activity in the methylcholanthrene-treated rabbit, which is consistent with the findings of Johnson et al. (1979). However, *in vitro* addition of _-naphthoflavone enhanced the activities of arylhydrocarbon hydroxylase and biphenyl 2-hydroxylase in liver microsomes from both control and methylcholanthrene-treated rabbits. The enhancement of mono-oxygenase activities by the *in vitro* addition of α-naphthoflavone might be caused by an interaction with the allosteric binding site(s) on the heme proteins. In contrast to the rabbit, *in vitro* addition of α-naphthoflavone selectively inhibited all four mono-oxygenase activities from the 3-MC-treated rat, mouse (C57BL/6N strain), and hamster.

Species differences in toxicity of a compound could be due to factors other than differences in its metabolism. Some of these factors include protein binding and biliary excretion in addition to absorption and renal elimination of drugs. When protein binding of some drugs (clofibric acid, etodolac, tolrestat, perrinone, benoxaprofen) was examined in the rabbit and other laboratory animals (rat, mouse, dog, rhesus monkey) as well as in humans, the binding in the rabbit appeared to be medium. In general, the binding was highest in human serum and weakest in the mouse. However, when the protein binding of prednisolone was studied in serum from rabbits, dogs, rats, and humans, the binding characteristics of the drug in rabbit serum were most similar to those in human serum (Rocci et al. 1980). A similar phenomenon was observed with a new cephalosporin, CL284635 (Bialer et al. 1986).

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CHAPTER 7

The Ferret

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CONTENTS

TOXICOLOGY

Interest in the ferret as a laboratory animal has grown in direct concordance with the escalating public opposition to the use of "domestic" animals in scientific research and, conversely, with the more recently identified need for a smaller nonrodent species to be used as a surrogate for the canine. To this end, conventional nonrodents such as the canine are being increasingly excluded from the testing of genetically engineered human biologics (for example) because a sufficient quantity of test material simply might not be available. The search for a surrogate nonrodent has, therefore, been focused primarily on smaller contemporary species considered more accommodating to the task at hand. One such small laboratory-adapted carnivore that has found increasing application in this present environment is the ferret.

Scientific Application of the Ferret and the Extent of Its Use in Toxicology

Research with the ferret falls into two main categories: that directed toward gaining basic knowledge applicable to human health, and the more recent efforts directed toward ensuring the safety of humans (and their environment) exposed to a multitude of chemical and biological agents. Much of the knowledge gained from the basic biomedical research studies with ferrets has, quite fortunately, found application in studies of the problems related to human toxicity assessment. In this respect, Thornton et al. (1979), Beach (1982), and Hoar (1984) have previously described the applicability of ferrets in the toxicology laboratory, and several symposia devoted to an analysis of uses of the ferret in preclinical safety studies and biomedical research highlighted important contributions (Fox 1987; Greener 1987; Haddad and Hoar 1981). The use of ferrets in such diverse biomedical research areas as cardiology (Breisch 1980; Marino et al. 1981; Marino and Severdia 1983), ophthalmology (Braekevelt 1982; Thorpe and Herbert 1976; Vinegar et al. 1982; Vinegar et al. 1985; Wen et al. 1985), virology (Bird et al. 1983; Chevance et al. 1978; Kauffman et al. 1982), bacteriology (Fox et al. 1983; Fox et al. 1982; Koshimizu et al. 1982), toxicology (Brantom et al. 1977; McLain, McCartney et al. 1987), and developmental toxicology (Beck 1978; Beck et al. 1978; McLain and Roe 1983; McLain, Harper et al. 1985) has become frequent and important.

The extent of the research effort with ferrets is further indicated by recent surveys of the scientific literature accessed through the BIOSIS and MEDLINE computerized database search systems, which cover entries from more than 8,000 journals in more than 70 different countries. The first selected list of references on the use of ferrets in biomedical research was published by Frederick and Babish (1985), and includes more than 569 entries of papers issued from 1977 to 1984. A later survey published by Clingerman and Fox (1991) contains more than 715 scientific citations dedicated to the use of ferrets as laboratory animals, although some of these are duplicate citations from the Frederick and Babish (1985) survey.

Earlier compendia of similar scientific literature were published by Marshall and Marshall (1973) and Shump et al. (1976), which span the years 1866 to 1974. As indicated by these literature reviews, the number of scientific publications specifically mentioning the ferret in their title or as a keyword prior to 1974 averaged approximately five per year, whereas from 1977 to 1983 the number increased to approximately 113 per year. Although only 23% of these latter citations were toxicological in content, they can be viewed as an indication of the ferret's increasingly important role in the area of toxicology research.

The increasing number of scientific applications of the ferret and the unprecedented growth of the biotechnology industry correlate highly with commercial sales of the animal over the last 10 years. Therefore, whether used as a surrogate nonrodent for testing the efficacy and safety of limitedly available human biologics, or as a simple alternative to more conventional laboratory carnivores, the present usage rate of ferrets suggests that information delineating the species'

applications and restrictions in the toxicology laboratory is duly warranted. This chapter, therefore, is an attempt to consolidate current information on the ferret that might be applicable to preclinical safety evaluations. More current information can be found by accessing the National Agricultural Library Web site (http://www.nal.usda.gov) and searching for "Information Resources on Ferrets, 1991–2002."

History

Taxonomy and Origin

The present-day domesticated ferret, *Mustela putorius furo* (see table 7.1 for taxonomic classification), is believed to have originated in North Africa more than 5,000 years ago (Hagedoorn 1947; Miller 1933; Thomson 1951). Some accounts credit the Egyptians with the domestication of ferrets (ca. 3000 B.C.) and their subsequent introduction to Europe, Asia, and Great Britain (Owen 1969; Pyle 1940). Most historical reviews describe the modern laboratory ferret as descending from the wild European ferret or polecat (*M. putorius* or *M. furo*) or the steppe polecat (*M. eversmanni*), with which they can breed and produce offspring (Fox 1988; Pocock 1932). The black-footed ferret (*M. nigripes*), an endangered species native to the western United States, is a distinctly different strain and is not used for research purposes (Boggess et al. 1980; Clark 1978; Moody et al. 1985).

Table 7.1 Taxonomic Classification of Ferrets

Kingdom	Animal
Subkingdom	Metazoa
Phylum	Chordata
Subphylum	Vertebrata
Class	Mammalia
Subclass	Eutheria
Order	Carnivora
Suborder	Fissipedia
Family	Mustilidae
Subfamily	Mustelinae
Genus	*Mustela*
Species	*Putorius*
Subspecies	*Furo*

The *Mustelidae* family of the ferret is considered to be the most primitive living group of terrestrial carnivores, with a total of 70 different species in 25 genera. Genera such as *Lutra* (river otter), *Pteronura* (giant otter), *Aonyx* (small-clawed or clawless otter), and *Enhydra* (sea otter) are aquatic, whereas most other members of the *Mustelid* family are land dwelling (fissipedia). In the widely distributed *Mustilinae* subfamily in particular, there are approximately 11 genera and 33 recent species. The more familiar relatives of the ferret include the weasel (ermine, sable), mink, marten, polecat, fisher, skunk, wolverine, otter, and badger. Less commonly known relatives include the zorille and ratel (honey badger). In Europe and Great Britain, the wild ferret is also known as a fitch, fitchew, fitchet, foul marten, or foumart.

Most mustilids have well-developed anal scent glands. The skunks, zorille, marbled polecat, stink badger, and ratel can forcibly eject the vile-smelling liquid as a spray or fluid. In contrast, the laboratory ferret is incapable of projecting its anal gland fluid, which many researchers also rate low on the odoriferous discomfort scale.

Economic Applications

Early Greek Ferreting and Falconry

Early Greek literature indicates that ferrets made their first social impact as hunters of rabbits (Thomson 1951). Similar to the use of grisons to flush chinchillas from their burrows, "ferreting" for rabbits consists of muzzling a ferret and placing it into one entrance of a rabbit burrow. The characteristic musk odor of the ferret would cause the burrow's occupants to flee by a rear entrance, whereupon they were duly dispatched by the hunter. The Greeks were probably the first to employ falconry as a method of capture for rabbits flushed from their burrow in this manner. However, many hunters simply held a net or clock sack over the rear entrance to the burrow, hoping that their strength could match the speed and force by which the rabbits exited the hole. Ferreting (or "rabbiting") with firearms is viewed as unsportsmanlike and is prohibited in many parts of the United States.

English Ferret-Legging

The English are credited with popularizing the notable sport of ferret-legging, which has seen a resurgence in recent years. In this contest, a competitor's trousers are tied off at the ankles and a ferret is inserted. After tightening the belt, the contestant proceeds to tolerate the ferret's repeated escape attempts. Katz (1987) described the "ideal" ferret for ferret-legging as "having claws like hypodermic needles and teeth like number 16 carpet tacks." Accordingly, the current record for ferret-legging is 5 hr and 26 min, a mark held by a 72-year-old Yorkshire man.

Use of the Ferret as a Rodent Exterminator

In the early part of the 20th century ferrets were popular as rodent exterminators in the United States and England. However, with the advent of chemical rodenticides, this specific application of the species has waned.

Pet Ferrets and Their Legal Restrictions

Currently there are an estimated 4 million to 5 million pet ferrets in the United States, with approximately 1 million in the state of California alone. Some states such as Georgia, Massachusetts, New Hampshire, and South Carolina, however, have banned the ownership of ferrets completely, and other states such as California, Minnesota, and New York have restricted ownership by requiring licensing, neutering, or leashing. Some localities, such as Carson City, Nevada, have gone as far as to prohibit the sale of ferrets to households that have children under the age of 3 years. These various restrictions have apparently resulted from controversial issues such as whether the ferret can be considered a truly domesticated species, media accounts of instances when young infants have been bitten, and the present lack of an FDA-approved rabies vaccine. Proponents of ferrets have, nevertheless, successfully argued their cause in the courts of Alaska, Maine, Pennsylvania, and West Virginia, and have had restrictions reversed or overruled in these states. Based on scientific information presented by lobbyists to individual state legislatures, the ferret is increasingly being categorized as a domesticated animal, thus exempt from control by individual state game commissions. Nevertheless, The Ferret Lovers' Club of Texas (2002) suggests checking local ordinances, or contacting the director of animal control or an equivalent authority to learn about the status of ferret ownership or use (in a given geographical area), as the animals might be prohibited by city or state ordinance or regulated by policy. With respect to domestication, a recent trade journal survey indicated that ferret bites are much less frequent (and severe) than dog bites when expressed relative to species population size, and laboratory researchers have found the ferret to be accommodatingly

docile and predictable. On the contrary, Applegate and Walhout (1998) caution that although the ferret is becoming an increasingly popular pet, the dangers of ferret ownership remain unrecognized by physicians and the general public. They described three incidents of ferret attacks in a 3-month period of time, concluding that the risk of attack is greatest for infants and small children, and that wounds caused by ferret attacks must be evaluated for injury, infection, and rabies prophylaxis.

Early Biomedical Research Studies with the Ferret

Pioneering biomedical research studies utilized the ferret in experiments concerned with the pathogenesis of human influenza virus (Moody et al. 1985; Pyle 1940; Smith et al. 1933), and because of its sensitive pulmonary vasculature, the species continues to be viewed as a valuable model for investigating pulmonary hypotension in humans (Andrews 1988; Vinegar et al. 1982). In a recent example of the ferret's utility in influenza virus research, de Jong et al. (2000) were able to demonstrate a mismatch between the 1997/1998 influenza vaccine and the major epidemic A(H3N2) virus strain as the cause of an inadequate vaccine-induced antibody response to this strain in the elderly. In that respect, the success of influenza vaccination depends largely on the antigenic match between the influenza vaccine strains and the virus strains actually circulating during the season. In the past, this match has proved to be satisfactory in most seasons. In the 1997/1998 season, however, de Jong and coworkers noted that hemagglutination inhibition (HI) assays with ferret antisera indicated a considerable mismatch between the H3N2 vaccine component and the most prevalent epidemic influenza A(H3N2) virus. The results from their antigenic analyses using pre- and postvaccination serum samples from volunteers of various ages, including residents of nursing homes who were more than 60 years of age, were in good agreement with the results obtained with ferret antisera. Homologous serum antibody responses to the H3N2 vaccine component as well as the cross-reactivity of the induced antibodies to the epidemic H3N2 strain declined with increasing age of the vaccinees. As a consequence of these two effects, 84% of the vaccinees over 75 years of age did not develop HI antibody titers ≥ 40 against the major H3N2 virus variant of 1997/1998, suggesting that they were not protected against infection with this virus variant. The authors concluded that these findings support the current policy of the World Health Organization (WHO), which is to base worldwide influenza virus surveillance on results predominantly obtained by antigenic analyses of influenza virus isolates with ferret antisera in HI tests. If an antigenic mismatch is observed, the protective efficacy of the vaccine, especially for the elderly, might be insufficient.

In the more contemporary area of DNA vaccine research, scientists at Merck Laboratories (Donnelly et al. 1995) used ferrets to demonstrate that DNA vaccines were more effective, particularly against different strains of virus, than inactivated virus or subvirion vaccines. They reported a comparison of DNA vaccines, using contemporary human strains of virus, and clinically licensed (inactivated virus or subvirion) vaccines in preclinical animal models, to better predict their efficacy in humans. Influenza DNA vaccines elicited antibodies in both nonhuman primates and ferrets and protected ferrets against challenge with an antigenically distinct epidemic human influenza virus more effectively than the contemporary clinically licensed vaccine. Along this same line, Webster et al. (1994) demonstrated that immunization of ferrets with a plasmid DNA expressing influenza virus haemagglutinin (pCMV/H1 DNA) provided complete protection from challenge with the homologous A/PR/8/34 (H1N1) influenza virus. They provided evidence that delivery of DNA-coated gold beads by gene gun to the epidermis was much more efficient than intramuscular delivery of DNA in aqueous solution. They also provided evidence that the antibody response induced by DNA delivered by gene gun was more cross-reactive than DNA delivered in aqueous solution or after natural infection, and concluded that this novel approach to vaccination against influenza might afford broader protection against antigenic drift than that provided by natural infection. Interest in the ferret as an animal model for influenza infection, and the use of plasmid DNA vaccines delivered to the human epidermis, have increased considerably since these early reports

were published. Ferrets have also been used quite successfully in assessments of reproductive toxicity (Beck et al. 1978; Gulamhusein et al. 1980; McLain and Roe 1983) and a Stanford University symposium (1981) and several review articles (Beck 1975; Gulamhusein and Beck 1977; Haddad and Rabe 1980) have highlighted the species' many advantages and contributions to this specific scientific discipline. Beck (1975, 1981) and Hoar (1984), for example, contrasted human, rat, and ferret placentas and concluded that the latter was most similar in structure and function to the human. Literature reports continue to demonstrate similar reproductive responses in humans and ferrets (Beck et al. 1978; Collie et al. 1978; Elizan et al. 1969; Gulamhusein et al. 1980; McLain and Roe 1983).

Husbandry

Specific husbandry practices for the ferret are summarized in table 7.2. Details of selected parameters are outlined in the following sections.

Table 7.2 Recommended Husbandry Practices for Ferrets Maintained Under Controlled Environment Conditions

Parameter	Units	Value
Cage dimensions (L × W × H)		
Breeding (2 adults)	cm	60 × 60 × 40
Growing (1–8 kits)	cm	40 × 40 × 30
Experimental	cm	Varies
Cage bar spacing	cm	< 2.5
Area temperature	°C	15 ± 5
Area humidity	%	50 ± 20
Room air changes	No./hr	10–15
Lighting (incandescent or fluorescent)		
Sexually inactive	hr/day	8 ± 2
Sexually active	hr/day	14 ± 2
Nest material (other than 3/8-in. grid flooring)		
All ages	—	Shavings
Pregnant females	—	Nest box
Food intake (not accounting for spillage)		
Energy (maint.)	Kcal/kg/day	200–300
Dry matter	g/day	50–70
Solid food, age at	Weeks	1–2
Diet peculiarities		
Growing	—	Ad libitum
Breeding, lactating	—	Ad libitum
Adult	Low body fat	Limit feed
Water requirements	ml/24 hr	75–100 or ad libitum

Caging and Bedding

The staff veterinarian or designee should be responsible for determining suitable housing for newly arriving ferrets. In addition, rooms in which ferrets are housed should be of adequate space, clean, ventilated, and environmentally controlled in accordance with government regulations and guidelines established for the care and use of laboratory animals, and in accordance with applicable facility standard procedures. Before modifying details of an established animal-care program, the staff veterinarian (or designee) should notify the research scientist whose animals are affected by the proposed change.

The recognition that ferrets adapt readily to the various types of caging routinely found in the small animal vivarium has undoubtedly contributed to the success of the species in the biomedical research laboratory. Stainless steel rabbit cages (2' L × 2' W × 1'3" H), for example, can house one

or two ferrets quite comfortably. Cat cages (2' L × 2' W × 2' H) are quite adequate when group housing is preferred. The most recent issue of the National Institutes of Health (National Research Council 1985) *Guide for the Care and Use of Laboratory Animals* did not specify standards for the size of ferret caging, but it is likely that either of the dimensions just discussed will more than satisfy any potentially forthcoming criteria. Both cage types described are low stress, providing for sufficient lateral (horizontal) and vertical (rearing) motion. In addition, they exceed the dimensions specified by Wilson and O'Donoghue (1982), and by the Royal Society of England (Andrews and Illman 1987). Wilson and O'Donoghue (1982) have described a mobile rack of cages for ferrets.

Group housing and opportunity for interaction suitable to ferrets should be provided whenever possible, but it should also be recognized that group housing of mature adults is frequently impractical (see following discussion). Therefore, wherever possible, caging should be provided that permits easy viewing of neighboring cages and their occupants.

Limitations of Cage Bar Dimensions

A variety of cage dimensions might be satisfactory for housing ferrets, as long as the interior dimensions provide for uninhibited movement and the cage bar spacing of the unit is less than 2.5 cm, or modified appropriately. With respect to cage height, behavioral observations indicate a natural rearing motion of this species (similar to cats and dogs), which is probably related to a strong dependency on smell when hunting and the need to extend their head above tall grasses to examine the upper air currents. Observations have also indicated that ferrets with body weights less than 700 g will escape through any cage bars spaced 2.5 cm or more apart, as well as through the popular J-type gravity feeder. Therefore, containment should be assured by employing commercially available stainless steel grid door inserts (Lab Products, Inc., Maywood, New Jersey, ¼-in. grid) and by locating feed dishes inside the cage bars. It is also possible to insert an appropriately shaped section of stainless steel grid (spacing approximately ¾" H × 1½" L) inside the J-type gravity feeder, which prevents escape and reduces the digging and scattering of feed (see following discussion).

Excretory Habits of Caged Ferrets

Ferrets are accommodating in that they characteristically deposit their wastes in distinct corners of the cage. Thus, cage floor grids should be large enough to allow excreta to fall through to the catch pans below, yet small enough so as not to cause the animal discomfort when walking or to allow escape. Commercially available wash-down rabbit caging with ⅜-in. grid flooring fits these criteria and requires little maintenance time for hygiene control. Alternatively, direct-contact bedding material (wood chips, paper strips, etc.) can be used with similar success, with soiled corner material removed daily and complete changes occurring as needed. For short-term housing or transportation, some investigators have employed the large polycarbonate rat breeding cage (9" H × 9" W × 16" L), covering the bottom with wood chips to facilitate cleaning. As indicated, however, these units should not be used for any extended housing period because of their small size. Moreover, the units should be dedicated to ferrets because they will tend to retain the musk odor, causing considerable consternation for the next small rodent inhabitant.

Nest Boxes for Pregnant Ferrets

A nest box (approximately 6" H × 6" W × 12" L) constructed of either metal or plastic, partially filled with wood chip or paper strip bedding material, and containing a 2- to 3-in. diameter entrance hole is a valuable cage addition for the pregnant jill and for increasing the comfort of animals subjected to chronic treatment protocols. Nontransparent nest boxes such as metal, however, are not conducive to recording detailed daily clinical observations because the animals will tend to spend a disproportionate amount of time inside them. The smaller sized polycarbonate rat and mouse cages have been

employed with greatest success. Entrance holes should be cut approximately 2 in. above floor level, which prevents the newborn kits from falling out while permitting easy access for the jill.

Individual Caging Requirements of Adult Ferrets

Littermates of both sexes can be group housed until sexual play or aggressive behavior becomes too violent. Thereafter, individualized caging is required, especially during periods of peak sexual activity and pregnancy, or when suppression of estrus is desired. Sexually active male ferrets, and to a lesser extent females, will fight when housed with others of the same sex. Similarly, although the species is not prone to cannibalism, jills that have just whelped should be allowed to recover and nurse their litter of kits undisturbed. Segregating by sex and by room will generally help to suppress the incidence and onset of estrus in females. Conversely, desegregation might promote the onset of sexual activity, and the sexual play of females might elicit ovulation or a pseudopregnancy.

For breeding purposes, it is recommended that receptive females be transferred to the sexually active male's home cage. When coitus is completed, the female should be returned to her own cage.

Reproductive sterilization apparently reduces aggressive behavior sufficiently such that animals can be group housed. However, a pecking order or individual domination might still become manifest.

Lighting

Similar to the effects of altered photoperiodism documented for mink, the light–dark cycle employed in the ferret vivarium can be manipulated to control the breeding cycle. Ferrets are seasonal breeders and will become conditioned for breeding with exposure to longer day lengths or periods of artificial illumination. To prevent the onset of sexual activity (or first estrus) in younger animals arriving at the vivarium during the natural breeding season (January–March), or to maintain a colony of stock animals in a sexually inactive state, lighting is optimally set between 6:18 and 10:14 light:dark to simulate the early winter (nonbreeding) months (Moody et al. 1985; Ryland and Gorham 1978). Conversely, increasing the period of illumination to between 12 and 16 hr might stimulate sexual activity in this species (Donovan 1967). Detailed discussions of the effects of day length on the coat-shedding cycles, body weight, and reproduction of the ferret have been published by Harvey and MacFarlane (1958) and Donovan (1966, 1967). Earlier but equally informative studies were reported by Bissonnette (1932), Hart (1951), and Hammond (1952).

It is noteworthy that loss of hair, which can proceed to the development of alopecia, is observed in ferrets (especially marked in estrus females) subjected to long exposure of 16 hr of light daily. Conversely, marked growth of hair takes place under short-day conditions. Because marked hair growth can help to confirm that animals are being maintained in a sexually inactive state, as opposed to pregnancy or pseudopregnancy (marked by hair loss), the condition of the coat should be monitored and included as part of the clinical observations protocol. A classic study of the molting and fur growth pattern in the adult mink, which applies to the ferret, was published by Bassett and Llewellyn (1949).

Temperature and Humidity

Because of their thick fur, ferrets will tolerate low environmental temperatures and, in fact, many commercial producers began their own business by group housing their sexually inactive animals in outdoor sheds throughout the year. Freezing winter temperatures were evident when the high-moisture diets fed to these animals froze on the cage floor pallets. Prolonged cold spells simply caused these animals to mold together into a single, continuous ball of fur buried deep beneath the straw bedding. This natural tolerance for cold temperature can be extrapolated to the vivarium.

For maximum thriftiness of mature, individually housed animals, environmentally controlled rooms should maintain an optimal temperature of 15 ± 5°C. Thickly furred adults will benefit from

the lower end of this temperature range. Conversely, lactating jills and their unweaned litters might require the slightly warmer temperatures at the upper end of this range.

As with most other species, high relative humidity is apparently not an important factor in ferret performance, except when accompanied by prolonged elevation in temperature. The recommended relative humidity for the vivarium is 50% ± 20%.

Clinical Signs of Heat Stress

Ferrets are particularly vulnerable to heat stress, and special care is necessary when moving them from one area to another during hot weather. Adult ferrets and breeding stock are especially sensitive owing to their subcutaneous fat cover and their inability to dissipate heat by sweating. Clinical signs of hyperthermia include panting and mouth breathing, prostration followed by eventual flaccidity, and emesis. When a tiered caging system is used, particular attention should be paid to animals housed in the tier closest to the ceiling, where the warmer air can concentrate.

Room Air Changes

Regardless of species, most modern vivariums are designed to maintain a range of 10 to 15 complete exchanges of room air (nonrecirculated) per hour. Ferrets do well under these conditions, but because of their musky odor and susceptibility to respiratory pathogens, the higher end of the range is frequently indicated. In the authors' experience, environmentally controlled animal rooms measuring 14' × 22', employing nine racks of nine cages per rack of wash-down caging (cleaned with water once daily), nonabsorbent wall and floor surfaces, and 15 air exchanges per hour will not retain an odor even when ferrets are housed two per cage at maximum room density.

Diet and Water

Natural Ingredient and Purified Diet Formulations

A recent survey (McLain, Harper et al. 1985) of a large commercial ferret colony (Marshall Farms, North Rose, New York) detailed a successful ingredient diet for ferrets (table 7.3). Reproduction data reported in this survey demonstrated that primiparous females fed this natural ingredient diet whelped an average of 10.3 ± 0.2 kits per litter (range = 1–18), successfully weaned 80% of these, and frequently produced three to four litters per year. Subsequently, the Marshall Farms natural ingredient ferret diet was analyzed and reproduced as a purified formulation (McLain and Roe 1983; McLain et al. 1988). Moreover, when prepared with animal fat and ≥ 50% moisture (using 2% agar), adequate weight gain is demonstrated for animals consuming this purified formulation.

Table 7.3 Composition of a Successful Natural Ingredient Ferret Diet

Ingredient	Approx. Wt. per Batch[a]
Wayne dog food cereal	100
Agway dog food cereal	50
Beef tripe	100
Beef lung	50
Beef liver	10
Fortified cod liver oil	1
Water	(varies)

[a] Total batch weight is approximately 1,000 lb. Dry ingredients are added to a mechanical mixer from bags or cans; water is added until desired consistency is achieved. Source: Courtesy of Mr. Gilman Marshall, Marshall Farms, North Rose, New York.

Commercial Diets

Two large commercial producers of animal feed presently market least-cost formulation ferret diets that also promote adequate weight gain. Ralston-Purina (St. Louis, Missouri) offers the Purina 5280 ferret chow, which is pelleted and available as a certified ration. Agway, Inc. (Ithaca, New York) also offers a pelleted ration that closely parallels the nutrient composition of the Marshall Farms natural ingredient diet.

Consumption Patterns and Acclimation of Ferrets to Commercial Rations. For maintenance of body weight, ferrets will require between 200 and 300 kcal/kg body weight daily. This translates to between 50 and 70 g/day of the pelleted commercial rations described previously, not accounting for spillage (McLain and Roe 1983; McLain et al. 1988). Bleavins and Aulerich (1981) reported food consumption to average 43 g/kg body weight in the ferret, and mean food passage time (as measured by a dye marker in the feed) was reported to average approximately 3 hr, indicating a relatively short digestive tract.

The study of food habits of ferrets living in the wild has resulted in several interesting and informative behavioral publications (Kaufman 1980; Roser and Lavers 1976). However, nothing has been published that will prepare the naive investigator for an unacclimated ferret's first encounter with a pelleted diet offered in a stainless steel or glass bowl. Initially, new arrivals will appear to immensely enjoy the sound of digging in metal or glass food dishes and subsequently spilling their daily allotment of food. To cure this habit, replenish the food only during designated feeding times. By approximately the third day, the animal will have lost body weight and be sufficiently hungry so that food pellets moistened slightly with water will be ravenously consumed. Gradually decrease the amount of water added to the pellets until dry food is consumed exclusively. This process might have to be repeated. Fortunately, the burden of pelleted diet acclimation has been assumed by the more conscientious animal vendors.

On rare occasions, an individual animal might appear to have chosen starvation rather than consuming a dry food ration. In these cases, canned (moist) cat or dog food or fresh organ meats might have to be fed to ensure survival. Although animals receiving these diets and special considerations might have to be excluded from controlled protocol studies, they can still be used subsequently for technical training purposes or exploratory protocols.

Automatic Watering Systems

Many commercial ferret producers use automatic watering systems in their facilities. Consequently, ferrets will readily adapt to the demand-controlled automatic systems present in the newer caging units, or to water bottles attached to the cage. As with other species, the valves of automatic watering systems must be checked routinely to ensure that they are functional, and water bottles should be changed daily.

Acclimation and Quarantine Procedures

Special Attention for New Arrivals to the Vivarium

It would be naive to assume that newly arriving ferrets will tolerate the type of handling that is afforded the more "conditioned" animals of this or any other species. In reality, ferrets arriving from most commercial breeders will have been selectively bred for an even disposition, but memories of pecking order and perhaps competition for food and water during shipping might still be fresh in their minds. Many of the new animals will be confused and frightened by the unfamiliar sights and sounds experienced during shipping. A small percentage of ferrets might, in fact, have become overly stressed and understandably resist (e.g., hiss, scream, back away from the hand)

any initial attempts to handle them. To reduce the risk of injury to both animal and technician, therefore, shipping crates containing newly arriving animals should be unpacked cautiously (preferably away from bright light or loud noise) and animals offered fresh food and water and dim lighting as soon as possible. Additionally, a qualified animal health technician or laboratory veterinarian should be available to assist in the identification and treatment of any animals that have succumbed to heat or shipping stress. Ferrets will generally respond rapidly to this initial attention paid to them by allowing handling and petting within only hours of arrival. Slower responding animals will require more patience, but will usually assume a more even disposition with additional efforts during acclimation.

Initial Physical Examination Parameters and the Observations Recorded During the Period of Quarantine

All animals should be subjected to a complete physical examination within 1 to 2 days of arrival. Examination should include inspection for disorders of the skin and fur (ectoparasites), nose and throat, eyes and ears, obvious physical impairments that would preclude the use of the animals in scientific research, fecal and urine screening (see later section on urinalysis), and the presence and condition of vendor-applied ear tags. The quarantine period for ferrets should be long enough to allow an evaluation of daily clinical observations, body weight change, and food and water consumption patterns for their individual contribution to the animals' physical health. Routine clinical observations and body weight changes by themselves, when recorded over a 1- to 2-week period, will usually enable the identification of animals infected with either respiratory or intestinal pathogens. Optimum prophylaxis requires that diseased animals be culled or isolated from healthy animals and administered appropriate professional health care.

Depending on the scientific application, blood samples might or might not be collected from animals during the quarantine period (see later section on blood sampling techniques).

A typical animal history chart for use during the quarantine periods of ferrets is shown in figure 7.1.

Veterinary Procedures and Common Diseases

General veterinary care of the ferret is similar in many aspects to that provided the feline and canine species. Approximate drug dosages, when required for specific veterinary procedures or prophylaxis, are listed in table 7.4 and table 7.5. Confirmation of these dosages is recommended for each laboratory before routine use.

Anesthetics and Drug Dosages

Anesthesia, sedation, or tranquilization, although not mandatory, will frequently facilitate initial, detailed physical examinations of the ferret and significantly reduce the potential for inadvertent injury to the examiner or animal. In addition, mild tranquilization will also facilitate examination of the eyes and have an indirect calming effect on the ophthalmologist as well. The following sections, therefore, provide general information concerning recommended agents, routes of administration, and doses of anesthetics, tranquilizers, and other various drugs frequently used in the ferret. These guidelines are intended to comply with appropriate sections of the Animal Welfare Act.

General Anesthetics

General anesthetics used successfully in ferrets include those drugs that produce controllable and reversible loss of consciousness with analgesia. The same general anesthesia principles apply to ferrets as would be practiced with most other species. For example, when possible, withhold food from ferrets prior to anesthetic administration. In addition, give a general physical examination

Facilities:
Address:

ANIMAL HISTORY

DATE RECEIVED	VENDOR	U.S.D.A. NO.	INITIAL WGT.	SCALE NO.

DATE OF BIRTH	BREED/DESCRIPTION	SEX	HISTORY

PHYSICAL EXAMINATION	DATE	BY	REVIEW	LAB
NA	GENERAL APPEARANCE:			
NA	INTEGUMANT: () ECTOPARASITES () ALOPECIA			
NA	DIGESTIVE: () ORAL () STOOL () AUSCULTATE () PALPATE			
NA	CV - RESPIRATORY: () AUSCULTATE () NASAL DISCHARGE			
NA	LYMPH NODES: () CERVICAL () AXILLARY () INGUINAL			
NA	MUSCULOSKELETAL:			
NA	UROGENITAL: () VULVAL SWELLING () DESCENDED TESTICLES			
NA	EYES: () NUCLEAR OPACIFICATION () CONJUNCTIVITIES			
NA	EARS: () OTITIS () ACARIASIS			

ASSIGNMENT HISTORY			FINAL DISPOSITION (CIRCLE ONE)		DATE
DATE	PROJ. NO.	STUDY DIR.	DIED	EUTHANIZED	
			CAUSE/METHOD		
			COMMENTS		INITIALS

COMMENTS:

VACCINATIONS (TYPE/DATES)

SPAYING/NEUTERING (DATE)	DESCENTING (DATE)
POSTQUARANTINE BODY WGT./SCALE NO.	FOOD HABITS/DIET FED
I.D. NUMBER	OTHER I.D.

Figure 7.1 Quarantine information chart for ferrets. Additional physical examination data should be added as required by each facility.

immediately prior to treatment to evaluate the general condition of the animals in terms of anesthetic risk. Once anesthesia is induced, monitor the animals every 15 min for heart and respiration rate, mucous membrane color, capillary refill time, and plane of anesthesia. During recovery from anesthetics, isolate the animal, place it in sternal recumbency until swallowing and jaw reflex return, and provide for maintenance of body temperature. Ferrets should be turned to the opposite side every 30 min during prolonged recovery to prevent hypostatic congestion of the lungs.

The various ketamine (KET) mixtures listed in table 7.4 are apparently the most frequently used anesthetics for minor surgical and noninvasive procedures, with a 25- to 35-mg/kg dosage inducing a desirable limpness and flaccidity to the animal body for approximately 30-45 min. Limited observations by one of the authors suggest that the albino strain of the ferret might be slightly more sensitive to KET-acepromazine (ACE) than the more common sable strain. Several

Table 7.4 Guidelines for Sedative, Preanesthetic, and Anesthetic Dosages in the Ferret[a]

Sedative	
Acepromazine (ACE)	0.2–0.5 mg/kg im, sc
Ketamine (KET)	10–20 mg/kg im
Xylazine (XYL)	1.0 mg/kg im, sc
Diazepam (DZP)	1.0–2.0 mg/kg im
Preanesthetics	
Acepromazine	.01–0.25 mg/kg im, sc
Atropine	0.05 mg/kg im, sc
Anesthetics	
KET	30–60 mg/kg im
KET + ACE	KET: 20–35 mg/kg
(100:1 mixture)	ACE: 0.2–0.35 mg/kg im
KET + Valium	KET: 25 mg/kg
(10:1 mixture)	VAL: 2.5 mg/kg im
XYL (followed by KET)	XYL: 2–3 mg/kg sc
	KET: 20–30 mg/kg im
DZP (followed by KET)	DZP: 2.0–3.5 mg/kg sc
	KET: 20–35 mg/kg im
Pentobarbital	25–35 mg/kg ip

[a] Dosages should be confirmed in each laboratory before application.

Table 7.5 Guidelines for Antibiotic, Hormone, and Steroid Dosages in the Ferret[a]

Antibiotics	
Albon (oral, inject)	Feline dose
Albon (coccidian Rx)	Feline dose
Amoxicillin (oral)	25–35 mg/kg bid
Ampicillin	10 mg/kg bid sc
Gentamicin	5 mg/kg im
Griseofulvin tabs	25 mg/kg
Ivermectin	1 mg/kg
Neomycin	10–20 mg/kg
Penicillin (Flo-Cillin)	Feline dose
Piperazine salts	Feline dose
Tetracycline (oral)	25 mg/kg bid
Hormones and steroids	
Dexamethasone	0.25 mg/kg
GnRH	20 ug
HCG	100 IU im
Insulin	1–2 units/kg
Oxytocin	0.2–3 U/kg sc, im
Prednisone (oral)	0.5–2 mg/kg
	Feline schedule

[a] Dosages should be confirmed in each laboratory before application.

informative discussions of ferret tranquilization, preanesthesia, and anesthesia have been published (Fox 1988; Moody et al. 1985; Moreland and Glaser 1985). Unfortunately, very little discussion is afforded to KET-ACE, a mixture with which one of us has had great success.

Preanesthetics. Preanesthetics include those drugs that facilitate effective anesthesia in the ferret. Atropine sulfate and acepromazine (table 7.4) are used effectively and are indicated to reduce excessive upper respiratory secretions associated with some anesthetics, and to decrease bradycardia (vagal effects). Atropine sulfate, for example, should be administered approximately 15 to 30 min

prior to anesthesia, and has a duration of action of approximately 15 to 30 min. It could cause dilation of the pupils and decreased lacrimation; therefore, use of ophthalmic ointments might be indicated.

Tranquilizers and Sedatives. These agents are frequently used to facilitate restraint for nonpainful procedures such as bandaging, radiology, parenteral injections, venipuncture, or examination (including ophthalmic); to decrease excitement in nervous or frightened animals; and, as preanesthetics, to decrease the dose of anesthetic required. Note that these agents are not analgesic or anesthetic in nature and should not be used as pain relievers.

The tranquilizing effect of acepromazine (table 7.4) can last several hours in the ferret. Moreover, when combined with ketamine, it is excellent for analgesia, sedation, and minor surgical procedures. However, because it can cause peripheral vasodilation, thermoregulation should be maintained.

Prophylaxis and Vaccination Schedules

Most commercial suppliers of ferrets routinely vaccinate kits against canine distemper virus (CDV) at approximately 6 to 8 weeks of age (2 weeks earlier if the jill has not been recently vaccinated), with boosters administered approximately 4 weeks later and every 3 years thereafter. A modified-live distemper virus of chick embryo tissue culture origin has been used successfully, as has the modified-live virus of canine origin. Killed vaccines might be ineffective (Ott and Svehag 1959) and unattenuated viral vaccines derived from ferret cell culture might be too virulent. Commerical breeders routinely vaccinate pregnant ferrets without harm to the fetus (Hagen et al. 1970). Unvaccinated ferrets exposed to the canine distemper virus for only 15 min will acquire the disease, and close to 100% of these animals will die. Because there is no cure for the disease once it is acquired, it is strongly recommended that the initial vaccination and subsequent booster be a prerequisite of animal purchase. A detailed description of the disease in ferrets was published by Ryland and Gorham (1978).

The incidence of rabies in domestic ferrets is very slight. The Centers for Disease Control (CDC) in Atlanta, Georgia, reports only five cases in the United States over a period from 1958 through 1983, and no recent cases have been confirmed. The U.S. Food and Drug Administration (FDA) has not yet approved a rabies vaccine, but the Canadian Council on Animal Care clearly states in its Guide to Care and Use of Experimental Animals (Vol. 2), that inactive ("killed") rabies vaccine is effective for ferrets. More recently, according to McBride (1989), Norden Laboratories of Lincoln, Nebraska, has finished preliminary testing on an inactivated ferret rabies vaccine and has begun the challenge test. A ferret rabies vaccine was approved in 1992. Testing has shown a slight difference in serological response from males to females, which could mean ferrets might require a two-dose initial vaccination (recommended vaccination age will be 12 weeks), with boosters (dosage = 1 cc) given every 12 months thereafter.

Surgical Sterilization Procedures and Anal Musk Gland Removal

Reproductive sterilization of laboratory ferrets is accomplished using surgical procedures and conditions typically described for the feline and canine species (Randolph 1986). Moreover, removal of the ferret's anal musk glands is similar to that documented for other mustelids (Creed and Kainer 1981). These procedures (spaying, castration, and descenting) are typically performed by vendors (on request) on animals as young as 8 weeks of age, apparently with minimal blood loss or tissue trauma when the proper anesthesia and surgical techniques are employed. When allowed by scientific protocol, these procedures are recommended for the chronic (repeated),

restrained, intravenous (caudal vein) treatment of the stronger smelling and more aggressive male ferrets especially, and to preclude estrous cycling of females.

Most Frequently Observed Diseases of Ferrets Maintained Under Controlled Laboratory Conditions

Although they are probably 10 breeding years behind the current virus-free and specific pathogen-free (SPF) rats, laboratory ferrets maintained under controlled conditions manifest a relatively low incidence of debilitating diseases. On the contrary, very few diseases with unique clinical signs that would enable early detection for prophylaxis have been documented in ferrets. Moreover, underlying pathological conditions appear to be rarely evidenced by more than a reduction in body weight and a reduction of appetite and food intake. Literature reviews of the various diseases observed in this species have been published by Ryland and Gorham (1978) as well as the very extensive review by Fox (1988). A more recent review has been published by Rosenthal (1994).

Influenza and Pneumonitis

As demonstrated by earlier by earlier classic studies (Bell and Dudgeon 1948; Fisher and Scott 1944; Smith et al. 1933; Smith and Stuart-Harris 1936) the human influenza virus can be readily transmitted from human to ferret, ferret to ferret, and ferret to human by way of aerosolized droplets containing infective viral particles. Infection is characterized by lethargy, elevated rectal temperature (40°C–41°C, lasting 1 day and then returning on the third day), and bouts of sneezing accompanied by a mucoserous nasal discharge. Some animals might also manifest a conjunctivitis or otitis. Clinical signs can persist for up to 2 weeks, with resistance to the same strain of influenza virus demonstrated on subsequent exposure. Congestion might be relieved by antihistamines suitable for other species.

Preventive measures include the wearing of facemasks by personnel exposed to outbreaks of the viral infection, and the isolation of infected animals. Limited prophylaxis has been reported with 6 mg/kg bid aerosolized amantadine hydrochloride (Cochran et al. 1965; Cusumano et al. 1965; Fenton et al. 1977) and to a lesser extent with 100 mg/kg ribavarin (Fenton and Potter 1977). An additional influenza-like disease includes the respiratory syncytial virus (RSV) infection of infant ferrets (Prince and Porter 1976; Tyrrell and Hoorn 1965). RSV also has significant health consequences in the human infant. In the absence of an adequate small animal model for testing the efficacy of adenovirus-vectored RSV vaccines, Hsu et al. (1994) established a ferret model for this purpose. In their studies, recombinant adenovirus types 4, 5, and 7 expressing the RSV fusion glycoprotein (F), the attachment glycoprotein (G), or both F and G were constructed previously. These recombinants contain a deletion of a large portion of the E3 region of the respective adenovirus vector. In addition, the researchers constructed an Ad7 (E3+) F recombinant virus that contains an intact E3 region to assess whether E3 region functions might enhance vaccine immunogenicity. Evaluation of these viruses in the ferret model demonstrated that Ad4 and Ad5 recombinants, administered intranasally to ferrets, induced stronger seroresponses to RSV than did Ad7 recombinant viruses. Ad7 (E3+) F did not show enhanced immunogenicity relative to E3-deleted recombinant viruses. However, measurement of RSV infectivity in nasal washes, following intranasal RSV challenge, showed that five different vaccination regimens, Ad7F/Ad4F, Ad7G/Ad4G, Ad7FG/Ad4FG, Ad4F/Ad7 (E3+)F and Ad5F/Ad4F, protected ferrets from RSV infection in a dose-dependent manner.

Bacterial pneumonias affecting ferrets are similar to those seen in other species. The list of reported strains includes *Bordetella sp.* (McLain 1989) and *Streptococcus sp.* (Andrews et al. 1979), as well as the various gram-negative bacteria (*E. coli, K. pneumoniae, P. aeruginosa*).

Interstitial pneumonitis in conjuction with focal mononuclear cell infiltrates can be caused by bacterial, viral, or protozoan infections, including *Pneumocystis carinii.* The latter protozoan parasite

is known to inhabit the lungs of various domestic and laboratory animals with compromised immune systems (Farrow et al. 1972; Long et al. 1986; Milder et al. 1980). Recently, Stokes et al. (1987) studied the disease in ferrets by immunosuppressing the animals with cortisone acetate (10–20 mg/kg subcutaneous for 9–10 weeks).

Aleutian Disease

Aleutian disease, a persistent and frequently fatal parvovirus infection first reported to be common in the Aleutian mink (Henson et al. 1961; Obel 1959), also occurs in the ferret (Kenyon et al. 1967; Kenyon et al. 1966; Ohshima et al. 1978). However, the strain of parvovirus infecting ferrets, although resulting in similar pathological lesions (e.g., hypergammaglobulinemia and plasma cell dyscrasia), is rarely fatal to them. Reviews have been published for Aleutian disease in mink (Porter et al. 1980) and in ferrets (Porter et al. 1982). Mild to moderate lymphocytic infiltrates of the liver (periportal localization), lung (pneumonitis), and kidney (polynephritis/glomerulonephropathy) are the predominant histological finding in ferrets with Aleutian disease. Occasionally, an animal will become anorexic and thin, and pass black, tarry feces.

Kenyon et al. (1967) have defined the hypergammaglobulinemia associated with Aleutian disease in ferrets as greater than 20% of total serum protein as γ-globulin. Moreover, Porter et al. (1982) demonstrated that ferrets with the highest Aleutian disease virus antibody titer also had the greatest increase in serum gamma globulin levels and the most severe tissue lesions. Because ferrets infected with Aleutian disease are generally asymptomatic, and the corresponding changes associated with the disease have potential for misinterpretation, McLain and Lin (1989) developed a method for predicting the degree of periportal lymphocytic infiltrates from routine blood samples. When both pretreatment and posttreatment blood samples are used in the "liver lymphocyte index" (LvLI) equation, the prediction or correlation was shown to have a 92% to 96% success rate (see later).

Proliferative Colitis

Fox et al. (1982) described proliferative colitis in ferrets and addressed the histopathological and bacteriological features of this disease entity, which, according to the authors, is fatal or requires euthanasia in almost all affected animals. The etiological agent is probably a *Campylobacter sp.,* with fecal-oral spread and food- and waterborne transmission apparently the principle avenues for infection.

Clinical signs of proliferative colitis include diarrhea, anorexia, and marked weight loss (≥ 10%–15%/week). Significant elevations in urine pH (≥ 8.0) and urobilinogen (≥ 2.0) might occur, compared with normal values of approximately 6.0 to 6.5 and 0.1, respectively. In the experience of one of the authors, the incidence of the disease has been 3 in approximately 1,000 animals (newly arriving only), with mortality occurring in only 1 of these. Antemortem fibrinogen was 442 mg/dl in the single fatality, compared with a normal value of 189 ± 78 mg/dl (95% upper tolerance limit = 387 mg/dl). It is noteworthy that in the same author's experience, no cases of proliferative colitis have occurred in resident animals released from quarantine.

Treatment for proliferative colitis should be instituted immediately on noting diarrhea and anorexia. Amoxicillin (oral suspension, 250 mg/5 cc), Pepto Bismol, and water should be given orally twice a day for 5 to 7 days in a dosage containing 1 cc amoxicillin, 1 cc Pepto Bismol, and 1 cc water.

Canine Distemper Virus (CDV)

Mortality in ferrets infected with pathogenic CDV approaches 100%. CDV was previously considered to have a host range restricted to the canid family. In 1994, the virus was associated with sporadic outbreaks of distemper in captive felids. However, after severe mortality occurred in the Serengeti lions (*Panthera leo*), attention focused on the pathogenesis of the virus and a concerted

effort was made to identify the virus as CDV or a closely related feline morbillivirus. Evermann et al. (2001) designed a study to explore the susceptibility of ferrets to challenge with two morbilliviruses isolated from lions and the protective effects of a modified-live mink distemper vaccine. Two strains of lion morbillivirus were used as a challenge, A92-27/20 (California lion isolate) and A94-11/13 (Serengeti lion isolate). The two strains of lion morbillivirus were antigenically related to CDV (Rockborn strain), and ferrets were susceptible to both of the viruses when inoculated intraperitoneally. The inoculated ferrets were anorectic at 5 to 6 days postinoculation (PI), exhibited oculonasal discharge at 9 to 12 days PI, and became moribund at 12 to 22 days PI. Severe bilateral conjunctivitis was the typical clinical sign. Inclusion bodies characteristic of morbillivirus (eosinophilic, intranuclear, and intracytoplasmic) were distributed in many epithelial cells, including those of the skin, conjunctiva, gallbladder, liver, pancreas, stomach, trachea, lung, urinary bladder, and kidney. Virus was reisolated from selected lung tissues collected at necropsy and identified by CDV-specific immunofluorescence. Ferrets vaccinated with the mink distemper vaccine (Onderstepoort strain) were protected from challenge with the two lion strains, adding further support to the premise that the viruses are closely related to CDV.

Bone Marrow Hypoplasia and Estrogen-Induced Anemia

Aplastic anemia has been associated with prolonged estrus in the female ferret (Kociba and Caputo 1981). Similarly, bone marrow hypoplasia in estrous ferrets has been attributed to prolonged exposure to estrogens. Because unbred female ferrets will remain in estrus for the duration of the normal breeding season (March–August), the potential for this pathological condition to occur would seem high.

Clinical signs of bone marrow hypoplasia include cutaneous petechiae and ecchymoses, GI hemorrhages, systemic bacterial infections, and pale mucous membranes (Kociba and Caputo 1981). Hematological findings include initial thrombocytes and leukocytosis followed by thrombocytopenia, leukopenia, and anemia. Decreased platelets (< 50,000/μl), hemorrhagic anemia, and death can occur in 40% to 50% of affected animals. Histopathological findings include bone marrow hypoplasia affecting all cell lines and decreased splenic extramedullary hematopoiesis (Sherrill and Gorham 1985). Prevention of the condition requires sterilization or breeding of estrous females, a controlled lighting regimen to preclude estrus cycling, or an intramuscular injection of human chronic gonadotropin (HCG; 100 IU; see table 7.5) administered at the peak of vulval swelling (diameter ≥15 mm) and repeated in 7 to 10 days if vulval regression does not occur.

Dosing

Gastric intubation is accomplished in fully conscious ferrets with a 10- to 16-gauge × 6-in. straight, stainless steel dosing needle with a 6.4-mm stainless steel ball on the end (EJAY International, Inc., Glendora, California) or, alternatively, with an appropriate diameter plastic tubing or French catheter. Because of the required length of the intubation needle or tubing, the animals should be held and treated in a vertical position. This is most easily accomplished by holding or resting the animal vertically across one's chest, allowing the intubation tube a straight, downward motion.

As with other test species, placement of the intubation tube within the stomach of the ferret is confirmed by visual examination of aspirate. McLain and Roe (1983) described the successful, daily intubation of approximately 40 pregnant female ferrets (20 doses/animal, 800 doses) without incident using this procedure.

Gag Reflex and Emesis in the Ferret

Similar to the dog and cat, the ferret is capable of a gag or vomit reflex and has received considerable attention as an alternative animal model for the study of the physiology of emesis

(Florczyk et al. 1981). Oral dosing of nausea-producing agents will elicit characteristic behavior, including licking, chin rubbing, walking backward, and slit eyes. Andrews et al. (1990) have published an excellent review of the abdominal visceral innervation and the emetic reflex in ferrets. Its similarity of responses to those of humans has made the ferret the model of choice for studying emesis (Sam et al. 2003; Schnell 2003).

Gut Physiology and Microflora

Poddar and colleagues (Poddar 1977; Poddar and Jacob 1977; Poddar and Murgatroyd 1976) and Pfeiffer (1970a, 1970b) have published rather extensively on the morphological and histological similarities of the GI tract of the human, ferret, and monkey.

Recent efforts have failed to identify any anaerobic gut microflora in the ferret (Gad, personal communication, August 1989). Identified enteric organisms include *Salmonella, Campylobacter sp., Cryptosporidia sp.,* and *Elimeria sp.* Moody et al. (1985) concluded that the IV administration of a test article to ferrets is virtually impossible without an indwelling catheter because the species lack easily accessible veins. More recently, Fox (1988) reiterated this and suggested that venous access (jugular, cephalic, or femoral) in the ferret might require sedation, local anesthesia, and a surgical skin incision for placement of a catheter for short-term drug or fluid administration.

However, McLain, McCartney et al. (1987) have reported on the subchronic (90-day, three times weekly), IV administration protocol in ferrets without the use of an indwelling catheter. Moreover, these authors routinely administer IV fluids repeatedly, and large volumes have been administered daily with prolonged infusion times by using either a 23-gauge, 1-in. needle, Angio- or Quik-Cath catheter. These procedures are possible with either mechanical or physical restraint of test animals and by access of the caudal vein.

The caudal veins of the ferret cannot be palpated, nor are they visible to the naked eye. To access these veins, view the dorsal surface of the shaved tail as the 12 o'clock position. Turn the tail in a clockwise or counterclockwise direction so that either of the large veins residing at the 10 o'clock or 2 o'clock position now assumes that 12 o'clock position. Insert a 23-gauge, 1-in. needle (or, e.g., Quick-Cath catheter) at a depth of angle similar to that used for rodents. The caudal artery assumes at 6 o'clock position when the animal is viewed dorsally. A diagram of a transverse section of the ferret tail, showing location of caudal artery and veins, was published by Bleakley (1980).

Typical IV infusion volumes and rates employed for ferrets depend, as with other species, on the glomerular filtration rate (GFR) and the physical characteristics of the specific test article. Ferrets can probably receive between 2 and 4 ml/kg/hr of IV fluids without a notable increase in urine volume or frequency. This compares with an approximate continuous infusion rate of 0.5 ml/kg/hr for dogs, and 8 ml/kg/hr for rats. Using the aforementioned ratios and "reference" body weights of 0.25, 1.5, and 10.0 kg for rat, ferret, and dog, respectively, the approximate total daily (24 hr) fluid volume that can be delivered to these test species without notable increases in the urine volume or frequency is, therefore, 50 ml (rat), 70 to 140 ml (ferret), and 120 ml (dog). Humans (70 kg) typically can receive volumes up to 3,000 ml/day, or approximately 2 ml/kg/hr. Increased fluid administration rates and volumes will generally be accommodated by all test species in direct relation to their individual GFRs.

Restraining Tube for Intravenous Dosing

Limited or initial IV dosing success might be possible with the crude ferret restraining device originally proposed by Curl and Curl (1985) for serial blood sampling. However, as the IV access capabilities of the laboratory improve, and more prolonged and frequent infusions are required in animals of different sizes, a more sophisticated restraint system will be necessary.

The typical ferret restraining device was designed for the purpose of accommodating the increasing skills of the laboratory's technical staff. Four different-sized restrainers have been developed and are proposed to accommodate the sexual dimorphism of ferrets, the seasonal deposition of body fat, and the various ages used in different research investigations. The inside diameters of the restraining tubes, and the corresponding weight ranges that they have accommodated include 6.4 cm, 400g to 700 g; 7.9 cm, 650 g to –1,100 g; 8.8 cm, 1,000 g to –1,750 g; and 10.1 cm, 1,500g to 2,300 g. Details of construction have been reported by McLain and McGrain-Dutson (1989).

Surgical Implantation and Maintenance of Indwelling Intravenous Catheters in Ferrets

Indwelling IV catheters can be implanted in ferrets when it is necessary to administer solutions at a very slow or constant rate using an infusion pump, or for chronic studies requiring many consecutive days of treatment.

Greener and Gillies (1985) have developed a method to ensure catheter patency throughout the course or acute of chronic studies without the use of heparin. These authors fill the catheter lumen with saline solution, and clamp the catheter with a rubber-shod hemostat at the end of each infusion. They then remove the syringe containing a blunt needle from the lumen of the tubing, insert an appropriately sized stainless steel blocking pin, then remove the rubber-shod hemostat. When the stainless steel pin is pushed slightly further into the catheter, the saline fluid is forced to fill the lumen tip at the venous end, discouraging clot formation. A detailed description of catheter implantation and maintenance in ferrets was published by Greener and Gillies (1985). An earlier method for the chronic jugular catheterization of the ferret was published by Florczyk and Schurig (1981). Animals can be treated in restraining tubes or hand-held and treated.

Inhalation Exposure of Ferrets

The pulmonary mechanics and physiology of the ferret lung as well as the potential of the species as an animal model for inhalation toxicology have been described quite thoroughly by Vinegar et al. (1979, 1982, 1985) and by Boyd and Mangos (1981). Noteworthy advantages of the use of ferrets in inhalation toxicology include the fact that because the ferret has more submucosal glands in the bronchial wall and an additional generation of terminal bronchioles, it is closer to the human lung than is the dog lung. Moreover, the disproportionately large lungs of the species can provide a useful tool for the studies of uptake, clearance, and deposition of aerosols; pulmonary blood flow; and diffusion. As suggested by Vinegar et al. (1985), they might also be good candidates for an isolated perfused lung preparation for metabolic studies and for regional lavage to look for markers of lung injury.

Physiological Measurements of the Ferret Lung

Physiological measurements were made by Vinegar et al. (1985) on anesthetized, tracheotomized, supine male ferrets. Six animals weighing 576 ± 12 g had tidal volumes (V_t) of 6.06 ± 0.30 ml, respiratory frequencies (f) of 26.7 ± 3.9/min, minute volume of 157.0 ± 14.8 ml/min, dynamic lung compliance (C-dyn) of 2.48 ± 0.21 ml/cmH_2O, and pulmonary resistance (R) of 22.56 ± 1.61 ml/cmH_2O/L/sec. Measurements on nine ferrets (including the six just mentioned) revealed a total lung capacity (TLC) of 89 ± 5 ml, vital capacity (VC) of 87 ± 5 ml, expiratory reserve volume of 16 ± 2, and a functional residual capacity (FRC) of 17.8 ± 2.0 ml. Maximum expiratory flow-volume curves showed peak flows of 10.1 VC/sec at 75% VC and flows of 8.4 and 5.4 VC/sec at 50% and 25% VC.

The total lung capacity of ferrets was estimated to be approximately 297% of what would be predicted for an animal of its size.

Miscellaneous Dosing Routes

Intramuscular, subcutaneous, intradermal, dermal, or intraperitoneal dosing procedures in the ferret are essentially no different than what would be practiced for other test species. Intramuscular administration, for example, is most easily given in the lateral aspect of the upper leg using a 28-gauge, $\frac{5}{8}$-in. needle. Subcutaneous or intradermal administration can be given just above the shoulders on the dorsal aspect of the neck, taking care not to deposit lipophillic test materials in subcutaneous fat pads, as they will be poorly absorbed. A dermal application site (for an uncovered test material) that is inaccessible to the ferret is the dorsal aspect of the neck, immediately above the shoulders.

Intraperitoneal administration to ferrets is generally easier when performed by two people. One person should restrain the animal by securing the neck, shoulders, and front limbs with one hand while using the other hand to secure the hind limbs and position the animal on its back or against the holder's chest. The injection can be administered with an appropriate gauge needle through an elevated, pinched portion of skin located around midabdomen. Because ferret skin is very thick, care should be taken to assure that the test material is not deposited subcutaneously or intradermally.

Less frequently used routes of administration in the ferret include intracerebral, intraduodenal, intrapleural, intranasal, intratracheal, intrathoracic, intravaginal, and rectal. Apparently, there are no unique anatomical features of the ferret that would preclude the use of any of these routes when the appropriate procedures are employed. In that respect, Chimes (1994) describes a method for surgical implantation of a catheter for repeated administration of liquid into the lungs of ferrets. By varying the size of the tubing, the method could be adapted for rodents or other animals. Furthermore, some of the techniques used to accommodate animal growth could be usefully adapted for other chronic catheterization or cannulation methods.

Important Physical Parameters

Developmental Milestones

At birth, ferret kits weigh between 7 g and 10 g (Shump and Shump 1978; Willis and Barrow 1971). The deciduous teeth begin to erupt at approximately 2 weeks of age, and emerge completely through the gums at 18 days of age (Fox 1988). Young ferrets will begin to consume moistened solid food as early as 2 to 3 weeks of age. They will begin to hear sound at approximately 32 days of age, and the eyes begin to open at approximately 34 days. The permanent canine teeth begin to appear at 47 to 52 days of age, and the deciduous canines are shed at approximately 56 to 70 days. Young ferrets are weaned at approximately 6 to 8 weeks postnatally.

Growth Curves and Typical Body Weight Ranges

At approximately 6 to 8 weeks of age a marked sexual dimorphism becomes apparent (figure 7.2), with adult male body weights (reached at approximately 4–5 months) eventually exceeding approximately twice that of adult females. Under optimum controlled environmental conditions, sexually inactive adult male ferrets, or hobs, will generally weigh between 1 kg and 2 kg at 5 to 7 months of age. In contrast, anestrous females (jills) will generally weigh between 0.5 kg and 1.0 kg at this same age. The observed range in body weight of sexually inactive animals maintained under controlled conditions is largely dependent on the specific husbandry practiced, although the strain and source of supply (specific vendor) can also contribute.

Figure 7.2 Growth curve for ferrets. A newborn kit's body weight will range between 6 g and 12 g at birth. At approximately 8 weeks the sexual dimorphism of the species begins, with male ferrets eventually attaining an adult body weight approximately twice that of females.

Marked, seasonal body weight fluctuations as high as 30% to 40% are commonly observed in both sexes and coincide with the natural breeding season of the species (March–August). Hobs and jills as young as 4 to 5 months of age will generally begin to deposit subcutaneous body fat in the fall (under natural lighting conditions) in preparation for the spring breeding season. Teleologically speaking, this conformational change probably evolved to facilitate propagation and survival of the species. Under natural conditions, for example, sexually active male ferrets might ignore food for extended periods in their concentrated search for receptive females. In addition, the prolonged coitus (several hours) of the species and the frequently fatal exchange following the chance encounter of two sexually active, territorial males can require tremendous amounts of energy. Males will generally return to, or remain at, a sexually inactive (undescended testicles) body weight under controlled lighting conditions. Females, on the other hand, because they are induced ovulators, can manifest a prolonged estrus with diminished appetite and subsequent marked body weight loss. Optimum environmental lighting (see earlier) and hormonal injections (see earlier) will preclude the onset of bone marrow hypoplasia or pometra associated with prolonged estrus in this species (Sherrill and Gorham 1985).

Age at Maturation

Male and female ferrets are generally assumed to be sexually mature 6 to 8 months of age. However, this will depend to a great extent on the photoperiodicity to which the individual animals are exposed (natural vs. controlled), or to the month (season) in which they are born. It is possible, for example, for animals as young as 4 months of age to conceive and produce viable offspring.

Although rats are sexually (i.e., physiologically) mature at 50 to 60 days of age (Rowett 1965), they are not routinely first-mated until approximately 100 to 120 days of age so that reproductive performance is maximized. McLain, Harper et al. (1985) have demonstrated that reproductive performance in primiparous ferrets is maximized at approximately 7 to 10 months of age, and decreases thereafter. Consequently, although physiological sexual maturity might occur earlier, 7 to 10 months is probably an optimum breeding age for ferrets.

Weight and Appearance of Testes

The male ferret's potential breeding season under natural lighting conditions extends from December to July, which precedes the female's breeding season of approximately March through August. Ryland and Gorham (1978) attribute this earlier sexual maturation of the male to a functional adaptation to allow for adequate sperm maturation.

Ishida (1968) and Basrur and Gilman (1968) also described the age and seasonal changes in the testes of the ferret. In ferrets born in June (natural lighting), bilateral testicular development started in December and reached maturity by February, with the functional period lasting from March until July and the period of quiescence being from August until December.

Infantile ferret testes are characterized by the lack of germinal cycle, by undifferentiated precursors of Sertoli cells, by the absence of a tubular lumen, and by small interstitial cells. The prepubertal testes become large as a result of the development of the germinal epithelium and tubular lumen, and by virtue of the growth of the Sertoli and interstitial cells (Boissin-Agasse and Boissin 1979; Ishida 1968). The testes can range in weight from 0.8 g/kg to 3.8 g/kg, with a mean weight of approximately 2.02 g/kg (Fox 1988).

The male ferret lacks seminal vesicles and vulvourethral glands, Moreover, the presence of a prostate gland in the ferret has been a subject of debate and, as in the majority of male mustelids, it has been classified as poorly developed (see Mead 1970 for reviews). However, during the first several months of postnatal development of the male ferret and during its sexual quiescence, prostatic tissue is difficult to locate except through histological examination. The prostate of the adult, sexually active ferret, on the other hand, is visible at gross autopsy.

The ventrally located penis is large for the animal's size, with the distal section curving dorsally so that it ends in a hook (Moody et al. 1985). The testes are located in the subcutaneous tissue of the caudoventral abdomen and descend into the scrotum only during the breeding season.

Curry et al. (1989) have published a comparison of sperm morphology and silver nitrate staining characteristics in the domestic ferret and the black-footed ferret. Shump et al. (1976) have previously described the semen volume and sperm concentration in the ferret.

Frequency of Estrus

Female ferrets are seasonally polyestrous and will generally reach sexual maturation at approximately the same age as males. Estrus is induced by increased day length, or by manipulation of the photoperiod with artificial illumination (described earlier). The onset of estrus in female ferrets is recognized by a continuous vulval swelling occurring over an approximate 2- to 3-week period. The vulva will increase in size approximately tenfold, measuring approximately 1.0 cm to 1.5 cm in diameter in primiparous females, and approximately 1.5 cm to 2.0 cm in diameter in multiparous animals at peak receptivity. Mating should occur at the peak of vulval swelling (approximately 2 weeks after the onset), when the vulva has a slightly pink and mucous-covered appearance. Ovulation is induced in the ferret approximately 3 to 36 hr after coitus, with implantation occurring approximately 12 to 13 days postcoitus. The vulva will begin to regress approximately 3 to 4 days after mating, regaining its preestrus size in approximately 2 to 3 weeks.

As indicated previously, female ferrets are induced ovulators and will remain in estrus for as long as 6 months if not mated. Prolonged estrus in this species can precipitate pyometra or complications of bone marrow hypoplasia and estrogen-induced anemia (described earlier). Females will generally return to estrus approximately 2 weeks after weaning of the litter (McLain, Harper et al. 1985). Occasionally, a lactation estrus will occur in females with less than five suckling young (Fox 1988).

Gestational Period and Litter Size

McLain, Harper et al. (1985) analyzed demographic data for 945 female ferrets from a commercial breeding colony for the effects of various maternal characteristics on subsequent reproductive

performance. In general, litter size ($M \pm$ SEM) was found to be greatest for young, primiparous females (10.3 ± 0.2) and decreased with advanced maternal age and parity to a cohort mean of 8.1 ± 0.1 for third-parity females 16 months of age. Gestational length ($M \pm SD$) was 41.3 ± 1.1 days and appeared to decrease with increasing day length and total litter size. The gestation period in this cohort ranged from 39 days (observed in 32 primiparous jills) to 46 days (observed in four multiparous females). Reproductive data for ferrets are summarized in table 7.6.

Table 7.6 Typical Parameters of Reproduction in Ferrets Maintained Under Controlled Environmental Conditions

Parameter	Units	Value
Age at pairing, M/F	Months	8–10
Breeding life, M/F	Years	2–5
Breeding season, M/F	By photoperiod	All year
Type of estrus cycle	—	Monoestrus
Duration of estrus	Days	Prolonged
Copulation time	Hours	Up to 3
Sperm deposition site	—	Posterior cervix
Sperm capacitation	Hours	3–11
Sperm viability	Hours in tract	36–48
Mechanism of ovulation	—	Induced
Time of ovulation	Hours, postcoitus	30–36
No. of ova	Average	12, range = 5–18
Ovum transit time	Days, postcoitus	5–6
Time of implantation	Days, postcoitus	12–13
Length of gestation	Days	41, range = 39–46
Litter size	Average	8–10, range = 1–18
Weight at birth	g	8, range = 6–12
Age at weaning	Weeks	6
Weight at weaning	Kg	0.2–0.4
Rebreeding	—	Immediately

Source: Data from Fox (1988), Marshall and Marshall, (1973); McLain, Harper et al. (1985), and Moody et al. (1985).

Normative Physiological Data

Studies of the effects and mechanisms of experimental treatments require that substantial normative physiological data for the species be established. Thus, as a contemporary animal model, the ferret continues to benefit from ongoing investigations contributing to this monumental task. Normative physiological data also aid in test species selection because in addition to its particular similarities with man, toxicologists will frequently select an animal model because the system of interest can be externally manipulated, or adapted to operate under extreme conditions. In this respect, much of the normative physiological data generated for ferrets has proven rewarding in that several potentially superior systems have been identified. On the other hand, the same contemporaries of ferrets continue to leave many questions unanswered.

Table 7.7 provides available normal cardiopulmonary data for the ferret.

Cardiovascular Parameters

Earlier discussions of the ferret's cardiovascular system were offered by Kempf and Chang (1949, macroscopic) and Borelli and Filho (1971, microscopic). Later reviews include those by Andrews et al. (1979), Baskin et al. (1981), and Andrews (1988).

Heart rate and blood pressure measurements have been made in both anesthetized and unanesthetized ferrets, but considerable more data have been generated for anesthetized animals. An apparent anesthetic effect on the heart rate is evident by the differences reported by Andrews et al.

Table 7.7 Selected Normative Cardiopulmonary Data for Ferrets Maintained Under Controlled Environmental Conditions

Parameter	Units	Value
Cardiovascular		
Blood volume	Ml/kg	60–70
Blood pressure		
Systolic	mmHg	140–164
Diastolic	mmHg	110–125
Cardiac output	Ml/min	139 (range = 82–200)
Circulation time		
Fluorescein	Sec	6.8 ± 1.2
Cyanide	Sec	4.5 ± 0.7
Heart rate		
Barbiturate	Beats/min	230 ± 26
Halothane	Beats/min	387 ± 54
Conscious, active	Beats/min	341 ± 39
Conscious, inactive	Beats/min	200–255
Pulmonary		
Tidal volume	ml	6.06 ± 0.30
Respiration rate		
Conscious	bpm	33–36
Pentobarbital	bpm	26.7 ± 3.9
Dynamic compliance	ml/cmH_2O	2.48 ± 0.21
Pulmonary resistance	cm$H_2$0/L/sec	22.56 ± 1.61
Total capacity	ml	89 ± 5
Functional residual capacity	ml	17.8 ± 2.0

Source: Data from Andrews et al. (1979), Thornton et al. (1979), Kempf and Chang (1949), Andrews (1988), and Vinegar et al. (1985).

(1979) for animals under urethane anesthesia (387 ± 54 beats/min), and that reported by Thornton et al. (1979) for animals under barbiturate anesthesia (230 ± 26 beats/min). In the unanesthetized animal, heart rates have been reported to range from 341 ± 39 beats/min (Andrews et al. 1979) to 200 to 255 beats/min (Thornton et al. 1979). Smith and Bishop (1985) have reported similar disparities in the heart rate (beats/min) of anesthetized (sodium pentobarbital) adult control ferrets (309, range = 250–380), anesthetized (sodium pentobarbital) adult ferrets with right ventricular hypertrophy (300, range = 245–380) and anesthetized (ketamine) weanling ferrets (280, range = 210–360). Andrews and Illman (1987) have suggested that the problem of variations in the heart rate of the ferret could be resolved by recording the animal's activity level and by viewing the results under different anesthetics as two ends of a spectrum, between which the heart rate of the unanesthetized animal would operate according to the animal's activity level.

Apparently, Kempf and Chang (1949) have provided the only measurement of cardiac output measured 139 ml/min (range = 82–200 ml/min) and circulation time measured 6.8 ± 1.2 sec (fluorescein) and 4.5 ± 0.7 sec (cyanide). Under urethane or barbiturate anesthesia, mean systolic blood pressure values between 140 and 164 mmHg have been reported from the ferret, with diastolic values of 110 to 125 mmHg (Andrews et al. 1979; Kempf and Chang 1949). In the conscious animal, systolic values have been reported as 161 mmHg (males) and 133 mmHg (females), with some animals presenting with blood pressure values as high as 190 mmHg (Thornton et al. 1979).

The electrocardiogram of normal ferrets and ferrets with right ventricular hypertrophy has been reported by Smith and Bishop (1985). These authors concluded that the normal ferret has a mean electrical axis of +86° ± 6.6 (*SD*), with a narrow range between +69° and +97°. All animals in their study exhibited a normal sinus rhythm, composed of the expected P wave, QRS complex, and T wave.

Experience from our laboratory has indicated that a maximum of approximately 50% to 60% of a ferret's total blood volume be removed by cardiac puncture technique (exsanguination).

Therefore, estimates suggest that the ferret's total blood volume is 6% to 7% of the body weight, or approximately 60 to 70 ml/kg.

Pulmonary Parameters

An early study by Barer et al. (1978) and a species comparison study by Peake et al. (1981) investigated the response of the blood-perfused ferret lung to hypoxia. Both groups of authors concluded that of several species investigated, the ferret has the most marked pulmonary vasoconstriction in response to hypoxia, with a maximum response occurring at approximately 25 mmHg. Vinegar et al. (1982) have postulated that the sensitivity to hypoxia in the ferret might be an adaptation to burrowing. The sensitivity of the ferret's pulmonary vasculature to hypoxia makes it a valuable model for the study of pulmonary hypertension in humans. A descriptive pulmonary vascular pressure profile in adult ferrets (*in vivo* and in isolated lungs) has been reported by Raj, Anderson, et al. (1990), and in 2- to 3-week-old, 5- to 6-week-old, and adult ferrets (Raj, Hillyard, et al. 1990).

In addition to the extensive data generated by Vinegar et al. (1985), discussed previously, various other investigators have examined the respiratory rate of ferrets. For example, Pyle (1940) reported the rate to be 33 to 36 breaths/min in the conscious ferret as compared to values of 31 ± 6 breaths/min in urethane-anesthetized animals (Andrews et al. 1979) and 43.5 ± 4.6 breaths/min in pentobarbital-anesthetized animals (Boyd and Mangos 1981). Similar to the results in the cardiovascular system, the disparity in reported respiration rates of the ferret might be the result of differences in type or dosage of anesthesia, or the age of the animals. Scientific protocols should, therefore, include requirements for documentation of these variables.

Normative pulmonary and cardiovascular values for ferrets are summarized in table 7.7.

Clinical Laboratory Parameters

Data for hematological serum chemistry determinations in ferrets have been reported by Thornton et al. (1979), Lee et al. (1982), Moody et al. (1985), and Fox (1988). However, all of these studies used small numbers of animals, and only Lee and colleagues described the variability in their sample of five females and eight males (three intact, five castrated). Fox (1988) contrasted the analytical results obtained in his laboratory from both orbital plexus and cardiac blood sample sites in ferrets. However, the data were not expressed statistically and an estimation of variability was not provided. To date, therefore, no studies have satisfactorily described the hemogram of laboratory ferrets with respect to sample variability and the distribution around the mean. Moreover, because Neptun et al. (1985, 1986) concluded that both sampling site and collection method are major sources of variation in clinical laboratory measurements of homogeneous laboratory rats, the potential for sample site and collection method differences must be adequately evaluated in ferrets. Obviously, when differences can be demonstrated, selection of an appropriate collection method and sampling site should include a consideration of which parameters are likely to be of major interest. For heterogeneous species such as the ferret, this would seem especially important.

In our experience, a significant amount of clinical laboratory data have been personally accumulated from ferrets used in acute and subacute GLP testing protocols. Moreover, the experimental results generated with these animals have been submitted to the FDA in fulfillment of the requirement for test data generated in a second species. In all probability, the sound statistical treatment of the data generated in these studies (McLain and Lin 1989), which is deemed necessary and appropriate for normalization of any heterogeneous species, was well received by the FDA reviewers and contributed significantly to the success of each submission process. Therefore, these data and discussion of their statistical treatment are summarized in the following sections.

Blood Sample Collection

Blood samples for clinical laboratory determinations in ferrets can be collected by several routes. Cardiac puncture and orbital plexus sampling of anesthetized animals might be the most rapid and efficient method when large numbers of samples are to be processed. However, alternative sites include toenail clipping, jugular venipuncture, and tail vein and artery for repeated sampling such as in pharmacokinetic analyses (McLain, Babish et al. 1985). Currently, the collection by the saphenous vein is the preferred methodology for repeat sampling during a study (Hem et al. 1998).

Hematological Parameters

In construction of the following database, blood for clinical pathology was collected in tubes containing either sodium citrate (3.8%) or potassium ethylenediaminetetraacetate (EDTA). Blood collected in tubes containing sodium citrate was used for the determination of prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen level. Blood collected in tubes containing EDTA was used for determination of all other hematological parameters.

Hematological determinations collected before experimental treatment in 370 adult (6–10 months) ferrets (187 males, 183 females) are summarized in table 7.8 and table 7.9. Reference values for the clotting factors (APTT, PT) were determined after IV saline treatment (single dose) of 64 animals (32 males, 32 females) from the preceding cohort, and from 32 additional animals (16 males, 16 females) that served as untreated controls in the above cohort. All ferrets were obtained from the same vendor. Fasted blood samples were collected from anesthetized animals (KET-ACE, 10:1, 35 mg/kg intramuscular) via cardiac puncture and assayed by conventional methods. Approximately 1.3% of the samples collected for hematology were not analyzed for various reasons. Approximately 13.5% of the platelet samples were "clumped" and not suitable for analysis. Only one of 370 fibrogen samples collected was not reported. Mortality associated with this blood collection procedure was generally low (approximately 2%), occurred largely in females, and was apparently correlated with the quantity of blood withdrawn from the animal (10 ml/animal, or approximately 10% and 20% of the total blood volume of males and females, respectively).

Methodological procedures used on the Coulter hemoglobinometer, Coulter ZBI, MLA 700, or by microscope for determination of hematology reference values included APTT (clotting time, cephaloplastin), PT (clotting time, thromboplastin), hemoglobin (HGB, Coulter Hemoterge II), hematocrit (HCT, calculated), erythrocytes (RBC, electrical resistance), mean corpuscular volume (MCV, electrical resistance), mean corpuscular hemoglobin (MCH, calculated), mean corpuscular hemoglobin concentration (MCHC, calculated), platelet count (Unopette, manual), fibrogen (optics), leukocytes (WBC, electrical resistance), and lymphocyte count, PMN count, eosinophils (EOS), monocytes (MONO), basophils (BASO), and band neutrophils (STAB) by Wright-Giemsa stain.

Noteworthy observation in sample collection included the fact that many citrate tubes were hemolyzed and a large percentage of platelet samples were clumped. Additionally, a manual method, which takes three times longer than the automated method, was necessary for analysis of platelets because of their large size. Finally, relative to other species, ferrets have many RBCs and blood samples will require a dilution before running on the Coulter ZBI. An additional 20% to 80% might require further dilution.

Table 7.8 lists the calculated reference values for selected hematology measurements of the ferret. The first column lists the various parameters and their units. The third through seventh columns list the descriptive statistics, which include the mean, standard deviation (*SD*), number of observations (*N*), minimum value (Min), and maximum value (Max). These descriptive statistics were based on the original units (scale) and were not affected by the transformation, if needed. The last column lists the transformation, if needed, for skewed distributions of measurement to become approximately normally (Gaussian) distributed. The eighth column lists the central tendency of each parameter. The

Table 7.8 Hematology Values for Adult Ferrets Maintained Under Controlled Environmental Conditions

Parameter	Sex	*M*	*SD*	N	Min	Max	Central Tendency	Tolerance limit			Sex effect	Transformation
								Lower	Upper	Method		
Hemoglobin (g/dl)	F	16.2	1.3	180	10.9	19.0	16.2	13.5	18.9	P	$p < .001$	None
	M	16.8	1.2	185	13.3	20.0	16.8	14.3	19.3			
Hematocrit (%)	F	48.4	4.0	180	33.2	57.8	48.4	40.0	56.8	P	$p < .01$	None
	M	49.8	3.7	185	39.6	62.0	49.8	42.0	57.5			
Red blood cells (mil/mm^3)	F	9.30	0.84	180	5.77	11.52	9.30	7.52	11.08	P	$p < .001$	None
	M	9.69	0.71	185	7.95	11.86	9.69	8.18	11.19			
MCV(μ^3)	F	52.2	2.0	180	42.5	60.3	52.2	48.0	56.4	P	$p < .001$	None
	M	51.4	1.4	185	47.9	54.9	51.4	48.4	54.4			
MCH (pg)	F	17.5	0.7	180	14.8	20.5	17.5	16.0	19.0	P	ns	None
	M	17.3	0.7	185	15.6	19.2	17.3	15.9	18.7			
MCHC (%)	F	33.5	0.9	180	30.5	36.7	33.5	31.6	35.4	P	$p < .05$	None
	M	33.8	0.9	185	31.5	36.1	33.8	31.8	35.8			
Platelet (10^3/mm^3)	F	764	246	157	330	1,520	726	364	1,447	P	ns	Ln(X)
	M	766	196	163	315	1,525	742	438	1,256			
Fibrinogen (mg/dl)	F	184	66	182	83	548	170	99	376	P	ns	Ln(X-63)
	M	189	28	187	93	657	173	100	387			
White blood cells (10^3/mm^3)	F	7.6	3.2	180	2.9	23.1	7.0	3.0	16.3	P	$p < .001$	Ln(X)
	M	9.2	2.7	185	3.7	18.2	8.8	4.6	16.6			
Lymphocytes (10^3/mm^3)	F	3.4	1.8	180	1.0	11.1	3.0	1.1	8.2	P	$p < .001$	Ln(X)
	M	4.2	1.6	185	1.2	8.3	3.9	1.7	9.0			
Polymorphic neutrophils (PMN) (10^3/mm^3)	F	3.8	2.0	180	1.2	13.3	3.4	1.2	9.4	P	$p < .001$	Ln(X)
	M	4.5	2.0	185	1.4	13.0	4.1	1.7	9.9			
EOS (10^3/mm^3)	F	0.25	0.22	180	0.00	1.39	0.18	0.00	0.78	N	$p < .01$	NA
	M	0.33	0.28	185	0.00	2.13	0.25	0.00	0.92			
MONO (10^3/mm^3)	F	0.09	0.13	180	0.00	0.86	0.05	0.00	0.48	N	ns	NA
	M	0.11	0.13	185	0.00	0.84	0.09	0.00	0.37			
BASO (10^3/mm^3)	F	0.03	0.07	180	0.00	0.46	0.00	0.00	0.23	N	$p < .05$	NA
	M	0.04	0.08	185	0.00	0.49	0.00	0.00	0.25			
STAB (10^3/mm^3)	F	0.01	0.03	180	0.00	0.22	0.00	0.00	0.13	N	ns	NA
	M	0.01	0.05	185	0.00	0.40	0.00	0.00	0.14			

Note: P = parametric; N = nonparametric; *ns* = not significant; NA = not applicable; Ln = natural logarithm; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; EOS = eosinophils; MONO = monocytes; BASO = basophils; STAB = band neutrophils.

Table 7.9 Whole Blood Clotting Factors, Serum Electrolytes, and Serum Enzyme Levels for Adult Ferrets Maintained Under Controlled Environmental Conditions

Parameter	Sex	*M*	*SD*	N	Min	Max	Central Tendency	Tolerance limit			Sex effect	Transformation
								Lower	Upper	Method		
Blood clotting factors												
PT (sec)	M+F	11.1	0.4	94	10.3	12.4	11.0	10.3	12.1	P	ns	Ln(X-9)
APTT (sec)	M+F	20.9	24.5	94	13.9	200.0	17.1	14.8	21.2	P	ns	Ln(X-11.8)
Serum electrolytes												
Na (mEq/L)	M+F	150	96	96	135	159	150	141	159	P	ns	None
K (mEq/L)	M+F	4.76	96	96	3.90	6.80	4.70	4.09	5.97	P	ns	Ln (X-3.5)
Cl (mEq/L)	M+F	114	96	96	96	124	114	108	120	P	ns	None
Serum enzymes												
Alk. Phos. (IU/L)	F	40	18	183	13	106	35	18	92	P	ns	Ln(X-10)
	M	37	18	187	15	99	33	17	88			
Lactic Dehydrogenase (LDH) (IU/L)	F	621	407	183	174	2,030	476	177	2,341	P	ns	Ln(X-120)
	M	595	505	187	143	3,030	426	163	2,309			
Serum glutamic oxaloacetic transaminase (SGOT) (IU/L)	F	97	48	183	34	271	82	41	282	P	ns	Ln(X-30)
	M	92	56	187	36	442	78	41	236			
Serum glutamic pyruvic transaminase (SGPT) (IU/L)	F	210	226	183	47	1,710	146	55	765	P	ns	Ln(X-40)
	M	205	207	187	45	1,850	153	60	670			

Note: P = parametric; N = nonparametric; *ns* = not significant; NA = not applicable; Ln = natural logarithm.

central tendency is the antitransformation of the transformed mean. For example, it is the geometrical mean if a logarithmic transformation is applied. If a transformation is needed, the central tendency better represents the norm of the population than the mean would. The next two columns list the tolerance limits. The tolerance limits contain 95% of the measurements (in the original scale) of untreated ferrets with 90% confidence. The next to the last column lists the test on the difference between means of males and females. The column to its left explains whether the tolerance limits and the sex effect were determined by parametric (P) or nonparametric (N) methods.

Reference values for the clotting factors (PT, APTT) are listed separately with the electrolytes and serum proteins (table 7.9). Unlike the values in table 7.8, these reference values are listed combining males and females. This was done because the database was much smaller, and no sex differences were detected for any of the parameters.

By comparison, the variance observed in ferret hematology is approximately one-half of that reported for humans (Conn 1963), minimally greater than the beagle dog, and considerably greater than the inbred SPF and virus-free rat (see specific chapters). Notable exceptions (increases or decreases) in mean values for hematological parameters in ferrets relative to humans include (males only, ferret/human): RBC (million/mm^3), 9.69 ± 0.71/5.4 ± 0.8; MCV (μ^3), 438–1236/150–450. Thornton et al. (1979) observed a higher HCT (and RBCs) in ferrets relative to rats and dogs and recommended a 20% longer spin time (due to a negligible RBC sedimentation rate of the ferret) for blood samples used for microhematocrit determination. These authors also reported a higher HGB level in males (55.4, $n = 28$) relative to females (49.2, $n = 11$), and no sex difference in number of WBCs (or differential). The present database confirmed a high HCT and HGB in ferrets. However, the sex difference in HGB, although still significant, is apparently not as large when cardiac blood samples are used or when the sample size is increased. Furthermore, the observations in this study that male ferrets have significantly greater numbers of WBCs (including lymphocytes, polymorphonuclear neutrophils, eosinohils, and basophils) than females is in contrast to what Thornton et al. (1979) have reported and in agreement with Lee et al. (1982).

Serum Chemistry Parameters

Reference serum chemistry values obtained from the same population of animals are listed in table 7.10. The blood collection procedure and table format are the same as previously described. Serum electrolytes and enzymes are included in table 7.9 with the clotting factors.

Methodological procedures used on the Hitachi 705 Chemistry Analyzer for determination of reference values include: albumin (bromcresol green), alkaline phosphatase (modified Bowers/McComb), SGOT and SGPT (modified Henry), BUN (urease), calcium (CPC), chloride (Beckman E4), cholesterol (esterase/oxidase), creatinine (Jaffe), glucose (hexokinase), LDH (modified Wacker), Na and K (Beckman E4), phosphorus (molybdate), total Bilirubin (DPD), total globulin and A/G ratio (calculated), total protein (Biuret), and uric acid (uricase).

Similar to the hematological findings, the variance observed in the ferret clinical chemistry is minimally greater than the beagle dog and considerable greater than the inbred SPF and virus-free rat (see specific chapters). Notable exceptions (increases or decreases) in the range in values for serum chemistry parameters in ferrets relative to humans (Conn 1963) include (males only, ferret/human): glucose (fasting, mg/dl), 81–142/60–100; BUN (mg/dl), 13–37/10–20; uric acid (mg/dl), 0.4–2.8/3.0–6.0; creatinine (mg/dl), 0.20–0.70/0.7–1.5; SGOT (IU/dl), 41–236/5–40; SGPT (IU/dl), 17–88/5–13. The considerable range in clinical chemistry parameters of the ferret, especially the liver enzymes, obviates the need for an analysis of the "change from baseline" rather than any particular absolute value in this species. Moreover, appropriate transformations should be applied whenever possible to all posttreatment values adjusted for pretreatment measurements.

Table 7.10 Serum Chemistry Values for Adult Ferrets Maintained in a Controlled Environment

Parameter	Sex	*M*	*SD*	N	Min	Max	Central Tendency	Tolerance limit			Sex effect	Transformation
								Lower	Upper	Method		
Calcium (mg/dl)	F	9.6	0.9	183	5.0	11.0	9.6	8.0	11.2	P	ns	None
	M	9.5	0.7	187	6.9	11.2	9.5	8.0	11.1			
Phosphorus (mg/dl)	F	6.5	1.0	183	4.0	8.7	6.5	4.4	8.5	P	$p < .001$	None
	M	7.0	1.0	187	4.9	9.5	7.0	5.0	9.0			
Glucose (mg/dl)	F	118	25	183	62	387	116	85	152	P	$p < .001$	Ln(X+96)
	M	110	15	187	28	164	110	81	142			
BUN (mg/dl)	F	27	7	187	12	76	20	13	37	P	$p < .001$	Ln(X-9)
	M	21	7	187	12	76	20	13	37			
Uric acid (mg/dl)	F	1.8	0.5	183	0.5	3.5	1.7	0.8	3.2	P	$p < .001$	Ln(X+0.74)
	M	1.3	0.6	187	0.2	3.0	1.3	0.4	2.8			
Cholesterol (mg/dl)	F	183	33	183	96	269	183	112	254	P	$p < .001$	None
	M	162	25	187	107	238	162	108	215			
Bilirubin (mg/dl)	F	0.2	0.1	183	0.0	0.4	0.2	0.0	0.3	N	ns	NA
	M	0.2	0.1	187	0.0	0.4	0.2	0.0	0.3			
Creatinine (mg/dl)	F	0.44	0.12	183	0.30	1.00	0.40	0.20	0.70	N	$p < .001$	NA
	M	0.48	0.10	187	0.20	1.00	0.50	0.20	0.70			
Total protein (g/dl)	F	5.9	0.8	183	3.5	9.7	5.9	4.3	7.6	P	ns	None
	M	6.0	0.8	187	3.9	11.2	6.0	4.6	7.4			
Albumin (g/dl)	F	3.4	0.5	183	1.8	5.7	3.4	2.4	4.4	P	ns	None
	M	3.5	0.4	187	2.2	4.5	3.5	2.6	4.4			
Globulin (g/dl)	F	2.5	0.5	183	1.2	5.5	2.4	1.8	3.8	P	ns	Ln(X-1.23)
	M	2.6	0.7	187	1.7	8.4	2.4	1.8	3.8			
A/G ratio	F	1.39	0.28	183	0.56	3.25	1.40	0.80	1.82	P	ns	X^2
	M	1.42	0.29	187	0.33	2.05	1.45	0.72	1.92			
Bilirubin (mg/dl)	F	0.2	0.1	183	0.0	0.4	0.2	0.0	0.3	N	ns	NA
	M	0.2	0.1	187	0.0	0.4	0.2	0.0	0.3			

Note: P = parametric; N = nonparametric; *ns* = not significant; NA = not applicable; Ln = natural logarithm; BUN = urease.

Effect of Sampling Site on Variations in Baseline Clinical Pathology Parameters of the Ferret

Table 7.11 and table 7.12 describe the influence of sample collection site on the variance of certain blood parameters of the ferret. In this analysis, the variances of select parameters of samples collected by the cardiac puncture technique described previously (n = 370) were compared to the variation in pretreatment blood samples, collected by orbital plexus technique, from 58 additional ferrets. The sex effect was removed in this comparison (i.e., the variance was pooled across sexes). The p value in table 7.11 and table 7.12 is for comparing variances between collection sites.

Table 7.11 Effect of Sampling Site on Variations in Baseline Hematology Parameters of Ferrets

		Cardiac Puncture		Orbital Plexus		
Parameters[a]	**No. of Samples[b]**	***M***	***SD***	***M***	***SD***	***p* Value**
Hemoglobin, g/dl	365/37	16.50	1.46	17.90	1.79	.00000
Hematocrit, %	365/37	49.10	3.82	54.80	6.39	.00000
RBC, 10^6/mm^3	365/37	9.50	0.78	10.53	1.01	.00000
MCV, mm^3	365/37	51.80	1.73	52.00	3.02	.00000
MCH, pg	365/37	17.40	0.71	17.00	0.77	.02304
MCHC, %	365/37	33.70	0.95	32.80	1.10	.00088
Platelet, 10^3/mm^3	320/27	6.60	0.28	6.30	0.37	.00000
WBC, 10^3/mm^3	365/37	2.06	0.36	2.48	0.28	.04223
Lymphocytes, %	365/37	45.90	12.15	47.80	14.07	.00274
STAB, %	365/37	0.14	0.44	0.34	0.95	.00000
MONO, %	365/37	1.16	1.31	2.6	1.83	.00000
EOS, %	365/37	3.41	2.42	2.90	2.59	.08827
BASO, %	365/37	0.41	0.74	0.05	0.23	.00000
POLY, %	365/37	49.10	12.28	46.50	13.57	.02803

[a] Transformed values include: platelet = Ln(X) and WBC = Ln(X).

[b] No. of samples = cardiac puncture plexus.

Table 7.12 Effect of Sampling Site on Variations in Baseline Serum Chemistry Parameters of Ferrets

		Cardiac Puncture		Orbital Plexus		
Parameters[a]	**No. of Samples[b]**	***M***	***SD***	***M***	***SD***	***p* Value**
Calcium, mg/dl	368/57	9.59	0.74	9.90	0.55	.00329
Phosphorus, mg/dl	370/57	6.72	0.97	8.60	1.12	.00346
Glucose, mg/dl	369/57	5.35	0.10	5.33	0.17	.00000
Cholesterol, mg/dl	370/57	173	29.7	185	30.60	.28056
BUN, mg/dl	369/57	2.51	0.59	2.53	0.69	.00169
Uric acid, mg/dl	370/56	0.80	0.25	1.17	0.18	.00222
Bilirubin, mg/dl	370/58	0.17	0.10	0.06	0.13	.00001
Creatinine, mg/dl	370/57	0.46	0.11	0.51	0.13	.00012
Total protein, mg/dl	369/57	5.96	0.73	6.20	0.40	.00000
Albumin, mg/dl	370/57	3.44	0.46	3.69	0.30	.00014
Globulin, mg/dl	368/57	0.19	0.36	0.24	0.26	.00241
A/G ratio	368/57	2.04	0.69	2.33	0.64	.24671
Alk. phos., IU/l	370/58	3.18	0.57	3.32	0.30	.00000
LDH, IU/l	370/58	5.80	0.90	6.67	0.62	.00064
SGOT, IU/l	370/58	3.91	0.72	4.24	0.52	.00158
SGPT, IU/l	370/58	4.70	0.87	4.61	0.60	.00053

[a] Transformed values include: glucose = Ln(X + 69); BUN = Ln(X-9); uric acid = Ln(X+0.74); globulin = Ln (X-1.23); A/G Ratio = X2, alk. phos. Ln (X-10); LDH = Ln(X-120); SGOT = Ln(X-30); SGPT = Ln(X-40).

[b] No. of samples = cardiac puncture/orbital plexus.

For all of the hematology parameters listed in table 7.11, except WBCs and basophils, the sample variance was significantly increased when collected by the orbital plexus relative to cardiac puncture. Moreover, when the critical level of significance is set at $\alpha = .05$, only eosinophils collected by each technique did not differ. Based on the number of clotted hematology samples in this analysis (12/58), however, as well as personal observations, it is recommended that each laboratory confirm its collection proficiency by this route in this species before protocol collection is initiated. In addition, the acceptability of each sample should be confirmed before an animal is returned to the home cage.

For the 16 clinical chemistry parameters in table 7.12, eight parameters (uric acid, calcium, total protein, albumin, globulin, alkaline phosphatase, LDH, and SGPT), especially the liver enzymes, showed significantly smaller variances when collected from the orbital plexus. Conversely, five parameters (BUN, creatinine, bilirubin, glucose, and phosphorus) showed significantly larger variance, and cholesterol, SGOT, and A/G showed no significant difference.

Although reference to previously published mean values for this species is inappropriate, the orbital plexus appears to provide hematological and clinical chemistry mean values similar to those reported by Thornton et al. (1979) for samples collected from the abdominal aorta. A much larger database would, however, have to be examined to confirm this.

Data from the foregoing analysis indicate that if protocols specify that blood samples are to be collected from multiple sites (i.e., cardiac puncture for hematology and orbital plexus for clinical chemistry), reductions in sample variance can be expected in most parameters, especially the liver enzymes. Alternatively, one can choose to optimize the ability to detect subtle changes in a select parameter by collecting a blood sample from the site affording the least variability.

Estimation of Ferret Sample Size Requirements for Optimum Statistical Power

McLain and Lin (1989) demonstrated that, when it is desired to achieve a statistical power in ferrets similar to that enjoyed with the less heterogeneous beagle dog, the needed sample size to detect a prespecified difference with prespecified statistical power and Type I error is proportional to the pure variance (random error). Therefore, the ferret's sample size requirements for any select parameter relative to the beagle dog's is, for practical purposes, the ratio of their pure variances adjusted (optimally) for pretreatment measurements using analysis of covariance (ANCOVA). When there is no a priori knowledge of target organ, or of a specific parameter's potential for change in response to experimental treatment, a weighted average of parameter variances can be used to estimate sample size requirements. The correlation coefficient between a parameter's posttreatment and pretreatment value should be used to assign weights, and the geometrical mean should be calculated. When the variance by cardiac puncture sample site is employed, the weighted geometrical mean of the sample size ratio of ferret to dog (based on the author's data) can be calculated to be 1.8, or approximately two ferrets for each beagle dog. When the variance and correlation coefficient calculations incorporate the effect of sample site, the weighted geometrical mean of the sample size of ferret to dog is reduced to 1.3, or approximately three ferrets for every two dogs. Table 7.13 and figure 7.3 illustrate the calculated variance ratios (ferret:dog) for serum chemistry and the various liver enzymes by different sample sizes and sampling sites. These data demonstrate that when a single ferret is used for each dog, the variance ratio for alkaline phosphatase, for example, is approximately 2.5; that is, it can be concluded that 2.5 times more ferrets are necessary to achieve a parameter variance that is approximately equal to that of the dog. Conversely, when alkaline phosphatase is calculated from an orbital plexus blood sample, the variance ratio drops to approximately 0.75; that is, it is now concluded that 1.25 dogs are necessary to achieve a parameter variance that is approximately equal to that of the ferret.

Table 7.13 Effect of Sample Size and Sample Method on the Adjusted (ANCOVA) Variance Ratios for Serum Chemistry Parameters of Ferrets and Dogs

	Variance Ratio (Ferret:Dog)			
	Cardiac Puncture		Orbital Plexus	
Parameter	**1:1**	**2:1**	**1:1**	**2:1**
Calcium	1.353	0.677	0.735	0.368
Glucose	4.272	2.136	—	—
BUN	2.777	1.389	—	—
Uric acid	0.640	0.320	0.419	0.210
Bilirubin	0.614	0.307	0.389	0.195
Creatinine	0.802	0.401	—	—
Total protein	1.864	0.932	0.568	0.284
Albumin	2.492	1.246	1.079	0.540
Globulin	1.091	0.546	0.631	0.316
A/G ratio	0.899	0.450	0.749	0.375
Alk. phos.	2.514	1.257	0.760	0.380
LDH	1.647	0.824	1.070	0.535
SGOT	4.244	2.122	3.395	1.698
SGPT	3.992	1.996	2.016	1.008

Note: Missing values show no improvement with orbital plexus collection.

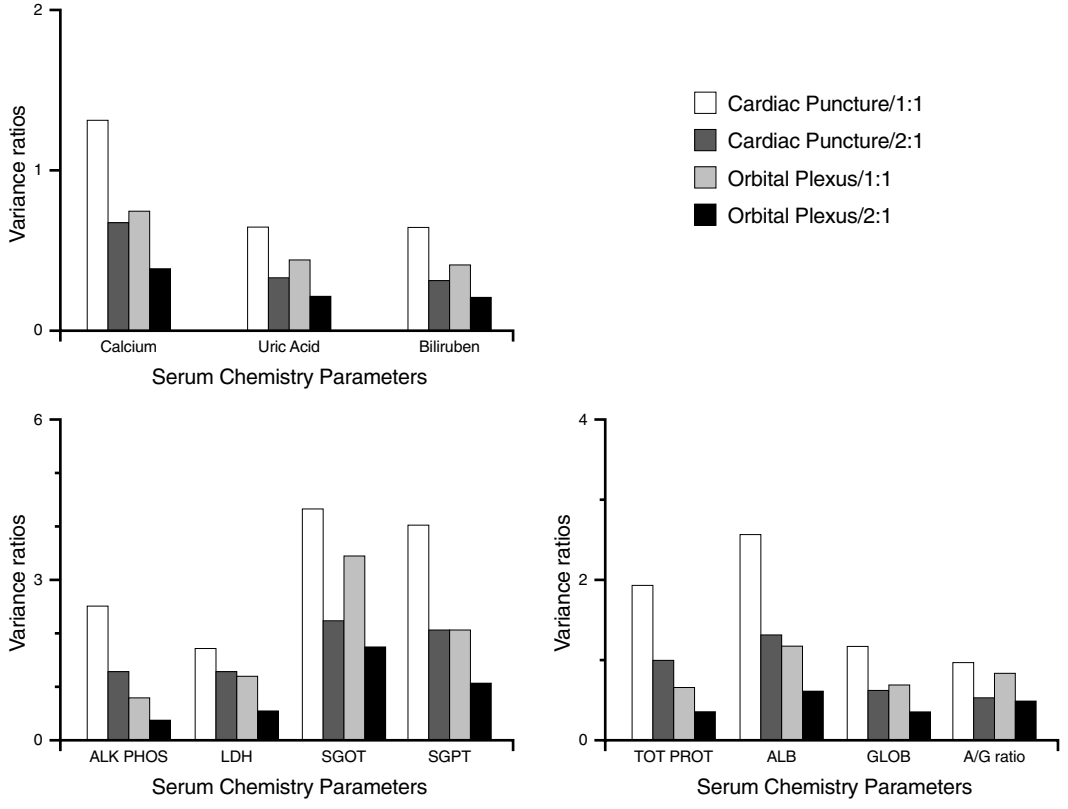

Figure 7.3 Serum chemistry parameter variance ratios for ferret relative to beagle dogs (ferret:dog) as a function of sample site (cardiac puncture or orbital plexus) and sample size (ferret:dog = 1:1 or 2:1). Ratios less than 1 favor the ferret.

Urinalysis

Urinalysis is a good general screening procedure for ferrets that can provide a wide variety of useful clinical information regarding an individual ferret's kidneys and the systemic disease that might affect this excretory organ. Collection procedures are similar to what would be performed for rodents, with a special emphasis placed on minimizing fecal contamination of the sample. Prestudy samples will help to identify pathological conditions that would justify eliminating an animal from consideration for testing. Additionally, prestudy urine samples can be subtracted from posttreatment samples to more precisely evaluate the response to experimental treatment.

Thornton et al. (1979) published the means and ranges for volume (ml), sodium (mmol), potassium (mmol), and chloride (mmol) for "feces-free" urine samples collected over a 24-hr period from 40 male and 24 female ferrets. In addition, they analyzed the urine samples for protein, ketones, blood, and bilirubin using reagent strips (Ames Bililabstix), and discussed the results of these findings.

The following database of macroscopic and microscopic urine parameters of the ferret was compiled in our laboratory from ferrets used in the GLP studies discussed previously. All of the ferrets were obtained from the same supplier (Marshall Farms, North Rose, New York) and were approximately the same age. Animals were placed in rodent metabolism cages for overnight urine collection. The particular type of metabolism cage employed minimized (but probably did not totally eliminate) urine and feces contact and precluded drinking water dilution of the urine samples.

Statistical Treatment of Urine Data

Analysis of variance (ANOVA, $\alpha = .05$) techniques were used in the following macroscopic and microscopic urine parameter databases to test for a sex effect for the continuous parameters (specific gravity and total volume). Sex differences were detected for total volume. Therefore, reference values must be considered separately for males and females for this parameter.

Scores were assigned to the responses for the categorical parameters. These scores are summarized in table 7.14. The assignment of scores was necessary to test for a sex effect and for calculating summary statistics. Table 7.14 must be used to interpret the categorical parameters in table 7.15 and table 7.16. The scoring method of Grizzle et al. (1969) was applied to test for a sex effect ($\alpha = 0.05$). Sex differences were detected with respect to pH, blood, epithelial cells, WBCs, RBCs, and bacteria. Therefore, reference values must be considered separately for males and females for these parameters. The parameters of urobilinogen, glucose, ketone, and crystals were constant.

Table 7.14 Scores Assigned to Categorical Responses in Ferret Urinalysis

	Assigned Score				
Parameter	**0**	**1**	**2**	**3**	**4**
Color	Pale yellow	Yellow	Light Amber	Amber	Brown
Turbidity	Clear	Slightly cloudy	Cloudy	Turbid	—
Protein, glucose, ketone, blood	Negative	Trace	1+	2+	3+
Bilirubin	Negative	Positive	1+	2+	—
Mucous, epithelial cells, crystals, Triple PO4, Ca oxalate, bacteria, Sperm	None	Rare	Few	Moderate	Many
Casts	None	Rare	1–4	5–9	—

Assigned scores for WBC and RBC									
0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
None	Rare	1–4	5–9	10–14	15–19	20–29	30–49	50+	TNTC

Note: TNTC = too numerous to count.

Table 7.15 Reference Semiquantitative Macroscopic Urine Profiles of Adult Male and Female Ferrets Maintained Under Controlled Environmental Conditions

Parameter	Sex	*M*	*SD*	N	Min	Max	Central Tendency	Tolerance limit			Sex effect	Transformation
								Lower	Upper	Method		
Volume (ml)	F	21.4	11.9	94	1	59	19.0	4.0	58.0	P	$p < .05$	Ln (X+5.27)
	M	31.8	21.0	98	6	114	27.4	6.1	88.5			
Color	F	1.0	0.4	94	0	3	1	0	3	P	ns	None
	M	1.0	0.4	98	0	4	1	0	2			
Turbidity	F	1.2	1.1	84	0	3		0	3	P	ns	None
	M	1.2	0.9	98	0	3	1	0	3			
Spec. Grav.	F	1.043	0.016	94	1.013	1.080	1.042	1.007	1.078	P	ns	None
	M	1.047	0.016	98	1.013	1.084	1.047	1.012	1.082			
pH	F	6.2	0.3	94	6.0	7.5	6.0	6.0	7.5	P	$p < .05$	None
	M	6.1	0.2	98	6.0	7.0	6.0	6.0	6.5			
Protein	F	1.0	1.0	94	0	4	1	0	3	P	ns	None
	M	0.9	0.9	98	0	4	1	0	3			
Glucose	F	0	—	94	0	0	0	0	0	P	ns	None
	M	0	—	98	0	0	0	0				
Ketone	F	0	—	94	0	0	0	0	0	P	ns	None
	M	0	—	98	0	0	0					
Bilirubin	F	0.1	0.4	94	0	3	0	0	2	P	ns	None
	M	0.1	0.4	98	0	2	0	0	2			
Blood	F	1.2	1.3	94	0	4	1	0	4	P	$p < .05$	None
	M	0.3	0.7	98	0	4	0	0	3			
Urobilinogen	F	0.1	—	94	0.1	0.1	0.1	0.1	0.1	P	ns	None
	M	0.1	—	98	0.1	0.1	0.1	0.1	0.1			

Note: P = parametric; N = nonparametric; ns = not significant; Ln = natural logarithm. The central tendency for urine volume: males = 27.4 ml; females = 19.0 ml.

Table 7.16 Reference Microscopic Urine Profiles of Adult Male and Female Ferrets Maintained Under Controlled Environmental Conditions

Parameter	Sex	*M*	*SD*	N	Min	Max	Central Tendency	Tolerance limit			Sex effect	Transformation
								Lower	Upper	Method		
Mucous	F	1.3	1.3	94	0	4	2	0	4	P	ns	None
	M	1.5	1.3	98	0	4	2	0	4			
Epithelial cells	F	1.7	1.2	94	0	4	2	0	4	P	$p < .05$	None
	M	2.5	1.1	98	0	4	2	0	4			
Crystals	F	0	—	94	0	0	0	0	0	P	ns	None
	M	0	—	98	0	0	0	0	0			
Triple PO4	F	0.2	0.8	94	0	4	0	0	4	P	ns	None
	M	0.1	0.4	98	0	3	0	0	2			
Ca oxalate	F	0.02	0.2	94	0	2	0	0	0	P	ns	None
	M	0.1	0.5	98	0	4	0	0	2			
Casts	F	0.1	0.5	94	0	3	0	0	2	P	ns	None
	M	0.2	0.6	98	0	2	0	0	2			
WBC	F	3.5	2.4	94	0	9	3	0	8	P	$p < .05$	None
	M	1.3	1.2	98	0	4	2	0	4			
RBC	F	1.7	1.6	94	0	8	2	0	6	P	$p < .05$	None
	M	1.0	1.3	98	0	6	1	0	5			
Bacteria	F	2.1	1.3	94	0	4	2	0	4	P	$p < .05$	None
	M	1.1	1.2	98	0	4	0	0	4			
Sperm	M	1.2	1.5	98	0	4	0	0	4	NA	—	—

Note: P = parametric; *ns* = not significant; Ln = natural logarithm

The references were calculated as statistical tolerance limits (90% confidence for 95% of the ferret population). These are expressed as a low and high value and are interpreted as follows: 95% of normal ferrets will have a parameter response between the low value and high value (with 90% confidence).

Nonparametric tolerance intervals were calculated for the categorical parameters. Categorical parameters are not normally distributed. The lower and upper limits for nonparametric intervals are of the form of "order statistics" of the categorical data. Examples of order statistics are: lowest value, highest value, third highest value, or sixth highest value. The selection of the order statistic depends on the sample size, the confidence level, and the proportion of the population that the interval is to include.

Tolerance limits based on the normal distribution were calculated for the continuous, normally distributed parameters. The lower and upper limits are of the form mean ± factor ∞ standard deviation. The selection of the factor depends on the sample size, the confidence level, and the proportion of the population that the interval is to include.

If a continuous parameter was skewed, an appropriate transformation was determined to normalize the distribution. The normal tolerance limits were then calculated for the normalized data. These limits and the mean of the normalized data were then expressed back in the original units. For example, the transformation for total urine volume (X) was found to be Ln(X+5.27). The mean, standard deviation, and normal tolerance limits (TL) for total urine volume of male ferrets, for example, was determined to be M = 3.48553, SD = 0.485852, lower TL (2.430259), and upper TL (4.540801). To express these in the original units of total volume, the inverse of Ln(X+5.27) is applied. The inverse is to exponentiate, then subtract 5.27. The resulting central tendency (CT), low and high limits for male ferret total urine volume are CT (27.4), low (6.1), high (88.5). The standard deviation of the normalized data cannot be expressed in the original units of total volume meaningfully.

Semiquantitative Macroscopic Urine Parameters

Thornton et al. (1979) reported that the mean 24-hr urine volume and range in volume was greatest in female ferrets (M = 28 ml, range = 8–140 ml) as opposed to males (M = 26 ml, range = 8–48 ml). In contrast, data listed in table 7.15 suggest that the opposite is true for overnight urine collection in ferrets, and that the distribution is skewed. Collection vessels employed during urine collection should be able to contain approximately 150 ml.

The color of normal ferret urine collected by ureter catheterization varies widely from colorless to deep yellow (unpublished observation from the author's laboratory). However, when metabolism cages are employed, the color can be altered by fecal chromogen contamination. Animals consuming Ralston Purina Ferret Chow 5280, for example, produce a semisoft, dark green-colored stool that is difficult to separate completely from urine and results in a yellow-green appearance. The fecal pellets, food pellets, and the greenish-tinged urine samples will all give a maximum positive response for blood, protein, and bilirubin with the Ames Multistix test strip. Consequently, when these parameters are key issues of a protocol, urine samples should probably be taken directly from the bladder by catheterization at or before necropsy.

Thornton et al. (1979) reported ketones in 50% of male urines, proteinuria in the majority of animals, and blood in larger amounts in females (attributed to estrus) than in males. In the present database, no ketones were detected in either males or females with the Ames Multistix. However, bilirubin was detected in some samples collected by metabolism cage (confirmed by bladder catheterization), and positive results were found for blood and protein in a number of animals. The presence of urine blood was significantly greater in females than in males and may, as indicated above, be associated with estrus. Alternatively, the propensity for urine to be contaminated with feces would seem greater in female ferrets because of their shorter anal-genital space.

Similar to the evaluation of different blood sampling sites, macroscopic urine data collected for ferrets suggests that a comparative study is warranted. This evaluation should include parameter values (by sex) obtained from bladder specimens, immediate versus delayed analysis, the contribution of various diets, and a comparison of estrous and anestrous females.

Reference Microscopic Urine Profiles

Significantly greater amounts of leukocytes, erythrocytes, and bacteria are observed in overnight urine samples of female ferrets when compared with males. However, the levels reported in table 7.16 are probably well below those that can be considered clinically important in this or any other species. Conn (1963) has indicated that normal human urine, for example, can contain a large range in leukocytes (0–650,000/24 hr) and erythrocytes (0–130,000/24 hr) when measured by the Addis count. Moreover, the presence of bacteria (in association with WBQ in the overnight urine samples described in table 7.16) is probably related more to storage conditions and time than to a manifestation of pyuria.

The mean number of RBCs observed in female ferrets corresponds to a table 7.14 classification range of between rare to 1 to 4 (per magnification field). To evaluate the contribution of estrus to this measurement, however, catheterization of the bladder would be required.

Organ Weights and Histology for Assessment of Toxicity

Prolonged toxicity testing (as opposed to acute testing) frequently involves the evaluation of all animals (or at least high-dose animals) for gross pathological and histological effects at least at the end of the experiment, but also for moribund animals sacrificed prematurely. The weights of various organs are usually included in this evaluation.

Organ Weights and Transformations

The absolute organ weights of the ferret (table 7.17), when divided by the body weight at necropsy, are routinely expressed as relative organ weights (table 7.18). This calculation assumes, however, that the organ weight increases in proportion to the body weight. This assumption is approximately valid for the liver weight and kidney weight but (as can be demonstrated in most species) is grossly violated for the brain weight. Consequently, a revised relative organ weight must be calculated (table 7.19) such that the calculation becomes independent of the body weight. Figure 7.4 demonstrates how ferret brain weight, for example, varies with respect to sex and body weight, and is then made proportional to body weight when the appropriate transformation is applied.

A species comparison of revised relative organ weights is listed in table 7.20. This particular control animal database includes 94 male and 87 female rats, 24 male and 24 female beagle dogs, and 48 male and 48 female ferrets. Table 7.20 demonstrates that (because the exponents are all less than 1) the ratio of organ weight (especially the brain) to body weight (unrevised) would be larger for smaller animals than for larger animals. The revised organ weight formulas remove the effect of body weight entirely and provide a valuable addition to the statistical protocol.

Table 7.17 Reference Absolute Organ Weights (g) of Adult Male and Female Ferrets Maintained Under Controlled Environmental Conditions

Organ	Sex	*M*	*SD*	N	Min	Max	Central Tendency	Tolerance limit			Sex effect	Transformation
								Lower	Upper	Method		
Brain	F	5.95	0.41	48	4.57	6.83	5.95	5.00	6.90	P	$p < .001$	None
	M	7.35	0.62	48	5.70	8.94	7.34	5.92	8.76			
Heart	F	4.02	0.52	48	3.01	5.80	4.04	2.84	5.64	P	$p < .001$	None
	M	6.62	0.81	48	3.88	8.25	6.62	4.76	8.48			
Lung	F	5.70	0.78	48	4.03	7.57	5.71	3.89	7.53	P	$p < .001$	None
	M	9.08	1.09	48	6.61	13.03	9.11	6.64	11.58			
Liver	F	21.9	4.5	48	12.5	35.8	22.1	12.1	32.2	P	$p < .001$	None
	M	37.8	5.0	48	19.7	44.8	37.8	26.4	49.2			
Spleen	F	4.73	1.63	48	1.99	8.62	4.77	1.03	8.50	P	$p < .001$	None
	M	7.92	1.73	48	3.26	12.06	7.92	3.92	11.91			
Left kidney	F	2.07	0.27	48	1.58	2.84	2.08	1.47	2.69	P	$p < .001$	None
	M	3.40	0.37	48	2.45	4.24	3.40	2.55	4.25			
Right kidney	F	1.99	0.27	48	1.46	2.62	2.00	1.38	2.63	P	$p < .001$	None
	M	3.27	0.36	48	2.26	4.23	3.27	2.44	4.10			

Note: P = parametric.

Table 7.18 Reference Relative Organ Weights (g/kg) of Adult Male and Female Ferrets Maintained Under Controlled Environmental Conditions

Organ	Sex	*M*	*SD*	N	Min	Max	Central Tendency	Tolerance limit			Sex effect	Transformation
								Lower	Upper	Method		
Brain	F	7.03	1.04	48	4.34	10.12	7.09	4.86	9.31	P	$p < .001$	g/kg
	M	4.75	1.21	48	3.39	11.60	4.60	3.06	6.15			
Heart	F	4.73	0.68	48	2.42	6.03	4.78	3.41	6.15	P	$p < .001$	g/kg
	M	4.19	0.49	48	3.51	5.78	4.19	3.05	5.33			
Lung	F	6.68	0.91	48	3.83	8.49	6.74	4.86	8.63	P	$p < .001$	g/kg
	M	5.80	1.10	48	4.27	10.81	5.69	3.80	7.58			
Liver	F	25.6	4.3	48	10.4	36.3	25.9	17.4	34.5	P	$p < .001$	g/kg
	M	23.9	2.6	48	19.5	30.3	23.9	17.9	29.8			
Spleen	F	5.56	1.92	48	2.34	10.1	5.61	1.21	10.0	P	$p < .001$	g/kg
	M	5.01	1.09	48	2.06	7.63	5.01	2.48	7.54			
Left kidney	F	2.44	0.35	48	1.30	3.32	2.46	1.74	3.18	P	$p < .001$	g/kg
	M	2.18	0.38	48	1.57	3.54	2.18	1.29	3.06			
Right kidney	F	2.34	0.34	48	1.19	3.14	2.37	1.68	3.06	P	$p < .001$	g/kg
	M	2.09	0.35	48	1.65	3.27	2.09	1.28	2.89			

Note: P = parametric.

Table 7.19 Reference Relative Organ Weights (g/kg) of Ferrets (Sexes Combined) Maintained Under Controlled Environmental Conditions

Organ	Sex	*M*	*SD*	N	Min	Max	Central Tendency	Tolerance limit			Sex effect	Transformation
								Lower	Upper	Method		
Brain	M+F	6.35	0.58	96	4.65	8.90	6.33	5.22	7.45	P	ns	$g/kg^{0.28}$
Heart	M+F	4.61	0.52	96	2.61	6.32	4.63	3.59	5.67	P	ns	$g/kg^{0.75}$
Lung	M+F	6.52	0.87	96	4.25	9.54	6.52	4.81	8.22	P	ns	$g/kg^{0.66}$
Liver	M+F	25.3	3.5	96	10.9	36.2	25.5	18.6	32.3	P	ns	$g/kg^{0.83}$
Spleen	M+F	5.40	1.45	96	2.36	9.66	5.44	2.34	8.53	P	ns	$g/kg^{0.79}$
Left kidney	M+F	2.41	0.32	96	1.44	3.15	2.42	1.75	3.08	P	$p < .05$	$g/kg^{0.68}$
Right kidney	M+F	2.31	0.30	96	1.31	3.05	2.32	1.69	2.95	P	$p < .05$	$g/kg^{0.68}$

Note: P = parametric; *ns* = not significant.

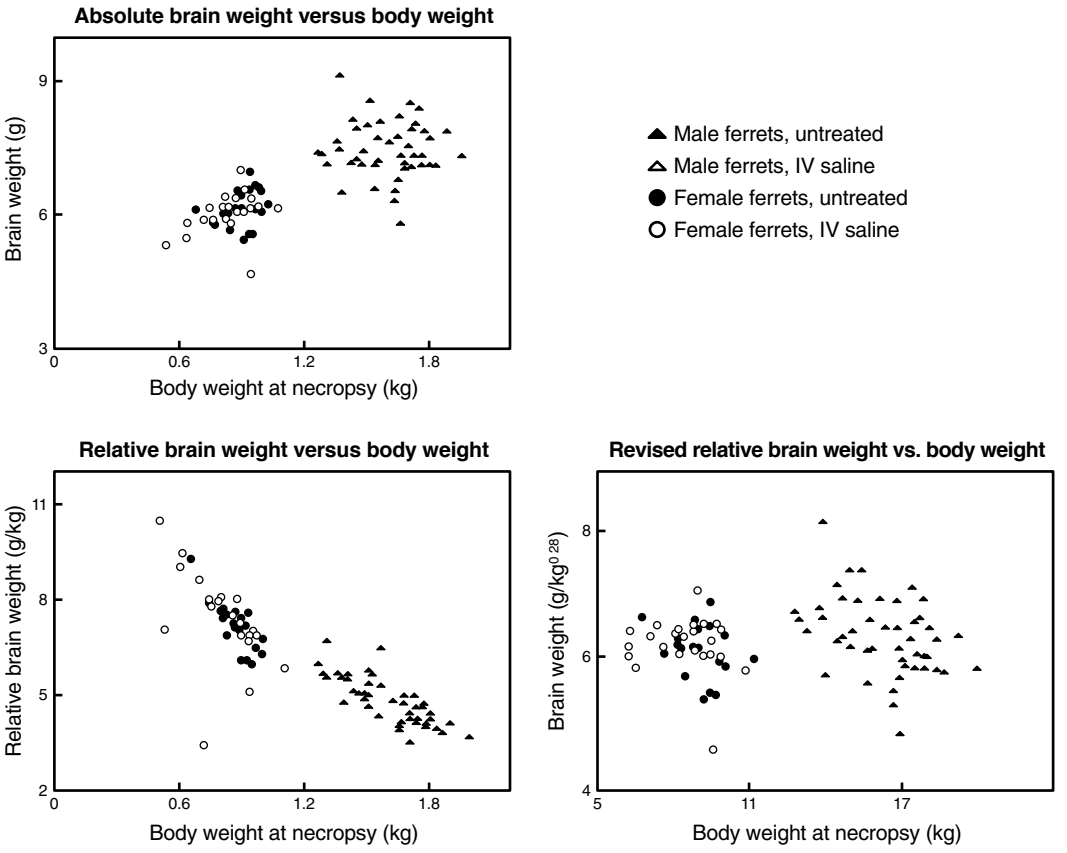

Figure 7.4 Scattergram of ferret brain weight expressed as the absolute weight (top left), relative weight (bottom left), and revised relative weight (bottom right). By applying the power transformation to body weight (i.e. revised relative organ weight) the effect of sexual dimorphism is eliminated, thus sample size (*N*) is increased accordingly.

Table 7.20 Revised Relative Organ Weight for Rats, Beagle Dogs, and Ferrets (Revised Relative Organ Weight = Organ Weight/[Body Weight]^{power})

	Power Factor (Exponent)		
Organ	Rat	Beagle Dog	Ferret
Brain	0.17	0.18	0.28
Lung	0.44	0.71	0.66
Heart	0.72	0.81	0.75
Liver	0.82	0.95	0.83
Spleen	0.40	0.97	0.79
Kidney	0.76	0.94	0.68

Common Histological Findings in Ferret Tissues

The histological findings detailed in table 7.21 provide a summary of what has been reported by two independent pathologists for 40 untreated control animals and 64 intravenously saline-treated control animals. Most noteworthy in this database is the fact that what one pathologist might consider reportable another pathologist might consider normal for the species (and thus not

reportable). This is evident by the contrasting frequency of histological citations for specific organs, with extramedullary hematopoiesis of the spleen being the most extreme example.

Table 7.21 Microscopic Findings of the Intravenous Saline-Treated (ST) and Untreated (UT) Male and Female Ferret's Major Organs as Reported by Two Independent Pathologists (Incidence [%], Severity[a])

	Pathologist		
Organ/Microscopic Finding	**A(ST)**	**B(ST)**	**B(UT)**
Brain			
Mononuclear cell infiltration, choroids plexus	15/32	0/32	0/40
	(46.9)	(0.0)	(0.0)
	NG	—	—
Mononuclear cell infiltration, meninges	2/32	0/32	0/40
	(6.3)	(0.0)	(0.0)
	1.0	—	—
Lungs			
Pneumonitis	26/32	26/32	33/40
	(81.3)	(81.3)	(82.5)
	1.7	1.5	1.5
Heart			
Fibrosis	0/32	4/32	5/40
	(0.0)	(12.5)	(12.5)
	—	1.2	1.2
Liver			
Mononuclear cell infiltration, portal or periportal	32/32	32/32	40/40
	(100)	(100)	(100)
	2.0	2.0	2.2
Kidney			
Mononuclear cell infiltration, interstitial	3/32	0/32	0/40
	(9.4)	(0.0)	(0.0)
	1.3	—	—
Nephritis, glomerulo- or interstitial	1/32	13/32	13/40
	(3.1)	(40.6)	(32.5)
	1.0	1.2	1.2
Spleen			
Extramedullary hematopoiesis	0/32	32/32	40/40
	(0.0)	(100)	(100)
	—	2.5	2.4
Stomach			
Mononuclear cell infiltration	1/32	1/32	2/40
	(3.1)	(3.1)	(5.0)
	3.0	2.0	1.5

[a] Severity: 1 = minimal; 2 = slight; 3 = moderate; 4 = moderately severe/high; 5 = severe/high; NG = not graded.

Mononuclear cell infiltration is apparently the most frequently observed histological finding in ferret tissues. This particular microscopic finding occurred in 100% of the livers examined and to a lesser and more variable degree in various other organs. The mean severity grade of slight assigned (by both pathologists) to the degree of mononuclear cell infiltration of the liver implies that moderate or greater infiltrations could have occurred in approximately one-third of the animals examined. Therefore, to minimize the number of moderate infiltrations and decrease the overall mean score, McLain and Lin (1989) devised an index method of severity prediction based on pre-and posttreatment blood samples (see later section). Use of this index to predict the degree of lymphocyte infiltration enables the culling of animals with unacceptable scores (defined by the investigator). Alternatively, the index can be used as a blocking factor to randomize animals into test groups such that lymphocyte infiltration scores between groups are similar.

Although the severity is slightly greater, the incidence of pneumonitis reported in table 7.21 for ferrets is not markedly different from what has been observed for beagle dogs (unpublished observations). On the other hand, the minimal to slight cardiac fibrosis observed, which is apparently a residual effect of the blood sampling procedure, is not a common finding in the beagle dog. The pathology section of this chapter provides a more detailed evaluation and overview of the toxicological histopathology of the ferret.

Derivation and Application of the Liver Lymphocyte Index (LvLI)

When using ferrets as a nonrodent animal model for safety assessment studies, the preexisting histological lesions of the species must be addressed. Obviously, improved hygiene and breeding efforts by the vendor will reduce the frequency of some lesions. Preceding this, however, statistical control can be used to accommodate or minimize their effects.

To control existing liver lesions common to the ferret, a database was constructed from pre- and posttreatment hematology and clinical chemistry measurements obtained by cardiac puncture from 152 male and 152 female ferrets. Tissues from all animals were evaluated microscopically for the presence and severity of lymphocyte infiltration in various organs. Organs evaluated included (but were not limited to) liver, lung, kidney, thyroid, stomach, colon, and brain. The histology scoring system used was 0 = none, 1 = minimal, 2 = slight, 3 = moderate, 4 = high, and 5 = severe. Attempts were made to predict the pathologist's score for liver lymphocyte infiltration, for example, by quantitative lesion indexes based on: (a) pretreatment clinical chemistry and hematology measurements of individual animals (pretreatment lesion index), and (b) all pre- and posttreatment measurements (including organ weights) of these same animals (final lesion index).

The benefits of using pretreatment lesion indexes for prediction of lesion severity and presence include the following: (a) Because an animal's organ tissue cannot be examined until the animal is sacrificed, the index can be used to screen for healthy animals based on measurements of their pretreatment blood samples. (b) The index can be used as a blocking factor to randomize animals such that all treatment groups (including the control group) contain animals with similar lesions. (c) The index can be used as a covariable in the statistical analysis to control for variation due to preexisting lesions. Similarly, the benefits of using the final index include the fact that it is an objective, quantitative, and continuous score instead of a subjective and ordinal score (pathologist score). In addition, it is statistically more powerful and easier to evaluate when comparing organ lesions across treatment groups.

The lesion index is the linear combination of all parameters that yield the best geometric separation between "less than moderate" and "moderate or higher" lymphocyte infiltration conditions. In other words, it is the weighted average of all parameters that yield the most power (or the least error) to predict a moderate or higher lymphocyte infiltration of an animal. The weight (coefficient) that is assigned to each parameter depends on how much that parameter contributes to the prediction power.

Table 7.22 lists the liver lymphocyte infiltrate index based on pretreatment parameters (LvLI-pre). The first column presents all parameters listed in order of decreasing importance of their contribution in prediction power. The third column lists the coefficient (weight) that was assigned to each parameter. The fourth column shows how each parameter correlated with the index. For this index, globulin is the strongest predictor, with a correlation coefficient of .583. After knowing the globulin level, the lymphocyte count provides more additional predictive power than any other parameter. Similarly, MCHC is the third highest predictor after we know the globulin and lymphocyte count. Total protein has a higher correlation (.478) with the index than does MCHC in this example, but it is given a lower priority because it is highly correlated with globulin. In other words, the joint information from globulin, lymphocyte count, and MCHC is more predictive than the joint information from globulin, lymphocyte count, and total protein. The same argument can be used for the rest of the information in table 7.22.

Table 7.22 Prestudy Parameters Used for the Calculation of a Liver Lymphocyte Infiltrate Index $(\text{LvLI-pre} = 6.2335 + \sum_{I=1}^{18} C_I Y_I)$

Parameter (X)[a]	Transformation (Y)	Weight Coefficient (C)	Correlation Coefficient (Rank)[b]
Globulin	Ln(X-1.23)	1.11690	.583 (1)
Lymphocyte count	Ln(X)	0.78835	.476 (3)
MCHC	X	0.25618	.218 (7)
LDH	Ln(X-120)	–0.47096	–.311 (5)
SGPT	Ln(X-40)	0.88691	.361 (4)
Uric acid	Ln(X+0.74)	–0.94505	–.093 (13)
RBC	X	0.30556	–.225 (6)
Basophil count	Ln(X+0.001)	0.13193	.156 (10)
Alk. phos.	Ln(X-10)	–0.64420	–.055 (17)
Cholesterol	X	0.01485	.092 (14)
Glucose	Ln(X+96)	–1.83530	–.158 (9)
Monocyte count	Ln(X+0.001)	0.06817	.178 (8)
STAB count	Ln(X+0.001)	–0.11888	–.088 (15)
Albumin	X	–0.85864	–.029 (18)
Total protein	X	0.41570	.478 (2)
BUN	Ln(X-9)	–0.44574	–.106 (11)
Creatinine	X	1.80620	.095 (12)
Fibrinogen	Ln(X-63)	–0.21236	–.056 (16)

[a] Parameters are listed in order (top to bottom) of additional predicting power.

[b] The correlation coefficient explains the predicting power of the parameter if all other parameters are unknown.

Table 7.23 lists the LvLI based on all information collected, including the organ weights at necropsy and all pretreatment data. The LvLI-pre explains 72% (.846) of this index. The posttreatment SGPT is a strong predictor. The posttreatment globulin is a weak predictor because it is highly correlated with LvLI-pre.

Success Rate of LvLI-Pre and LvLI

Table 7.24 shows the success rate of LvLI-pre and LvLI For those ferrets with LvLI-pre greater or equal to 3, we would expect a moderate or high severity score (degree of lymphocyte infiltration) when their liver tissue is evaluated by the pathologist at sacrifice (2–3 weeks later). Among the 74 ferrets in the present database that were judged to have a moderate or high severity score by the pathologist, 65 were judged to have moderate or high scores with the LvLI-pre. Therefore, we have 88% confidence that the index (based on pretreatment blood samples) would agree with the pathologist's opinion 2 to 3 weeks later. Similarly, among the 224 ferrets that were judged to have a slight or less severity score by the pathologist, 196 (88%) were judged the same by the LvLI-pre.

LvLI is shown to have a 92% (68/74) success rate in predicting moderate or high liver lymphocyte infiltrate severity scores and has a 96% (212/221) success rate in predicting slight or moderate scores as judged by a pathologist.

Table 7.23 Poststudy Parameters Used for the Calculation of a Liver Lymphocyte Infiltrate Index $(LVLI = 8.5207 + \sum_{I=1}^{21} C_I Y_I)$

Parameter (X)[a]	Transformation (Y)	Weight Coefficient (C)	Correlation Coefficient (Rank)[b]
LvLI-pre	X	0.44589	.846 (1)
SGPT	Ln(X-40)	0.72639	.630 (2)
A/G ratio	X^2	0.06011	–.409 (5)
Creatinine	X	0.85106	.192 (11)
Potassium	Ln(X-3.5)	–0.49440	–.196 (10)
Heart, rev.-rel.	X	0.38091	.096 (15)
Kidney, rev.-rel.	X	–0.47868	.004 (21)
Spleen, rev.-rel.	X	0.08443	.388 (6)
A/G ratio-pre	X^2	0.15299	–.381 (7)
MCV	X	–0.07150	–.081 (16)
Glucose	Ln(X+96)	–1.05990	–.151 (12)
HCT-pre	X	–0.03010	–.226 (9)
STAB count-pre	Ln(X+0.001)	–0.06265	–.081 (17)
Phosphorus-pre	X	0.09743	.138 (13)
Lung, rev.-rel.	X	–0.05949	–.044 (19)
Cholesterol-pre	X	0.00204	.076 (18)
EOS count	Ln(X+0.001)	–1.05796	.008 (20)
Liver, rev.-rel.	X	0.01964	.356 (8)
BASO count-pre	Ln(X+0.001)	0.02406	.126 (14)
Globulin	Ln(X-1.23)	1.48610	.551 (3)
Total protein	X	–0.51067	.479 (4)

[a] Parameters are listed in order (top to bottom) of additional predicting power.

[b] The correlation coefficient explains the predicting power of the parameter if all other parameters are unknown.

Table 7.24 Performance Measurement of LvLI-Pre and LvLI

	Scored by Pathologist	
	Slight or Less	**Moderate or High**
Based on LvLI-pre		
Slight or less, LVLI < 3	196	9
Moderate or high, LvLI > 3	28	65
Total	224	74
Based on LvLI (final)		
Slight or less, LvLI < 3	212	6
Moderate or high, LvLI > 3	9	68
Total	221	74

Typical Protocols

Acute Toxicity Testing

Traditionally, the single test that is conducted on essentially all chemicals that are of any biological interest is the acute toxicity test. In the classical sense, the test consists of administering the compound to the animals on one occasion, for a time period that is usually less than 24 hr. The purpose of the test is to determine the symptomology consequent to administration of the compound and, as it is used less frequently today, to determine the order of lethality of the compound.

Essentially all initial acute toxicity tests are performed in rodents because of their small size, availability, and the abundance of reference toxicological data generated for the species. Subsequent testing using similar procedures is performed in other species such as the ferret for the purpose of contrast and confirmation.

The ferret has proven especially useful in the screening type of acute toxicity study. These studies aid in identifying compounds of such low toxicity that, when considered in relation to a proposed use of low exposure, extensive investigations to make a judgment of safety are not justified. In the screening type of acute toxicity study the amount of test agent to which an animal is exposed is usually so massive that it generally bears no practical relationship to the expected human exposure.

Acute Oral Testing

Acute oral toxicity testing in ferrets, commonly performed subsequent to rodent testing, is designed to elicit the qualitative and quantitative nature of the toxic effects from a one-time oral exposure to a large dose of a chemical or test agent. Whether the purpose of testing is to provide data for estimating the lethal dose for 50% of a group of animals, or for demonstration that some large multiple of the potential human dose does not result in an irreversible manifestation of toxicity, the number of animals employed should be sufficient for a sound statistical evaluation. Typically, six to eight adult ferrets/sex/group are sufficient when there is no a priori knowledge of the raw effects resulting from exposure to the test agent. Procedures for testing would be similar to those prescribed for rodents, with the exception that emesis should be included in the physical and observational examination protocol. In addition, phonation should be monitored as it is more common in the ferret than it is in rodents.

Acute Dermal Testing

The ability of some chemicals to penetrate intact and abraded skin and produce systemic toxicity is well known, and steps should therefore be taken to evaluate this possibility when appropriate. The albino rabbit is, of course, the animal most frequently used in assessing dermal toxicity. However, the mouse, rat, guinea pig, and dog have also been used. The ferret would most likely parallel the dog in this type of evaluation, with its thick skin severalfold less permeable than that of the rat or rabbit (thus closer to human skin).

An ideal dermal application site in the ferret is the dorsal surface just above or at the level of the shoulders. Large “rat jackets” or vests can be fitted to the animals when it is necessary to cover a test material. After an appropriate exposure period, excess material is removed, and the local changes and any gross signs of toxicity are noted. Animals should be observed for an appropriate period of time and postmortem studies performed.

Acute Inhalation Toxicity

Test article exposure by inhalation is probably the most time consuming and expensive of all toxicological dosing procedures. Vinegar et a l. (1985) have convincingly argued, however, that the ferret is a less expensive substitute for the dog in acute inhalation toxicity testing. Moreover, because the ferret has more submucosal glands in the bronchial walls and an additional generation of terminal bronchioles, these authors have stated that the ferret lung is closer anatomically to the human lung than is the dog lung (see earlier discussion). Protocols for acute inhalation toxicity in ferrets should parallel those used for rodents, with the possible exception of perhaps increasing the effect sample size to accommodate the increased heterogeneity of the species. In addition, because pulmonary changes are likely manifestations of response to experimental treatment, ferrets selected for study should be thoroughly screened during quarantine for the absence of respiratory disease. Application of an LvLI, as described previously for the liver, can detect ferrets with moderate or higher infiltrates

with an 88% success rate when using LvLI-pre, and a 94% success rate when all variables are used in the calculation.

Subchronic Toxicity Testing

Subchronic toxicity procedures are designed to determine the adverse effects that might occur during repeated exposure over a period of a few days to usually 3 months (90 days). The subchronic procedures usually include the routes of exposure expected for man, with exposure levels lower than in the acute toxicity protocols. A high exposure level that is judged to be sufficiently large to produce adverse effects and at least one lower exposure level that is not expected to produce adverse effects are used. Intermediate exposure levels should be introduced when they are considered necessary. The number of ferrets used should be sufficient for statistical confidence (usually six to eight adult ferrets/sex/group). Observations should include overt signs of toxicity, food consumption (when appropriate), body weight change, hematology, clinical chemistry, urinalysis, organ weights, and gross and microscopic pathology. To increase the statistical power, clinical pathology should be evaluated as the change from baseline (i.e., pretreatment samples must be collected from all animals).

Clinical observations of test animals should include daily cageside evaluations as well as detailed examinations performed at least once per week. The detailed physical examination can be scheduled with the body weight measurement. Standard procedures employed for other test species are acceptable. Additional clinical notations should include inspections for excessive fur or hair under the cage, swelling of the vulva in females, and testicular prominence in males. To maintain all animals in a sexually inactive state, they should be segregated by sex (when possible) and subjected to shortened photoperiods. Recovery studies can be included if the changes observed indicate that this type of procedure is necessary. As with the other types of toxicity procedures, subchronic toxicity data generated with ferrets are most complementary when expressed as a contrast to subchronic rodent data.

Long-Term Bioassays for Chronic Toxicity and Carcinogenesis

The classic approach to the study of chronic toxicity and the carcinogenic potential of test substances involves studies in two or more species of animals (one of which should ideally be a nonrodent) for periods of time ranging from many months to several years. Rats and mice have, of course, been the primary test species for these protocols. However, long-term testing with ferrets would seem to be a plausible and welcomed alternative.

Irrespective of the variability observed in the normal ferret's clinical or microscopic profile, with increasing dosage in the continuum of the dose–response relationship, a region is generally entered where the effects are clearly adverse.

The detection of adverse effects in chronic toxicity protocols begins with gross observations of the intact animal in terms of growth, appearance, and activity. The next point of discrimination is at the organ-system level, wherein changes of a biochemical and physiological nature are assessed. These are followed by an examination of morphological changes at the gross and cellular levels in sacrificed animals or biopsy material. Clearly, adverse effects that occur in ferrets or any other species are those that result in impairment of functional capacity (as determined by anatomical, physiological, and biochemical or behavioral parameters), or in a decrement of the ability to compensate for additional stress; are irreversible during exposure or following cessation of exposure; and enhance the susceptibility of the individual to the deleterious effects of other environmental influences.

Developmental Toxicity Assessment

It is evident that there is no one animal species that can be considered ideal for evaluating human developmental toxicity. Ferrets are no exception to this dilemma, of course, and provide

just a higher level of evaluation for embryo toxicity than what is offered with rodents. The ferret's chief advantage in reproductive toxicity assessment is its small size and the fact that it does not have the atypical yolk-sac placenta that is common to the rat.

Protocols for evaluation of developmental toxicity in the ferret follow guidelines similar to those that would be used for rodents, with the exception that animals obtained from a reputable supplier should be vaccinated for distemper and subjected to a thorough veterinary examination on arrival at the vivarium. Estrous females at their peak of vulval swelling should be placed with sexually active males of proven fertility and observed for successful coitus on several successive occasions. The first successful coitus is considered day 0 of pregnancy because ovulation generally occurs approximately 30 hr postcoitus. The examination of vaginal lavage for motile sperm after observed mating would be helpful in determining successful insemination. Presumed-mated jills can also be shipped from the supplier (Marshall Farms, North Rose, New York) the day following coitus (day 1 of gestation) with no apparent effect on reproductive parameters if transportation is completed on gestation day 1. Acclimatization to the vivarium is easily accomplished between arrival and day 12 of gestation when dosing should begin. Vulval regression and obvious weight gain would indicate successful conception. The critical period in the ferret is generally accepted as days 12 through 30, with implantation occurring on approximately day 12.

Litters should be taken by cesarean section on gestation day 35 and subjected to the same examination procedures used for rats and rabbits. That is, living fetuses should be weighed, sexed, and examined externally and internally by gross dissection of all cavity organs or by the Wilson or Staples techniques. Skeletal visualization by potassium hydroxide (KOH) clearing should be done on a predetermined number of fetuses. Other parameter evaluations and reporting are similar to what would be done for rodents.

Routine acceptance of the ferret in developmental toxicity studies is still to be established within the various governmental agencies. It is recommended, therefore, that a positive control group (15 mg/kg all-trans retinoic acid on day 14 of gestation) be included with every study.

Summary

Review of Advantages and Disadvantages

The most obvious advantages to the use of the ferret as an animal model in toxicology include their relatively low cost, small body size, ease of handling (mild disposition) and maintenance, and ability to adapt to most existing facilities and laboratory equipment. Less obvious advantages include the current lack of opposition to their use (traditionally voiced by antivivisectionists), and their apparent physiological and biochemical similarities with humans. The increasing popularity of ferrets in biomedical research correlates highly with growth of the biotechnology industry. In this respect, the ferret requires only about one-tenth of the limited test article that would be needed for the more conventional beagle dog.

Disadvantages of the ferret include their large relative heterogeneity, current lack of extensive databases, lack (temporary) of an approved rabies vaccine, lack of "virus-free" ferrets, limited availability, and the small number of vendors selling the animal.

Steps That Can Be Taken to Minimize Disadvantages

A significant portion of this chapter is devoted to the presentation and discussion of material that could be viewed as a beginning database for the ferret. In addition, novel methods for decreasing the level of heterogeneity in this species (or any other species) and eliminating undesirable animals from testing consideration have been proposed. New information has been presented with respect to the pending approval of a rabies vaccine for ferrets. However, only the continued use of and

demand for ferrets will cause the number of vendors available to increase, and only concentrated research and breeding efforts will provide the scientific community with a virus-free ferret.

PATHOLOGY

The ferret (*Mustela putorius furo*) is thought to have originated from the wild European polecat or ferret and might also be related to the steppe polecat. Ferrets share many anatomical, metabolic, and physiologic features with humans, which has promoted their use as an animal model. Ferrets are used in biomedical research in a wide variety of studies including cardiopulmonary, neurological, and GI research. Ferrets have been used in cardiovascular research to examine ischemia and ion exchange in the heart muscle, pulmonary mucus secretion related to asthma or influenza, neurological changes associated with brain and spinal cord injury, and gastric infections and ulcerations. Ferrets have also been used as a model for the demonstration of medical procedures such as pediatric tracheal intubation.

Infectious and Parasitic Diseases

Canine Distemper

This is a morbillivirus disease with essentially 100% fatality. Disease progression ranges from 12 to 42 days. The disease is profoundly immunosuppressive, and surviving animals succumb to neurologic dysfunction. Treatment is not recommended (Hoover et al. 1989; Kaufman et al. 1982). Gross lesions include photophobia, oculonasal discharge, bronchopneumonia, hyperkeratosis of the planum nasale and footpads, and a papular rash beginning on the chin and progressing to a generalized form. Microscopically there is pneumonitis with syncytia, multifocal dermatitis, hyperkeratosis, and eosinophilic, intracytoplasmic, and intranuclear inclusions in a wide variety of epithelial cells and neurons. A nonsuppurative encephalitis with demyelination can be seen in animals with neurologic disease (Fox et al. 1998; Williams et al. 1988).

Parvoviral Infection (Aleutian Disease)

Aleutian disease is caused by an antigenically related parvovirus that causes Aleutian disease in mink. In ferrets, the disease is much more insidious with hypergammaglobulinemia, CNS disease, wasting syndrome, proteinuria, and, in late stages of the disease, an immune complex glomerulonephritis (Alexandersen et al. 1994; Ohshima et al. 1978; Oxenham 1990; Porter et al. 1982; Welchman et al. 1993). Gross lesions observed late in the course of the disease include splenomegaly, lymphadenopathy, petechial hemorrhage, and hematuria. Microscopic findings include prominent plasmacytic infiltrates (plasmacytosis) in the renal interstitium, hepatic portal areas, splenic red pulp, lymph nodes, and the bone marrow. In most cases, there will be marked membranous glomerulonephritis and numerous ectatic protein-filled tubules as a result. Vasculitis can be seen in almost any organ (Alexandersen et al. 1994; Daoust and Hunter 1978; Palley et al. 1992; Wolfensohn and Lloyd 1995).

Coronavirus-Associated Epizootic Catarrhal Enteritis (ECE)

ECE is a diarrheal disease that causes epizootics of high morbidity (up to 100%), but low mortality. The diarrhea is rapidly dehydrating and most mortalities occur in older animals with concurrent illness. Symptoms include vomiting and passage of a dark green stool with abundant mucus. Grossly the intestines might be flaccid with a moderate amount of watery ingesta. Microscopically, lesions include

lymphocytic enteritis, vacuolar degeneration and necrosis of villus enterocytes, villus atrophy, fusion, and blunting (Williams et al. 2000).

Rabies

The incidence is low with fewer than 25 confirmed cases since 1954. The disease can result in both furious (less common) and dumb forms, and might present as a progressive hindlimb paralysis. There are no gross lesions and microscopically intracytoplasmic eosinophilic viral inclusions (Negri bodies) might be demonstrated on H&E stains or on standard fluorescent antibody tests (Fox et al. 1998).

Influenza

Ferrets are the only domestic animal species that is susceptible to the human influenza viruses (Renegar 1992; Smith and Sweet 1988). For this reason, they are often used as animal models in influenza research, and often infected by their human owners. The disease is quite similar to that in humans, with clinical signs being photophobia, a catarrhal nasal discharge, sneezing, coughing, pyrexia, anorexia, and malaise. Gross lesions are generally minimal, with congestion and exudation of the nasal mucosa and mild reddening of the tracheal mucosa. Microscopically, there is mild subacute inflammation and occasional necrosis of the nasal mucosa. A mild subacute interstitial pneumonia might be present (Fox et al. 1998; Renegar 1992; Smith and Sweet 1988).

Helicobacter mustelae

Helicobacter mustelae associated gastric disease in ferrets over the age of 4 years resembles *Helicobacter pylori* in man and nonhuman primates (Fox et al. 1990, 1991; Otto et al. 1990). The bacterium causes disease via two mechanisms: (a) the stimulation of a marked lymphoplasmacytic inflammatory response, resulting in loss of glandular epithelium, most prominently in the pylorus; and (b) the ability to increase the pH of the stomach (Fox et al. 1990, 1993; Gottfried et al. 1990). Gross lesions in advanced cases might be limited to gastric ulcers. Microscopically, gastritis is accompanied by the presence of bacteria in the superficial mucus or within the gastric glands and these can be demonstrated with Warthin-Starry stain (Fox et al. 1990, 1991).

Proliferative Colitis

This is an uncommon, sporadic disease that is usually seen in young male ferrets under 1 year of age affecting only one or two animals in a large colony (Finkler 1992; Fox et al. 1982). Clinical signs include tenesmus and frequent painful defecation with frank blood and mucus. The disease is caused by an intracellular campylobacter-like organism bacteria belonging to *Ileobacter (Desulfovibrio)* sp (Fox et al. 1988, 1989, 1994). Grossly there is marked thickening of the colonic wall and the mucosa has a cobblestone appearance. Microscopically, the mucosa is multifocally thickened due to proliferation of immature epithelial cells and mixed inflammatory cell infiltrate. Bacteria in the apical cytoplasm of epithelial cells can be demonstrated with Warthin-Starry stain (Finkler 1992; Fox et al. 1994; Krueger et al. 1989).

Clostridium perfringens

Gastroenteritis associated with *Clostridium perfringens* type A has been reported in black-footed ferret kits. Grossly, there is gastric bloat and multifocal intestinal hemorrhage. Microscopically, there is marked coagulative necrosis of the intestinal mucosa with numerous adherent bacilli. In addition to perfringens, botulism due to toxins produced has been reported (Schulman et al. 1993).

Tuberculosis

Ferrets are susceptible to human, bovine, and avian mycobacteria. Mycobacterium avium-intracellulare infection is a rare condition in ferrets that is most commonly seen in the GI tract and mesenteric lymph nodes. Mesenteric lymphadenopathy is the most common gross lesion. Microscopically, there is lymphadenitis with large foamy macrophages containing acid-fast bacilli (Schultheiss and Dolginow 1994).

Mastitis

Pregnant jills in the first few weeks of lactation can develop mastitis. Hemolytic *E. coli* is the most commonly isolated organism, and results in a syndrome of gangrenous mastitis. If untreated, jills rapidly become septic or endotoxemic. *Staph aureus* is occasionally cultured from cases of mastitis and produces a more suppurative, less necrotic form of mastitis. Grossly, affected teats are swollen, necrotic, black, firm, and nonpainful. In *Staph aureus* mastitis, the mammary glands are hot, painful, and reddish in color; purulent exudate might be expressed from the lactiferous ducts. Microscopically, the primary lesion in *E. coli* mastitis is diffuse severe coagulative necrosis, hemorrhage, and edema that extends into the adjacent adipose tissue and muscle. In staphyloccoccal mastitis, there is less evidence of infarction. A purulent galactophoritis and mastitis is present. Bacteria might be present in the tissues in both cases (Fox et al. 1998; Liberson et al. 1983).

Intestinal Parasites

With the exception of coccidia, intestinal parasites are uncommon in ferrets. *Cryptosporidium sp.* infection can be prevalent in a colony but it does not appear to be very pathogenic (Rehg et al. 1988). Three species of coccidia have been seen in ferrets: *Eimeria furo, Eimeria ictidea,* and *Isospora laidlawii.* Although most coccidial infections are subclinical, lethal coccidial infections are occasionally seen in young kits. Grossly, digested blood might be present in the GI tract of several affected kits. Microscopically, numbers of parasites range from very low to extremely high in severe infections and all stages of the parasite, including micro- and macrogametocytes can be seen (Blankenship-Paris et al. 1993).

Dirofilariasis

Canine heartworm infection is uncommon, due to the fact that most ferrets are kept indoors. Due to the small size of the ferret heart, as few as two heartworms can result in fatal cardiac insufficiency (Miller and Merton 1982; Moreland et al. 1986; Parrott et al. 1984). Grossly the presence of heartworms within the right ventricles and pulmonary artery can be construed as the cause of death. Microscopic lesions are similar to those observed with cardiomyopathy and consist of an increase in fibrous connective tissue around myocardial vessels that extends into the interstitium, atrophy, and loss of myocytes. Secondary hepatic congestion might be present (Campbell and Blair 1978; Miller and Merton 1982; Moreland et al. 1986; Parrott et al. 1984).

Dermatomycosis

This is an uncommon disease and cases occur either in very young animals kept in poor conditions or in older, immunosuppressed animals. Both *Microsporum canis* and *Trichophyton mentagrophytes* have been seen in ferrets.

Grossly animals have areas of crusting alopecia with brittle hair and numerous broken hair shafts. In immunosuppressed animals, the rash can become generalized.

Microscopically, skin from affected sites is generally covered with a thick layer of keratin debris, degenerate neutrophils, and entrapped fungal arthrospores and hyphae. There is ulceration of the skin, and follicles often contain numerous fungal arthrospores that occasionally invade the hair shaft. There is generally a neutrophilic or lymphoplasmacytic dermal infiltrate in perivascular and periadnexal areas. *Pneumocystis carinii* can be induced by cortisone treatment but natural disease has not been reported (Fox et al. 1998).

Ectoparasites

Sarcoptic mange comes in two distinct forms in ferrets: a very pruritic whole-body form, and a variably pruritic form localized to the feet characterized by swollen feet, evidence of self-mutilation, and nail loss (Phillips et al. 1987). Microscopically, there might be ulceration and hyperkeratosis of the skin and a few cross-sections of mites in the epidermis or deep under the overlying crust. Demodectic mange is generally seen in older or immunosuppressed ferrets. Skin scrapings can demonstrate the presence of nymphs or adults and skin biopsies reveal few cigar-shaped mites within the hair follicles. Ferrets are commonly infected with ear mites (*Otodectes cynotis*) and fleas (*Ctenocephalides sp.*). Most young ferrets and many older ones have clinical cases of ear mite infection that require periodic treatment. Grossly, ferrets with ear mites have copious amounts of a thick brown-black wax and adult mites and eggs can be found microscopically (Fox 1988).

Other sporadic bacterial infections reported in ferrets include diarrhaeal disease due to *Campylobacter coli* (Larson and Hoffman 1990) and abortion due to *Campylobacter jejuni* (Bell and Manning 1990). *Staphylococcus aureus, Streptococcus pyogenes,* and *Corynebacterium spp.* are infrequent causes of subcutaneous and mammary gland abscesses, infected bite wounds, genital infections in females in prolonged estrus, and oral infections secondary to trauma.

Neoplastic Diseases

Ferrets have an incidence and spectrum of neoplastic disease similar to other mammalian species. Endocrine, hemolymphatic, integumentary, and digestive systems were most commonly affected. Tumor incidence is highest in ferrets between 4 and 7 years old. The most common tumor types are pancreatic islet cell, adrenocortical cell tumors, and lymphoma (Li et al. 1998).

Islet Cell Tumors

Pancreatic endocrine tumors are common in the ferret. Clinical signs include lethargy, stupor, ptyalism, and ataxia that can progress to coma and death (Jergens and Shaw 1989; Lumeij et al. 1987; Marini et al. 1993). Metastasis to visceral organs is rare. Grossly these tumors are well-defined nodules that range in size from 2 mm to 1 cm and they can be multiple. Microscopically, these tumors are unencapsulated, and resemble normal, albeit greatly enlarged islets of Langerhans. These neoplasms stain strongly for insulin with scattered glucagon staining (Fix and Harms 1990).

Adrenal Gland Tumors

Proliferative lesions of the ferret adrenal gland are common in the cortex and they fall into three categories: hyperplasia, adenoma, and carcinoma. The histological features of these lesions often overlap and the incidence of nodular hyperplasia is more common than adenoma or carcinoma. The presence of necrosis, cellular atypia, and an increased mitotic rate are strong indicators of malignancy. The presence of a single nodule in the adrenal cortex without factors associated with malignancy indicates adenoma, whereas the presence of multiple nodules is evidence of

nodular cortical hyperplasia (Fox and Marini 1998; Lawrence et al. 1993; Rosenthal 1993). In addition to cortical tumors, adrenal teratomas are reported in ferrets. The tumors contain tissues from ectodermal, mesodermal, and endodermal germ cell layers including rudimentary teeth (Williams et al. 2001).

Lymphosarcoma

This is the most common malignant tumor in the domestic ferret. It is speculated that lymphosarcoma in the ferret might be the result of a retroviral infection (Erdman et al. 1995). A viral agent has not, as of yet, been isolated from cases of lymphosarcoma in the ferret. Gastric lymphoma resembling gastric mucosa-associated lymphoid tissue (MALT) lymphoma linked with *Helicobacter pylori* infection in humans was observed in ferrets infected with *H. mustelae* (Erdman et al. 1997). Different forms of the disease have been recognized. Older ferrets have the well-differentiated lymphocyte form, young ferrets less than 2 years of age have the large blastic form and a third form that resembles that of the lymphocytic form with a subpopulation of atypical large cleaved, multinucleate lymphocytes and occasional Reed-Sternberg-like cells. Grossly adults with lymphocytic form have diffuse lymphadenopathy, splenomegaly, and metastatic nodules can be present in a number of visceral organs. The presence of a large thymic mass is strongly suggestive of the juvenile form (Batchelder et al. 1996). Microscopically, in the adult form there is effacement of the normal nodal architecture by an infiltrate of small noncleaved lymphocytes. In the liver, neoplastic infiltrates are primarily seen extending from portal areas, whereas in the spleen there is an expansion of the periarteriolar lymphoid sheaths. Nodal involvement is observed late in the disease in the juvenile form and the population consists of large cleaved and noncleaved lymphoblasts. In the liver, neoplastic cells are more commonly seen as discrete nodules distending sinusoids and replacing hepatocytes, whereas in the spleen, the periarteriolar lymphoid sheath is totally replaced and expanded by a monomorphic lymphoblast population (Erdman et al. 1992).

Chordoma

Chordomas arise in or adjacent to vertebra from remnants of primitive notochord, and are most commonly seen at the tip of the tail and rarely in the cervical spine (Dunn et al. 1991; Williams et al. 1993). Metastasis has not been seen in neoplasms arising in the tail. Grossly, they are seen as clublike swellings at the tip of the tail that involve the last caudal vertebra. Cervical chordomas present as lytic neoplasms in the neck of animals with posterior paresis. Microscopically, the neoplasm is composed of foamy "physaliferous cells" that are separated by a moderate amount of myxomatous matrix with areas of well-differentiated cartilage and bone (Allison and Rakich 1988; Dunn et al. 1991).

Skin Tumors

The two most common cutaneous neoplasms in ferrets are sebaceous epithelioma and mast cell tumor. Sebaceous epitheliomas appear as warty, verrucous lesions with a predilection for the head and neck and mast cell tumors appear as pruritic flat, alopecic, hyperkeratotic plaques. Microscopically epitheliomas are composed of basal cells, of which a small percentage exhibit sebaceous or squamous differentiation whereas mast cell tumor is composed of well-differentiated mast cells with low numbers of eosinophils (Li et al. 1998; Parker and Picut 1993; Stauber et al. 1990).

Miscellaneous Tumors

Other uncommon neoplasms encountered in ferrets include osteomas of the flat bones, leiomyosarcoma, interstitial cell tumors of the testes, ovarian germ cell or stromal cell tumors, and

adenocarcinoma of the pancreas and intestine. Apocrine gland cystadenomas and carcinomas are not uncommon and can occur around the head, neck, prepuce, and vulva, due to the large numbers of scent glands in these regions. Hemangiomas and low-grade hemangiosarcomas are occasionally seen; metastasis has not been reported. Squamous cell carcinoma has been reported several times in the ferret and has a predilection for the face, where it is locally destructive with a low metastatic potential (Li et al. 1998).

Endocrine and Miscellaneous Diseases

Adrenal-Associated Endocrinopathy

This is a common endocrine disorder of middle-aged to older ferrets. The syndrome is the result of proliferative lesions in the adrenal cortex that secrete excess amounts of estrogenic hormones but rarely cortisol. As a result of excess estrogen, affected ferrets exhibit a range of cutaneous, behavioral, and reproductive signs (Fox et al. 1987; Rosenthal 1993). Grossly, lesions are characterized by bilaterally symmetrical alopecia beginning over the tailhead and progressing forward over the flanks and abdomen. Spayed females can have enlarged vulva. Microscopically the proliferative lesions of the adrenal cortex can be hyperplasia, adenoma, or carcinoma (Lawrence et al. 1993; Rosenthal 1993).

Estrus-Associated Aplastic Anemia

Ferrets are induced ovulators and intact unmated jills will develop marked bone marrow suppression as a result of high levels of circulating estrogens. Initially, there is a mild thrombocytosis and leukocytosis, but the condition soon progresses to a nonregenerative anemia, leukopenia, and thrombocytopenia. The anemia can remain nonregenerative anemia up to 4 months past ovariohysterectomy in affected animals. Hemorrhage is reported to be the most common cause of death. Grossly female ferrets in estrus have prominently swollen vulvas, and signs of hyperestrogenism include pale mucus membranes, alopecia, melena, thin watery blood, hemorrhages throughout the body, hematuria, pyometra, bronchopneumonia, and vaginitis. Diagnosis of aplastic anemia is most commonly based on the presence of a low PCV (< 20%) in an estrus jill. Microscopically, the most characteristic lesion in affected jills is hypocellularity of the bone marrow (Fox et al. 1998).

Cardiomyopathy

This is a common disease in the American lines of ferrets, which has a presumed genetic basis. Several forms of this condition can be seen: dilatative, hypertrophic, and a restrictive form in which there is marked replacement of myocardium by fibrous connective tissue, with minimal change in chamber area. Signs of cardiomyopathy can be seen as early as 1 year of age in severely affected animals, but are more common between 5 and 7 years of age. Grossly, the heart might appear enlarged, and the right ventricle might appear thin or flabby. With progressively severe cases, there is often an accumulation of a serosanguinous ascitic transudate in the abdominal cavity or the pleural cavity; congested, occasionally nodular liver as a result of chronic passive congestion; and in severe cases, the lungs are atelectatic and compressed by the presence of a globose heart and abundant pleural effusion. Microscopically, early lesions consist of an increase in fibrous connective tissue around myocardial vessels, which extends into the interstitium. As the condition progresses, there is atrophy and loss of myocytes. Focal areas of myocyte degeneration might be present, with an infiltrate of moderate numbers of inflammatory cells. In some cases of cardiomyopathy, there might be marked focal misalignment of myocytes, suggesting orientation in several different planes (Fox 1998; Greenlee and Stephens 1984; Lipman and Fox 1987).

Gastric Ulcers

Ferrets are extremely susceptible to stress-related gastric ulcers. This is a common finding in animals with other systemic diseases and often contributes to debility in older animals. Gastric ulcers are often seen in association with gastric *Helicobacter mustelae* infection, however, a definitive cause-and-effect relationship has not been proven in this species. Grossly, there can be digested blood with multiple pinpoint ulcers or a single large focus usually in the pyloric stomach (Fox 1998).

Splenomegaly

This is an extremely common finding in middle-aged to older ferrets and the cause is unknown. Enlarged spleens are prone to rupture. Grossly there is diffuse enlargement of the spleen and microscopically most cases of splenomegaly have marked congestion, florid extramedullary hematopoiesis, and occasionally large areas of coagulative necrosis (Erdman et al. 1998).

Aspiration Pneumonia

This is a common cause of pneumonia in the ferret either due to orally administered medicants or vomitus. Ferrets often resist liquid oral medication by fighting and squirming during administration, and often involuntarily inhale part of the medication. Grossly, there might be consolidation of the cranioventral lung lobes, either unilaterally or bilaterally. Microscopically, the lesion is centered around bronchioles and acute lesions contain neutrophils, sloughed epithelial cells, bacterial colonies, and in long-standing cases, there might be a pronounced granulomatous response, with multinucleate giant cells. In cases of aspiration of vomitus, the lesion is characterized by extensive necrosis of the airway and surrounding alveoli, with sloughing of the bronchiolar epithelium and coagulative necrosis of the adjacent alveolar septa. Aspiration pneumonia should be differentiated from endogenous lipid pneumonia also known as foam cell foci or subpleural histiocytosis, a common incidental finding at necropsy. This lesion is of no clinical significance and consists of multiple to coalescing white to yellow foci within the subpleural pulmonary parenchyma. Microscopically, the basic lesion is simply an aggregate of lipid-laden macrophages in the alveoli immediately subjacent to the pleura (Fox 1998).

Chronic Interstitial Nephritis and Urinary Tract Infections

This is a common finding and early lesions can be seen as early as 2 years, and advanced cases resulting in renal failure can occur as early as 4.5 years. Lowering of protein levels after 3 years of age is recommended. Grossly kidneys are generally pitted and large focal depressions can be seen in the outer cortex as a result of scarring. Microscopically, there are linear bands of fibrosis, infiltrates of inflammatory cells, tubular atrophy, and glomerulosclerosis. In addition, bacterial urinary tract infections due to *E. coli* or *Staphylococcus aureus* are commonly seen in female ferrets. Bladder infections are often subclinical in female ferrets, and ascending infections resulting in pyelonephritis are not uncommon. Renal failure can result from severe pyelonephritis in this species. Grossly long-standing cases have hydronephrosis and hydroureter and microscopically there is ulcerative cystitis or a suppurative tubulointerstitial nephritis (Fox et al. 1998).

Diabetes mellitus is a poorly defined, uncommon disease characterized by polydipsia, polyuria, glucosuria, and loss of body condition. Blood glucose levels in affected ferrets generally range into the 500s, but levels as high as 725 g/dl have been reported. Microscopically glycogenic vacuolation of the islets of Langerhans and glycogen accumulation in renal tubular epithelium could be seen.

Other sporadic conditions have been reported occasionally in ferrets. Neural tube birth defects are reported in kits. The lesions range from simple cranioschisis to spina bifida, to craniorachischisis

(Williams et al. 1994). Prostatic squamous metaplasia due to excess estrogens from adrenal lesions has been recognized as a common cause of dysuria and urethral blockage in the ferret. Older ferrets can have broken upper canines, accumulation of dental calculi (moist diet), and tooth root abscess (Andrews and Illman 1979). Idiopathic megaesophagus occurs in middle-aged to older ferrets with marked dilation of the intrathoracic esophagus, secondary *Candida* infections and aspiration pneumonia (Blanco et al. 1995). Eosinophilic enteritis is a wasting disease seen in young male ferrets under 14 months of age. The lesion is characterized by eosinophilic infiltrates in the small intestine, mesenteric lymph nodes, and rarely with eosinophilic vasculitis (Fox et al. 1992; Palley and Fox 1992). Young or bored cage-bound ferrets can have GI foreign bodies (Mullen 1992), renal cysts (Dillberger 1985), urolithiasis (Nguyen et al. 1979; Palmore and Bartos 1987), and cataracts (Miller et al. 1993).

METABOLISM

The ferret is a relatively new species in toxicological and pharmacological research. There has been increased interest in this species since the early 1970s (Hoar 1984), moreso in Europe than in North America. Interestingly, however, the pace of research specifically on xenobiotic metabolism in this species has declined from a relative flurry of papers published in the late 1970s to relatively few papers published in the late 1980s. As a result, xenobiotic metabolism has not been very well characterized. For example, with regard to disposition, there is no published information on the blood–brain barrier, the blood–testis barrier, or plasma protein binding in the ferret. One has to assume that the ferret is not radically different from other species with regard to these parameters. This review concentrates on the published literature, which tends to fall into three main groups: (a) Characterizations of the microsomal mixed function oxidase (MMFO) system, (b) studies to characterize *in vivo* pharmacokinetics of various chemicals, and (c) studies on species-related differences in conjugation reactions.

Peculiarities of oral absorption in the ferret should be mentioned. The ferret has no cecum, and the transition from small to large intestine can only be detected by histological examination (Hoar 1984). The intestine is a relatively short tube, and recent work has failed to isolate any anaerobic GI bacteria. Hence, oral bioavailability of chemicals tends to be less in the ferret than in rodents.

Some indexes of xenobiotic metabolism in the ferret are summarized in table 7.25. Ioannides et al. (1977) published the first, and one of the few, extensive examinations of the MMFO system. They established that there were definite age-related (day 0–56) increases in, for example, cytochrome P-450 and aniline hydroxylase activity. Hence, the data given in table 7.25 are drawn from adult animals of the age or size that most investigators report using. Ioannides et al. (1977) also reported on slight quantitative strain differences between the polecat ferret and the albino ferret for various parameters. For example, both strains had essentially the same activity toward biphenyl as a substrate, but the albino had much greater activity toward ethylmorphine. Sexual dimorphism was also identified only for the albino ferret, with the males having higher amounts of microsomal protein, cytochrome P-450, and ethylmorphine N-demethylase activity (Ioannides et al. 1977). In contrast, Shull et al. (1982) reported that although the concentrations of cytochrome P-450 were not different, females had higher microsomal activity toward benzo(a)pyrene, hexobarbital, ethoxycourmarin, and ethylmorphine. Given this information, one should assume that there is the potential for strain- and sex-related differences in xenobiotic metabolism in the ferret, and one should consider establishing baseline data for the ferrets being used in specific studies. This is further reinforced by the variability displayed in the sets of clinical chemistry data reported elsewhere in this chapter. P-450 levels in the ferret liver are only 30% of those in rat, and have been characterized as including 1A1, 1A2, 2D6, 2C19, 2E1, and 3A1 isozymes (Lin and Lu 1997; Liu et al. 2003).

Table 7.25 Summary of Hepatic Xenobiotic Metabolizing Enzymes in Ferrets

Enzyme	Concentration or Activity	Comments and References
Cytochrome P-450	0.16–0.73 nmol/mg-m	Ioannides et al. (1977); Lake et al. (1979); Shull et al. (1982); Costello and Chengelis (1989). Strain, sex, and sex variability identified.
Cytochrome b_5	0.15–0.22 nmol/mg-m	Ioannides et al. (1977); Shull et al. (1982); Costello and Chengelis (1989).
NADPH: Cytochrome C reductase	60–113 nmol/min/mg-m	Ioannides et al. (1977); Lake et al. (1979); Costello and Chengelis (1989).
MMFO activities		Ioannides et al. (1977); Lake et al. (1979); Shull et al. (1982). Good agreement between papers. Costello and Chengelis (1989).
Ethylmorphine demethylase	1.2–3.3 nmol/min/mg-m	
Aniline hydroxylase	0.3–1.3 nmol/min/mg-m	
Benzo(a)pyrene hydroxylase	0.95 ± 0.13 nmol/min/mg-m	
Epoxide hydrolase	No data identified	No applicable publications identified
UDP-glucuronosyl transferase	Limited data available using these substrates	Lake et al. (1979); Shull et al. (1982). Available papers use conflicting data presentations, making comparisons difficult.
1-naphthol		
4-methylumbeliiferone		
Glutathione S-transferase		
p-nitrobenzyl chloride	18.2 ± 4.6 nmol/min/mg-c	Costello and Chengelis (1989)
Chlorodinitrobenzene	485 ± 107 nmol/min/mg-c	
Protein content estimates		
Microsomal	10–30 mg/g tissue	Ioannides et al. (1977), Costello and Chengelis (1989); Lake et al. (1977).
Cytosolic	110–150 mg/g tissue	

Note: Mg-m = mg microsomal protein; mg-c = mg cytosolic protein.

Depending on the age, sex, and strain of the ferret, relative liver weight ranges from 3.0% to 5.0% of body weight. There is also some variability in microsomal protein from adult animals: Values as high as 33.8 mg/g liver (Lake et al. 1977) and as low as 12.1 mg/g (Ioannides et al. 1977) have been reported. Concentrations of P-450 are likewise varied, ranging from 0.19 nmol/mg microsomal protein to 0.73 nmol/mg (values inferred from data presented by Lake et al. 1977, and Ioannides et al. 1977, respectively). However, the values of 0.29 (Lake et al. 1979) and 0.19 nmol/mg microsomal protein (Shull et al. 1982) would appear to be better estimates. Ferrets tend to have less microsomal protein and less cytochrome P-450/mg protein than rats. Hence, on a gram liver basis, ferrets have considerably less cytochrome P-450 than rats. In general, however, ferret cytochrome P-450 has a higher rate of substrate turnover.

The activity on a microsomal protein basis toward common model substrates, such as aniline and ethylmorphine, has been reported to be about the same (Ioannides et al. 1977) or somewhat less (Lake et al. 1979) than the rat. The exception to this observation is that Ionnides et al. reported that the male albino ferret had much higher activities than the rat toward ethylmorphine. This high activity toward ethylmorphine was not confirmed by Lake et al. (1979) or Shull et al. (1982), and the reason for this difference is not clear. These observations again underscore the potential variability in xenobiotic metabolism in the ferret and the importance of establishing baseline values in one's own laboratory.

Only Ioannides et al. (1977) have discussed the developmental aspects of the MMFO. At birth, the hepatic content and concentration of the MMFO are very low. Different enzyme activities develop differently thereafter. Biphenyl 4-hydroxylation parallels the activity-of NADPH reductase, and reaches adult levels (on a gram liver basis) in 14 days. Other activities tend to follow the development of cytochrome P-450, reaching adult levels approximately 2 months after birth. The largest increases in the components of the mixed function oxidase occur after weaning (4–6 weeks of age). Unlike the rat, the mature adult ferret retains measurable biphenyl 2-hydroxylation and p-nitrobenzaote reduction activities.

Few in-depth studies on the inducibility of the ferret MMFO system have been reported. There are no reports on the effects of phenobarbital on xenobiotic metabolism in the ferret. Lake et al. (1979) have demonstrated, however, that Aroclor 1254 (single intraperitoneal dose, 500 mg/kg) will cause large increases in microsomal protein, the content of cytochrome P-450, and the specific activity toward aminopyrine, benzphetamine, ethylmorphine, and aniline. In contrast, Shull et al. (1982) have reported that Aroclor 1016 and 1242 (by dietary admixture, 20 ppm, for 28 days) caused increases in relative liver weight without increases in the microsomal concentration of cytochrome P-450. In a shorter term experiment, two doses of Aroclor 1242 (100 mg/kg on day 0, 200 mg/kg intraperitoneally on day 5, and sacrifice on day 10) caused fivefold increases in cytochrome P-450, with a shift in the absorbance maximum toward 448 nm. These authors (Shull et al. 1982) concluded that the ferret is weakly inducible. The ferret differs from the rat in that the polychlorinated biphenyl (PCB) induction results in large increases in cytochrome P-450 with only modest increases in benzo(a)pyrene hydroxylase.

Lake et al. (1977) reported that a plithalate ester ([di-(2-ethylhexyl)phthalate]) caused increases in liver weight in the ferret, but no increases in microsomal protein. The concentration of cytochrome P-450 and MMFO activity actually decreased. Here is an example of how an increase in liver weight does not necessarily mean microsomal induction. It has since been established that phthalate esters induced peroxisome proliferation (Reddy and Lalwani 1983). Lake et al. (1977) did not examine the more common peroxisomal marker enzymes. Hence, the suitability of the ferret as a model for studying peroxisomal proliferation, or in examining toxicity in the absence thereof, remains to be established.

Very few, if any, papers have been published on the activities of epoxide hydrolase or glutathione S-transferase in the ferret. Traditionally, these enzymes are considered protective in that they react with and deactivate reactive potentially toxic metabolites. Early data discussed by Williams (1972) would suggest that the ferret is capable of forming mercapturic acids in a fashion similar to rodents, at least with chlorobenzene. Preliminary data from our laboratory would suggest that ferret glutathione 5-transferase activity with p-nitrobenzyl chloride and chlorodinitrobenzene (table 7.25) are less than those seen in rats. An interesting paper by Frederick and Babish (1984) might provide some clues as to the activities of these enzymes relative to those of the rat. These investigators compared the mutagenic activity of uninduced rat and ferret S-9 liver fractions in the Ames assay. Ferret liver fraction had more mutagenic activity (with 2-acetylamidofluorene, cyclophosphamide, and 7,12-dimethylbenzanthracene) than that of the rat. When calculated on a cytochrome P-450 basis, ferret S-9 has 5 to 10 times the activity. The higher mutagenic activity in ferrets could be due to lower detoxification activity, but this remains to be established.

Although glutathione S-transferase reactions have not been extensively studied in the ferret, other conjugative processes have been examined. These include glucuronide, sulfate, glycine, and taurine conjugate formation. In general, as a carnivore, the ferret is more likely to form amino acid conjugates (glycine and taurine), whereas rodents are more likely to form glucuronide and sulfate conjugates. This area was extensively reviewed by Hiram et al. (1977). With regard to the ferret, there has been limited activity in this field since. In general, these investigations were of similar design in that model chemicals, usually an aromatic acetic acid, were administered to a variety of species and the different conjugates identified. For example, Emudianaughe et al. (1978) examined the metabolism of 2-naphthyl-acetic acid. In the ferret, the main urinary metabolite was the taurine conjugate, whereas this was undetectable in the rat where the glucuronide was the main metabolite. There was also a large species difference in the percentage of dose excreted in the urine, 3% in the rat versus 30% in the ferret. This is not surprising as glucuronides in the rat tend to be actively excreted in the bile. Idle et al. (1978) compared the metabolism of 10 aromatic acids in the rat and ferret. The ferret generated substantial (greater than 5%) amounts of taurine metabolites with seven of the test chemicals, whereas the rat did so only with one (2-naphtylacetic acid). Qualitatively, both species had essentially equivalent ability to elaborate glycine conjugates, although the rat tended to have higher activity for any specific chemical. In contrast, the rat

produced little glucuronide with benzoic acid, whereas this was the major pathway for benzoic acid metabolism in the ferret. In their studies on phenoxybenzoic acid, Huckle et al. (1981) reported that the glycine and taurine conjugates were the major metabolites in ferrets, whereas the rat produced no detectable taurine conjugate and the sulfate was the major metabolite. These investigators have further shown that the majority of the glycine conjugative ability lies in the kidney, not the liver (Huckle et al. 1981). Therefore, as a general rule of thumb in working with ferrets, one should expect that amino acid conjugates will be the main metabolites of aromatic acids, and that these will be primarily excreted in the urine.

The conclusion of the previous paragraph not withstanding, the ferret does have the ability to form glucuronides with aromatic alcohols (phenol, naphthols, etc.). Conjugation with this class of substrates simply has not been well studied. Ioannides et al. (1977) examined glucuronide formation using 4-methyl-umbelliferone, and detected relatively low activity. Lake et al. (1979) detected much higher activities with 1-naphthol as the substrate. For both these substrates, the reported activity is less than that reported for rats. As in other species, glucuronosyl transferase is largely a hepatic enzyme, and is inducible by treatment with Aroclor 1254. Hence, glucuronosyl transferase activity in the ferret has been established, but in-depth study remains to be completed.

Although hepatic metabolism normally accounts for the majority of xenobiotic metabolism, extrahepatic pathways can sometimes be substantially involved in certain pathways. For example, the importance of the kidney in glycine conjugation has been mentioned. Extrahepatic pathways can sometimes be important with regard to toxicological mechanisms. Extrahepatic metabolism has been only marginally explored in the ferret. Lake et al. (1979) reported measurable amounts of benzo(a)pyrene hydroxylase, 7-ethoxycoumarin O-deethylase, and 1-naphthol glucuronyl transferase activities in the intestinal mucosa, kidney, lung, and testes of ferrets. As with the rat, these activities were present at much lower (two to three orders of magnitude) concentrations than in the liver. Extrahepatic activities of benzo(a)pyrene hydroxylase and 7-ethoxycoumarin O-deethylase tended to be similar between the rat and ferret, but rats had tenfold higher benzo(a)pyrene hydroxylase in the kidney. Rats consistently had higher 1-naphthol glucuronyl transferase activities. Extrahepatic benzo(a)pyrene hydroxylase, 7-ethoxycoumarin O-deethylase activities were induced by Aroclor 1254. Hence, extrahepatic metabolism in the ferret is not dissimilar to that in the rat, and the potential involvement of these enzymes in metabolism and disposition and toxicity should be kept in mind.

The disposition and metabolism of a specific chemical in the ferret has been examined in a few papers. Bleavins et al. (1982) compared the disposition of hexachlorobenzene in pregnant and nonpregnant ferrets. Clearance was greatly accelerated in the pregnant and nursing dams. This was attributed to a high degree of placental and mammary transfer of the chemical to pups. This could suggest that the ferret would be a good nonrodent model for multigenerational studies.

Gorrod and Damani (1980) examined the *in vivo* N-oxidation of 3-substituted pyridines in various animal species. Interestingly, the ferret more closely resembled the other species examined, with regard to N-oxide formation, than the rat. The ferret excreted approximately 34% of the dose as the N-oxide, whereas this figure was only 10% in the rat. Treatment with 3-methylcholanthrene radically changed this relationship, decreasing the amount of N-oxide to 0.43% in the ferret but increasing it to 4.2% in the rat. This is an example of not only species differences in metabolism, but species differences in response to hepatic MMFO induction.

Ioannides et al. (1982) examined glyceryl trinitrate (GTN) metabolism in the ferret as part of a broader examination of the effects of species, sex, age, and route of administration on the elimination of this chemical. They observed that there was an excellent correlation between body weight and plasma half-life of GTN (the higher the weight, the higher the half-life). The results from the ferret were in line with this conclusion. Ferrets, however, had lower than expected volumes of distribution, which the authors attributed to the smaller proportion of body fat in the ferret in comparison to other species.

Ideally, one would like to know or be able to compare xenobiotic metabolism in the model species to that in humans. Predictive comparisons between humans and the ferret, however, are particularly difficult because xenobiotic metabolism in the ferret is not as well characterized as that for other species. Very few papers have been published comparing the disposition of specific chemicals in both species. In general, there are few hard rules in anticipating species similarities in metabolism. For example, Williams (1972), in a classic work, noted that the human more closely resembled the rat than the ferret in the metabolism of phenol and benzoic acid. In contrast, the rabbit resembles the ferret in the metabolism of phenol, but the human in the metabolism of benzoic acid. Perhaps, as the MMFO of the ferret becomes better characterized, out ability to predict similarities in xenobiotic metabolism between humans and ferrets will also improve.

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CHAPTER 8

The Dog

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TOXICOLOGY

The canine model in biomedical research has served a dual role by helping to provide information on biomedical problems in humans as well as fundamental knowledge of benefit to dogs themselves. Advantages of using the dog in the laboratory were recognized by researchers as early as the 17th century. The dog's internal system, organs, and muscles are similiar to those of man, a fact that has stimulated the development of canine models in numerous areas such as circulation and cardiovascular research. The relatively large size and longevity of the dog and the high incidence of malignancies inherent in the species makes the dog advantageous for sequential studies in

individual animals related to the etiology, pathogenesis, and therapy of malignancies (Shifrine and Wilson 1980). Also, the extensive database available on canine immunohematological parameters has made the dog an extremely valuable animal model for organ transplantation studies, especially in the areas of kidney preservation and the evaluation of immunosuppressant drugs (Shifrine and Wilson 1980).

History

The history of the domestic dog (*Canis familiaris*) is obscure. Although the genus Canis also includes wolves, jackals, dingos, coyotes, and more distant relatives such as various foxes (Alopex, Vulpes, and Urocyon) and the Cape Hunting dog (Lycaon), there is no wild species from which the dog is definitely known to derive. The wolf and the jackal have been considered as likely ancestors, although an alternative suggestion has been made that the dog as a species might have arisen as a result of a hybridization between some doglike ancestor and the wolf (Fox 1965a).

The dog is thought to be the oldest of domesticated animals, a fact that has contributed to the difficulty in attempting to identify its wild ancestors. Skeletal remains place its domestication as far back as 8000 B.C. (Andersen 1970). It is likely that for many centuries the early dog remained of one general type that was similar to the modern-day Australian dingo (Andersen 1970). The ability to selectively breed dogs through several generations for specific traits is believed to have been a relatively early human discovery, and was probably triggered by the recognized benefit of using the dog for hunting and scavenging activities. Examples of early breeding efforts include a Pekingese type that has existed in China for about 4,000 years, a mastiff breed depicted in Assyrian sculpture about 600 B.C., a greyhound type in Egypt of similar antiquity, and the powerful hunting dogs of early Britain and Gaul.

The division into a multiplicity of breeds is a more recent development of the last 300 years. Great Britain has historically been the country most active in the selective breeding of a great variety of dogs; by the 19th century, a full complement of breeds was developed for a variety of tasks, including sporting (setter, pointer, spaniel, and the smaller bull and fox terriers), coursing (greyhound), shepherding (Old English and Scottish collie), sight and smell hunting (including wolfhound, foxhound, and beagle), guarding (mastiff), and for use as pets (King Charles spaniel, pug, and Pomeranian).

In scientific research, the use of experimental animals that have an unknown history and are of questionable health is a major concern. From a clinicopathological standpoint, heterogeneous populations of mongrel dogs do not appear to be more variable than homogeneous populations; however, laboratory bred animals generally show less clinical, gross, and microscopic evidence of disease (Pick and Eubanks 1965). Because of such concerns, researchers have moved in the direction of using laboratory-bred dogs.

Of the many breeds available, only a limited number possess the qualities (e.g., moderate size, even temperament, and ease of handling) desired in an experimental dog. Specific breeds that have been used in biomedical research include the greyhound for its well-defined muscles and nerves and large chest cavity, the dalmatian for its humanlike excretory functions, and the German shepherd for its good bone and joint configuration.

In the general area of toxicological research, the beagle is probably the most frequently used species of dog, although the Pembrokshire corgi has been advocated as a satisfactory alternative (Noel 1970). Because of the predominant use of the beagle, much of the following discussion specifically refers to that species.

Normal Parameters

In toxicological research, determining whether a statistically significant change is biologically relevant can often be difficult. Establishing the existence of an adverse effect is in great part

Table 8.1 Feed Consumption in Beagles
Mean Feed Consumption (95% CL) (kg dry diet/dog/week)

Approximate Age Range (Months)	Males[a]	Females[a]
4.5–5.0	2.27 (1.6—2.8)	2.05 (1.4–2.7)
5.5–6.0	2.62 (2.1–2.8)	2.39 (1.8–2.8)
6.5–7.0	2.68 (2.3–2.8)	2.45 (1.9–2.8)
7.5–8.0	2.73 (2.4–2.8)	2.55 (2.0–2.8)
9.5–10.0	2.76 (2.7–2.8)	2.60 (2.2–2.8)
10.5–16.0	2.77 (2.6–2.8)	2.59 (2.1–2.8)

[a] $N = 130$.

Source: From Noel (1970); used with permission.

accomplished by making comparisons between treatment and control groups. A great deal of reliance is placed on control data that, in the case of most nonrodent toxicology studies, are often collected from a relatively limited number of animals (usually four to six for a 2- or 4-week study). Because of this it is extremely important to have a well-established database of normal physiological parameters with a good estimate of the expected magnitude of variability.

Growth Curves and Feed Consumption

Table 8.1 and figure 8.1, respectively, show beagle feed consumption and weight data over a 1-year period starting at approximately 5 months of age. The biggest relative increase in feed consumption is seen between 4.5 and 6 months of age (table 8.1). A slower rate of increase is observed over the following 3 to 4, months, and by about 10 months of age, feed consumption levels off and remains stationary thereafter This pattern coincides fairly well with the rapid fall in the rate of body weight gain, which begins between 6.5 and 7. 0 months of age (figure 8.1). By about 12 months of age, animals have almost attained full adult weight. In the beagle, there appears to be an increase in the variability in body weight with increasing age. The average male gains more weight with age than the average female; by puberty, males are about 2 kg heavier than females.

Reproductive and Physiological Parameters

Normal physiological and reproductive parameters are shown in table 8.2 and table 8.3, respectively. The three phases of the estrus cycle are proestrus, a preparatory phase that leads to estrus; metestrus, which is dominated by the influence of the corpus luteum; and anestrus, a period of sexual quiesence (Christie and Bell 1971). Whereas most mammals can be classified as seasonal or continuous breeders, the dog fits neither category. The observations of cyclical ovarian events and accompanying behavioral and morphological changes in genitalia at one heat period that continue until the next suggest that the bitch has an extended estrus cycle (Engle 1946; Jochle and Andersen 1977).

In the male beagle dog, the reproductive organs appear to reach a peak of development at about 32 weeks of age (Tsutsui et al. 1986). This sexual maturation is preceded by the rapid development of the anterior pituitary gland, which in turn stimulates testicular development by gonadotropin release.

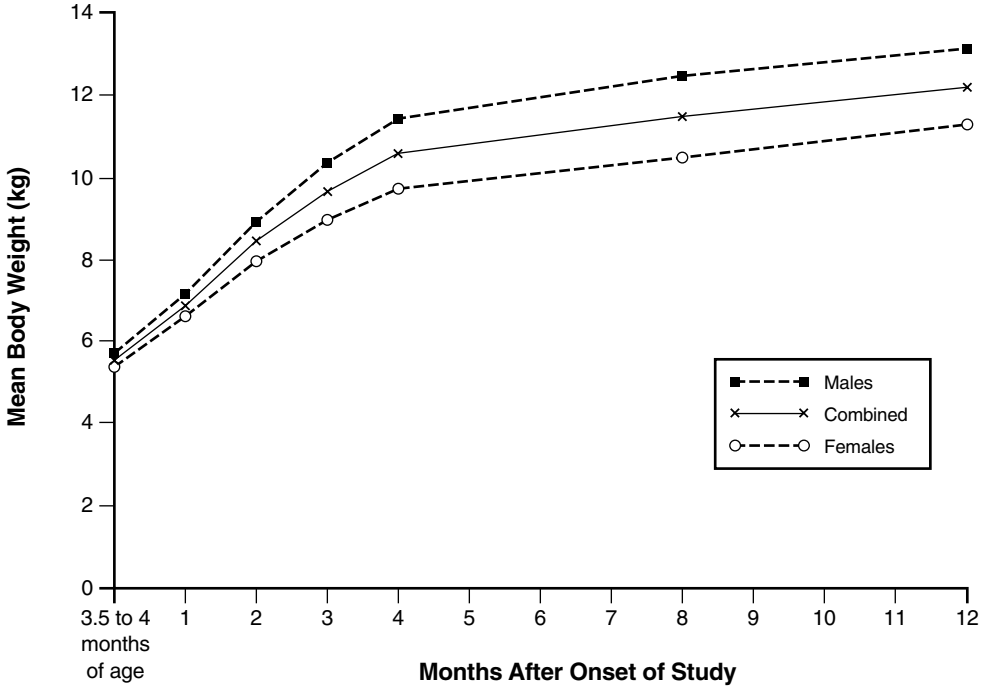

Figure 8.1 Growth weight curves in the beagle (adapted from Noel 1970).

Table 8.2 Normal Physiological Parameters

Average life span	12.5 years [a]
Daily food consumption	25–40 g/kg body weight
Daily water consumption	Ad libitum; approximately 600 ml
Rectal temperature	37.8°C ± 0.13 (*SD*)[c]
Respiration rate	20/min (10–30)[b]
Oxygen consumption	0.36 ml 0_2/g/hr[b]
Tidal volume	24 ml/kg (18–35)[b]
Minute volume	4.5 L ± 0.2[c]
Functional residual capacity	367 ml (248–540)[b]
Lung compliance	117 ml ±5.6 (5 CM H_20)
PO_2	73.7 mmHg (61–87)[b]
PCO_2	36 mmHg (29–46)[b]
pH	7.44 (7.37–7.51)[b]
Basal metabolism	2 cal/kg/hr [d]

[a] In dogs raised under "protected" environmental conditions (Andersen and Rosenblatt 1974).

[b] Measured in beagles weighing from 6.8 to 11.5 kg (Pickrell et al. 1971).

[c] Measured in beagles weighing 13 kg (Andersen 1970).

[d] Michaud and Elvehjem (1944).

Table 8.3 Reproductive Parameters

Puberty	6–12 months of age
Breeding age	
Males	10–12 months
Females	9–12 months
Gestation	60–65 days
Estrus cycle:[a]	
Proestrus	5–15 days
Estrus	5–15 days
Metestrus	60–65 days
Anestrus	Length variable
Weaning age	5–8 weeks
Litter size	1–11 pups; 5–6 average
Breeding age (males)	10–12 months
Peak testis development	32 weeks[a]
Peak testis weight	8.6 g[a]
Peak epididymis and deferens gland, penis, and prostate development	32 weeks

[a] Tsutsui et al. (1986).

Husbandry

Proper handling and care of the experimental dog is essential for its general physical and psychological well-being. Although dogs have many of the same general requirements as other experimental animals, special consideration must be given to factors such as space adequacy and degree of socialization.

Housing

Housing is one of the most important features in the physical environment of the laboratory animal and usually consists of a microenviromnent, or primary enclosure (the cage or pen), and the macroenvironment, or secondary enclosure (the room containing the cage or pen). The housing system employed should allow adequate space for freedom of movement and postural adjustment and provide a comfortable resting area for the animal. It should also be escape-proof, provide adequate ventilation and access to feed and water, keep the animal dry and clean, and meet biological needs. Ideal housing can be turned into metabolism cages by the addition of trays and movable partitions, thus causing minimal disturbance during feces and urine collection. The most common types of housing for experimental dogs are cages and pens (or runs). The current space recommendations of the National Research Council (NRC) for laboratory dogs, as published in the 1996 revision of the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health [NIH] 1996), are shown in table 8.4.

Table 8.4 Minimum Space Requirements for Laboratory Dogs [a]

Weight	Type of Housing	Floor Area/Animal (m^2)	Height (cm)[b]
< 15 kg	Pen/run	0.72	—
15–30 kg	Pen/run	1.08	—
< 15 kg	Cage	0.72	81.28
15–30 kg	Cage	1.08	91.44

[a] These recommendations might require modification according to the body conformation of individual animals and breeds.

[b] From the resting floor to the cage top.

Source: Adapted from NIH (1996).

The Animal Welfare Act states that primary enclosures for dogs must meet a set of minimum requirements (see Code of Federal Regulations, Section 96FR3.6) including a minimum amount of required floor space, [calculated as (the length of the dog in inches + 6 inches) × (the length of the dog in inches + 6 inches) divided by 144 and again divided by 144].

Indoor Pens

Indoor pens are excellent for the maintenance of dogs over an extended period of time under controlled conditions. Such kennels are usually constructed with concrete flooring and a drain gutter outside the runs. Typical dimensions for pens that can house up to four dogs are 3' 8" wide, 8' high, and 10' long. Care needs to be taken to ensure that the surface is not so rough that it abrades the dog's footpads, nor so smooth that the floor becomes slippery when wet (due to water or urine) and causes injury to the animals or handlers.

Caging

When purchasing caging, special attention must be given to provisions for cage ventilation, drainage, and durability. The most commonly used and recommended materials for dog cages are fiberglass or stainless steel, both of which are durable and easy to clean. Most cages used in research facilities are equipped with automatic watering systems and modified doors with feed bowl holders as well as mobile bases to allow easy movement of the cage racks in and out of animal rooms.

In general, the current standard-size cages (generally 35" × 30.5" × 33.75" or 35" × 40.5" × 33.75" for larger dogs) used in most research facilities should only be used to maintain dogs for limited periods of time. An alternate to the use of pens or runs are modular and expandable stainless steel dog kennels, which allow the investigator to increase the cage floor space as needed.

Exercise

Although the dog can adapt to a wide variety of environments the question has repeatedly arisen as to whether the degree of physical activity possible in a standard laboratory cage is sufficient for maintenance of normal physiological activity.

The Animal Welfare Act mandates that research facilities must develop, document, and follow an appropriate plan to provide dogs with the opportunity to exercise (Code of Federal Regulations, Section 9CFR3.8). Additionally, the plan must be approved by the attending veterinarian and must include written standard procedures to be followed in providing the opportunity for exercise. The difficulty arises, however, in establishing a program that will be of most benefit to the dogs.

There are several reports in the literature that indicate there are no differences in clinical laboratory determinations, growth weight, and immunological, electrocardiographic, and ophthalmic parameters for dogs housed continuously in standard-sized cages, large cages, or those released for exercise (Campbell et al. 1988; Hite et al. 1977; Namand et al. 1975). Newton (1972) has also demonstrated that, in terms of the musculoskeletal system, the physiological well-being of cage-confined dogs (relative to dogs housed in pens or those cage-confined with access to a pen) is unaffected.

The Animal Welfare Act notes that dogs housed individually in cages, pens, or runs that provide twice the minimum floor space or group-housed in cages or runs that provide at least 100% of the required space for each dog if housed separately, should provide adequate opportunity for exercise (Code of Federal Regulations, Section 9CFR3.8). Analysis of activity patterns of dogs confined in cages of different sizes, however, indicates that cage size has no effect on the amount of time spent sitting, standing, or lying down (Campbell et al. 1988). Regardless of the size of the cage, dogs do

not exercise unless humans are in the room. The most beneficial effects are seen when dogs are released and exercised as a group rather than alone. Dogs released alone tend to spend most of their time walking or investigating the area rather than running and jumping.

Socialization

Whatever the kennel design or exercise program used by a research facility, the needs of the confined laboratory dog for a certain level of socialization should be recognized. Both dog–dog and human–dog contact are important. Generally, laboratory dogs are more content if they can share a cage with a companion. One approach adopted by some facilities has been to allow two dogs of the same sex and treatment group daily access to each other's cage from early evening to early morning. This approach, however, is not always logistically possible or scientifically feasible. In situations in which experimental design dictates that animals be housed separately, normal behavior can be maintained by permitting visual, auditory, and olfactory access to roommates by appropriate spatial cage arrangement. Early handling of young animals to familiarize them with the laboratory environment is extremely important, as lack of early socialization can result in abnormal behavior later in life (Fox 1965b). One of the most effective measures to prevent such problems in dogs is frequent human contact, which should continue throughout the animals' adult life. The Animal Welfare Act notes that dogs housed individually in cages or maintained at a facility without sensory contact with another dog must be provided with positive physical contact with humans on a daily basis (Code of Federal Regulations, Section 96FR3.7).

Sanitation

Good sanitation is essential in an animal facility. The macroenvironment as well as the microenvironment need to kept free of dirt, debris, and contamination. Bacterial, viral, and parasitic problems can be magnified by an improperly maintained facility. Special attention should be given to cracks, rough areas, and depressions that can harbor moisture and provide shelter for parasite ova and other infectious agents (Bebiak et al. 1987).

Animal rooms, corridors, and storage areas should be routinely cleaned and sanitized. Pens and caging should be mopped or hosed out daily to prevent accumulation of fecal material and general debris. Cages and pens should be scrubbed down with a sanitizing solution about every 2 weeks; cages are rotated and thoroughly sanitized (e.g., soaking the cage in a 5% acid solution for about 20 min and then passing them through a 170°F cage wash). Watering systems also need to be cleaned regularly, either through flushing with water or cleansing with an antibacterial agent.

Another area of special attention is the storage of feed. Although most laboratory chow is of good quality and free of contamination when it leaves the manufacturer, biological and chemical spoilage can result during transit or by improper use. A dry, cool environment is recommended for feed storage to prevent mold formation and insect infestation. Feed should be stored off the floor either in unopened bags or in waterproof, cleanable containers after opening.

Temperature, Relative Humidity, and Ventilation

Special attention must be given to regulation of the macroenvironment as well as the microenvironment in which the laboratory animal resides. Environmental stress in humans and animals has been shown to affect physiological status, which in turn can alter susceptibility to infections and toxic chemicals (Baetjier 1968; NIH 1985).

Recommended dry-bulb temperatures and relative humidity ranges for dogs are 64.4°F to 84.2°F (16°C–27°C) and 30% to 70%, respectively (NIH 1985). Fluctuations in temperature and humidity can affect factors such as behavior and metabolic rate in dogs (Lusk 1931). Increases in temperature and high humidity are a particular concern because of the dog's limited capacity to dissipate heat

(Norris et al. 1968). Sweat glands in the dog are confined to the footpads, and the animal primarily responds to high temperatures by panting and, to a lesser extent, by radiation and conduction. It has been shown that a stress zone for dogs, defined as a 1.1° increase in rectal temperature for all subjects tested, can be created at dry bulb temperatures greater than 32.6°C (Besch et al. 1984). Exposure to higher temperatures could lead to decreases in feed consumption in normal animals, and it is possible that exposing animals to toxic agents in the presence of elevated temperatures could result in an increase in the incidence or severity of toxicity. Norris et al. (1968) have noted that the dog probably cannot survive for extended periods of time in environments appreciably in excess of 40°C and 40% relative humidity.

Temperature and humidity are also considered to be coincident factors in many diseases caused by chemicals and infectious agents in contact with the skin and can alter cutaneous absorption of compounds, local reaction of the skin to irritants, and reaction to sensitizing agents.

Ventilation control is necessary to minimize variations due to climatic conditions, provide adequate oxygen, remove thermal loads, and dilute gaseous and particulate contaminants. The necessary volume of air change per room will depend on its maximum holding capacity (i.e., number of animals in the room and room size). The number of air changes per hour is normally between 10 and 20 (Munkeldt 1948; Runkle 1964). It has been suggested that recirculated room air can be utilized as long as all airborne contaminants have been removed (NIH 1985); however, this concept has not proven to be successful, in part because of improper or insufficient maintenance of recirculation systems (Gorton 1978). Special care should also be paid to the positioning of the room intake and outlet grills so that air is evenly distributed around the room and to each cage irrespective of its position.

Diet

Although the dog is classed as a carnivore, it readily adapts to an omnivorous diet. The digestive tract of the dog is relatively short (compared to other mammalian species), which facilitates the rapid passage of food. Dogs also lack intestinal diverticula, or bacterial harboring sacs that prevent foods that require microbial breakdown (prior to absorption) from being used effectively. Holding times of food in the beagle stomach, small intestine, and large intestine have been reported to be about 3 to 5 hr, 1 hr, and 10 hr or more, respectively, with a total passage time throughout the GI tract of 24 hr (Andersen 1970).

Dogs require daily dietary sources of energy, amino acids, glucose precursors, fatty acids, minerals, vitamins, and water. A summary of nutrient requirements for dogs (based on the recommendations of the National Research Council 1985) is presented in table 8.5. Dogs do not appear to have a specific requirement for carbohydrates; however, in a well-balanced diet, they can utilize large amounts of carbohydrates (up to 70%, dry basis; Andersen 1970).

Adult laboratory beagles maintained in a laboratory environment function well with one feeding of standard laboratory chow per day. Dogs tend to bolt their food, and animals on a once-a-day feeding regimen will often eat all their food within a 30- to 45-min period. However, to ensure that the slow eaters have sufficient opportunity to finish most of their food, most laboratories allocate a longer period (2–4 hr) for access to feed.

Although commercially available dog chows provide sufficient amounts of all necessary nutrients, some test compounds might induce deficiencies through loss of appetite, malabsorption, or vomiting that can complicate interpretation of toxic effects. Protein deficiency in the dog results in depressed food intake, growth retardation, hypoproteinemia (associated with edema), and a rough, dull hair coat. Deficiencies in any of the essential amino acids can result in similar clinical signs. Diets low in fat can cause dry, coarse hair and flaky dermatitis (Michaud and Elvehjem 1944). Inadequate intake or loss of essential minerals (e.g., calcium, phosphorus, potassium, and sodium chloride) can result in problems such as spontaneous fractures, bone loss, osteoporosis, and osteomalacia (calcium and phosphorus); restlessness, muscular paralysis, and lesions of the heart and

Table 8.5 Nutrient Requirements for Dogs

Nutrient	Unit	Growth Requirements	Adult Maintenance Requirements
Fat	g	2.7	1
Linoleic acid	mg	540	200
Protein			
Arginine	mg	274	21
Histidine	mg	98	22
Isoleucine	mg	196	48
Leucine	mg	318	84
Lysine	mg	280	50
Methionine cystine	mg	212	30
Phenylalanine-tyrosine	mg	390	86
Threonine	mg	254	44
Tryptophan	mg	82	13
Valine	mg	210	60
Dispensable amino acids	mg	3,414	1,266
Carbohydrate minerals			
Calcium	mg	320	119
Phosphorus	mg	240	89
Potassium	mg	240	89
Sodium	mg	30	11
Chloride	mg	46	17
Magnesium	mg	22	8.2
Iron	mg	1.74	0.65
Copper	mg	0.16	0.06
Manganese	mg	0.28	0.10
Zinc	mg	1.94	0.72
Iodine	m	0.032	0.012
Selenium	m	6	2.2
Vitamins			
A	IU	202	75
D	IU	22	8
E	IU	1.2	0.5
K			
Thiamin	mg	54	20
Riboflavin	mg	100	50
Pantothenic acid	m	400	200
Niacin	mg	450	225
Pyridoxine	mg	60	22
Folic acid	mg	8	4
Biotin[a]	mg	—	—
B_{12}	mg	1	0.5
Choline	mg	50	25

[a] Dogs have a metabolic requirement, but a dietary requirement was demonstrated when foods from natural ingredients were fed.

Source: Adapted from National Research Council (1985).

kidney (potassium); and fatigue exhaustion, inability to maintain water balance, dryness of the skin, and loss of hair (sodium chloride). Vitamin deficiencies, exacerbated by test compound effects, can also induce significant pathology.

Water

The body of the adult dog contains about 60% water and has a limited capacity to store water (Gaebler and Choitz 1964). The dog can normally cope with a large fluid intake owing to a readily adjustable urine volume, but the unsalvagable water losses of the body dictate the minimum water

intake. Average daily water intake in adult dogs has been determined to be approximately 600 ml (Richter 1938), and the amount consumed daily has been shown to closely approximate total daily water loss (Gaebler and Choitz 1964). The individual dog's requirements for water appear to be self-regulated and depend on factors such as the type of feed consumed, ambient temperature, amount of exercise, physiological state, and temperament. For these reasons, in most cases dogs should be permitted free access to water at all times.

Drinking devices for dogs include pans, water bottles, and, most commonly, automatic watering devices. The water source is usually either tap water or filtered or sterilized water. As with other species, if an automatic watering system is used, the water lines and valves need to be routinely checked to ensure they are working properly and the animal has an uninterrupted supply of water. Whatever the water delivery system used, dogs need to be observed on a daily basis for signs of dehydration so that prompt steps can be taken toward rehydration. Dehydration can usually be detected by checking the elasticity of the skin at the back of the neck. A common rehydration procedure is to subcutaneously administer lactated Ringer's solution (200–400 ml, depending on the size of the animal).

Disease

The four diseases of most concern in maintaining a dog colony are canine distemper, infectious canine hepatitis, leptospirosis, and rabies. Although various immunization programs can be undertaken, the most common approach is to administer a single multigenic vaccine.

Some of the most common infectious and parasitic diseases observed in dogs are listed in table 8.6. The most common ectoparasites include fleas, lice, ticks, and mites. Otodectic mites especially can be a problem if neglected; however, treatment can easily be managed by the use of an oil containing a nonirritating insecticide. The most serious mange mite is *Demodex follicularum,* which in some colonies has been detected as a facultative pathogen in about 80% of the animals (Greve and Gaatar 1964).

Table 8.6 Some Common Parasitic and Infectious Diseases of the Dog

Disease	Pathogenic Agent	Clinical Picture of Infection	Common Source
Coccidiosis	Isospora	Primarily a disease of young animals. Diarrhea and dehydration	Ingestion of sporulated oocysts
Campylobacter Enteritis	Camphylobacter fetus subsp. jejuni	Watery diarrhea (puppies) and intestinalis	Direct contact with infected animals, ingestion of contaminated food, placental transfer, exposure at delivery
Giarrdia	Giarrdia trophozoites	Disease most important in young dogs. Intermittent or chronic diarrhea which may persist for several months. Signs associated with malabsorption of nutrients (reduced growth rate, weight loss, dull coat, etc.)	Direct contact with feces
Brucellosis	Brucella canis	Females: Abortion in third trimester; generalized lymphadenitis and persistent bacteremia Males: Orchitis, epididymitis, and prostatis	Contact with the urine of infected animals
Leptospirosis	Leptospira interrogans	Often a sudden onset with anorexia, vomiting, fever, and conjunctivitis followed in a few days by hypothermia, depression, dypsnea, muscle soreness, and oral mucosa hemorrhagic areas. Death (10% of cases) usually related to nephritis.	Direct contact with urine of infected animals

Table 8.6 Some Common Parasitic and Infectious Diseases of the Dog (Continued)

Disease	Pathogenic Agent	Clinical Picture of Infection	Common Source
Listeriosis	Listeria monocytogenes	Abortion, perinatal infections with CNS signs.	Unknown
Tuberculosis	Mycobacterium bovis M. tuberculosis	Coughs of long duration; lung and pleural lesions almost exclusively exudative in nature.	Exposure to bovine or human tuberculosis
Dermatomycosis (ringworm)	Microsporum canis M. distortum (most common agents)	Frequently no clinical signs. If lesions occur, commonly seen as circular alopecic areas surrounded by vesicles, pastules, erythema, or scaling.	Direct contact with the lesions of affected animals
Demodicosis (de modectic: mange, red mange)	Demodex mites	Most common in dogs 3 to 5 months old. Pruritic, alopecia, red lesions around eyes or muzzle. Immune deficiency. Severe infestation can become systemic and prove fatal.	Direct contact with infected animals
Heartworm (canine filariasis)	Dirofilaria immitis	Early and moderate heartworm disease: Early tiring on exercise. Advanced disease: Lungs exhibit large emboli, thrombi, and pneumonia, and right heart is enlarged and worm filled. Signs consistent with right heart failure.	Exposure to infected mosquitoes.
Cutaneous larval migrans (creeping eruption)	Ancylostoma braziliense A. caninum A. duodenale Necator americanus	Normally the parasite is hookworms in dogs. Generally young dogs show clinical disease. Pale gums, weight loss, failure to grow properly. Tarrry feces in heavy infections.	Infection through direct contact with feces of an infected animal or contaminated sand or soil
Dipylidium caninum	Dipylidium caninum (common tapeworm of dogs)	Generally asymptomatic. In clinically recognized cases, diarrhea and pruritus around anal area.	Ingestion of infected fleas.
Leishmaniases	Leishmania donovani L. tropica L. brasiliensis	May be harbored in bone marrow, liver, or spleen, resulting in macrophages containing the parasite. In more advanced cases, dermal lesions, emaciation, alopecia, keratitis, and seborrhea. Fatality is common.	Animal bitten by infected sandflies
Salmonellosis	Salmonella sp. (many serotypes)	Acute gastroenteritis with diarrhea, vomiting, and resultant dehydration.	Ingestion of contaminated foods or water
North American blastomycosis	Blastomyces dermatitidis	A chronic disease in dogs. Depression, fever, anorexia, leading to chronic weight loss, and nonproductive dry coughs. Nodules and abscesses through the lung. Dissemination by hematogenous route leads to destruction of peripheral lymph nodes, bones, and meninges.	Unknown
Rabies	Rabies virus	Two clinical forms in dog: (1) Paralytic form characterized by paralysis of the muscles of the throat and masseter region. (2) Furious form in animals will attack humans, moving or inert objects, or other dogs. Death usually follows within 5 days of these signs.	Contamination of bite wounds by saliva of infected animal in the terminal stages of the disease

Source: Adapted from Bekaert (1982).

Of the endoparasites found in dogs, whipworm is of most concern because of its location in the cecum and colon, which makes treatment difficult.

Coccidiosis (*Isospora spp*.) is a common cause of diarrhea and even death in dogs. Most species of coccidia are self-limiting and nonpathogenic; diffuse hemmorhage of the intestinal mucosa, however, can be seen in dogs infected with *Isospora bigemina* (Andersen 1970). In dogs, as in many other species, adults are generally more resistant to this disease than young animals.

Giardia species are flagellate protozoa that inhibit the small intestine of a wide range of vertebrates. In dogs (usually young animals), the main sign is intermittent or chronic diarrhea. The disease can often be difficult to detect because animals might harbor the organism and not shed it in the feces until triggered by a factor such as stress, diet or environmental change, or treatment with a test article.

Heartworm (*Dirofilaria immitis*) can be hazardous in dog colonies because of the toxic side effects associated with administering filaricides. This disease is endemic to the eastern and southern states, and dogs entering a facility should be tested for the presence of microfilaria in the blood. It has been suggested that microfilaria counts above 25/ml of blood warrant euthanasia (Andersen 1970).

Finally, it should be noted that susceptibility to disease can be enhanced in dogs by factors such as poor sanitation, improper environmental control, number of animals in the primary enclosure and the animal room, or inadequate or poor-quality diet and insufficient water intake. Personnel who handle dogs must also be trained in proper handling techniques to avoid cage-to-cage or dog-to-handler transmissions of infections.

Quarantine and veterinary care procedures conducted on arrival of dogs at a research facility should include the performance of a gross physical examination to look for signs of disease or physical defects, measurement of body weight and rectal temperature, and blood collection for hematological (including microfilaria testing) and clinical chemistry analysis. Whereas colony-bred animals should be fully vaccinated by the breeder, random-source animals will likely need to be vaccinated at the receiving facility. If animals are intended for long-term studies (of more than 1 year in duration), they will need to be vaccinated annually for rabies. Most facilities will also prophylactically treat dogs for ear mites. Depending on animal health status, newly acquired dogs are generally maintained in quarantine for at least 2 to 3 weeks for colony-bred animals and 3 to 4 weeks for random-source animals. Random-source animals should be well separated from colony-bred animals. All dogs should be regularly examined during the quarantine period and observed frequently for signs of any potential health problems.

Dosing Techniques

It is essential that personnel who are responsible for the dosing of dogs be confident in the handling and manipulation of the animals The first step is to establish a sense of confidence and security between the dog and its handler. During this conditioning period, the handler should crouch down to reduce his or her height and slowly bring his or her hand toward the muzzle so that the dog can sniff it. Next, the hand should move along the neck and hindquarters, and the response of the dog should be assessed. Most dogs, especially beagles, respond well to such handling.

If possible, it is better during a study to use the same team of people who were involved in the conditioning process. Efforts should be made to distinguish potential "fear biters" prior to selection of dogs for a study. To remove a dog from its cage or pen, the handler should hold the animal by the nape of the neck while keeping the forearms in line with the spine and supporting the animal under the abdomen with the other hand.

Oral

The two oral dosing procedures most commonly used with the dog are gavage and capsule administration. Dietary administration, as commonly used with rodents, is another possible oral

route; however, this is not an optimal route of administration in dogs for several reasons. The increased activity of the dog, especially when humans enter or are present in the animal room, can result in a continual problem with feed spillage. Another concern is that dogs are often finicky eaters and sometimes will consume little or nothing during the course of a day and compensate by eating more the next day, which can result in erratic blood levels of test compound.

Capsule

The general approach in the administration of a gelatin capsule to a dog is to first wet the capsule with water, open the animal's mouth, and then place the capsule as far back on the animal's tongue as possible. The muzzle is held closed, the head lifted, and the throat stroked until the animal swallows the capsule. The person dosing the animals should look for evidence of swallowing; almost all dogs will lick their lips after swallowing a capsule. After animals have been dosed, cages should be checked for spit-out capsules and any undamaged capsules readministered. Many facilities include a 1- to 2-week prestudy conditioning period in which dogs are dosed daily with empty capsules to help them become familiarized with the dosing procedure. It is not uncommon for dogs treated with certain test compounds to exhibit salivation prior to daily capsule dosing; this is probably a conditioned response in which capsule dosing is related to adverse systemic or local effects of the test article.

Gavage

The first step in gavaging a dog is to place a rubber bit between the teeth. An alternate method that can be more comfortable for the dog is to place towels in the animal's mouth. A flexible intubation tube is then inserted into the mouth, back into the esophagus, and down toward the stomach. Correct placement of the tube can be ascertained by inserting the free end of the gavage tube into a beaker of water and checking for the absence of bubbles. Dosing volumes for gavage are generally in the range of 5 to 10 ml/kg, and fluid should be administered slowly to prevent gastric reflux.

Capsule administration is generally the preferred method of oral administration in dog studies, because gavaging is more labor intensive and there is always the chance of gavage error or aspiration. One approach in dealing with compounds that cause extensive vomiting in fasted dogs either by capsule or gavage administration is to allow the dogs access to feed for a few hours prior to dosing. Because the incidence of daily vomiting in control dogs can be as high as 50% (see the later section on species peculiarities), it is often useful to sham dose animals with a water solution for several days before actually starting a gavage study. This will frequently acclimate animals to dosing and reduce emesis during the study.

Subcutaneous

For subcutaneous dosing, the skin over the side of the neck anterior to the scapula is picked up in a triangular fold and a needle (usually 21 gauge) is inserted into the base of the skin fold parallel to the body wall. The plunger of the syringe is then pulled back gently to ensure that the needle has not gone through both sides of the skin fold or that a blood vessel has not been penetrated. In either case, the needle should be repositioned. The volume administered should generally be in the range of 5 to 10 ml/kg. In general, the irritation produced by subcutaneous administration is relatively moderate, and the route can be used for limited repeated dose studies. The site of injection can be regularly moved from left to right and front to back in a predetermined pattern of injection (e.g., either side of the neck, either side of the midback, and on either side of the far back close to the rump).

Intramuscular

For intramuscular administration, the injection area (usually the meaty part of the hindleg) is swabbed with alcohol. The injection is made with a 20- or 21-gauge needle, and the maximum volume used should be in the range of 2 to 5 ml. It is important not to insert the needle too deeply to avoid hitting the sciatic nerve. The plunger of the needle is pulled back to ensure that a blood vessel has not been penetrated; if blood is present, the needle should be repositioned. The site of injection should be rotated during a repeated-dose study. An important consideration is the potential for a test compound to induce intramuscular irritation. Because of this, muscle irritation studies should be conducted prior to initiating a repeated-dose study.

Intraperitoneal

For intraperitoneal injection, the dog is usually held in a lateral position, and a small needle (usually a 23-gauge, 3/4-in. needle) is inserted about 1/4 in. into the lower right quadrant of the belly, taking care to avoid damage to the internal organs. The midline should be avoided, so as to reduce the risk of penetrating the bladder. The needle should be checked for proper placement and repositioned if necessary. The volume given should be in the range of 3 to 5 ml/kg of body weight.

Intravenous

Bolus

The femoral, cephalic, and saphenous veins are commonly used for IV administration in the dog. With the dog restrained by a second person, the hair over the vein is shaved (or clipped), swabbed with an alcohol solution, and the vein dilated (by pressure or heat) proximal to the injection site. Usually a 21- or 22-gauge needle is used, and the maximum volume for an adult dog should be about 3 to 5 ml/kg. For repeated-dose bolus or infusion studies, injections should be kept as low on the vein as possible throughout the study to prevent the formation of scar tissue.

The use of a butterfly catheter, to which the syringe is attached, is recommended to help avoid slippage of the needle from the vein in case of movement by the animal. The needle should be inserted in the direction of the blood flow and checked for proper placement by pulling back gently on the plunger of the syringe. The contents of the syringe are slowly injected (over 2–4 min). As with intramuscular administration, the potential for IV irritation should be determined prior to starting IV toxicity studies.

Infusion

For IV infusions lasting up to 2 or more hours, dogs should be restrained by the use of a device such as a sling (or a stainless steel horse) and a jacket and tether. As with bolus injections, the vein to be infused is dilated by the application of pressure or heat, and an infusion catheter (usually 23 gauge) is inserted into the vein. The needle is withdrawn when blood flows out (thus ensuring proper placement of the catheter), the catheter and tubing are loosely anchored to the leg with tape, and the catheter is connected to the tubing from the prepared syringe. The infusion pump is preset to the appropriate rate divided by total volume, and turned on. During the infusion, the limb being infused should be periodically checked for the presence of swelling or bulge. If this occurs, the infusion should be stopped, the catheter repositioned higher on the vein (or another leg used), and the infusion recommenced. Sufficient slack should be maintained in the catheter so that if an animal moves during the infusion, the catheter will not be pulled out.

For continuous IV infusions, a more effective approach might be the use of a vascular access port system, although Perkin and Stejskal 1994, report successful use of an externalized medical

catheter. A subcutaneously placed rigid multipuncture reservoir with an indwelling catheter is inserted into the jugular vein. The test compound is accessed via catheter tubing encased in a swivel/tether apparatus connected to a jacket harness. The advantage of such a system is that it allows the animal greater freedom of movement during the infusion procedure.

Rectal and Vaginal

Compounds administered rectally to dogs can be delivered in the form of suppositories (emulsion and suspension types), rectal gelatin capsules (solutions and suspensions), or microenemas. The most frequently used dosage form, however, is the suspension suppository (De Boer et al. 1982). Microenemas are generally prepared as either a gelatin and saline solution or a simple physiological saline solution and administered in a volume of 0.5 to 1.0 ml. Suppositories are commonly in the weight range of 0.5 to 1.0 g, should be short and blunted in shape, and are inserted about 4 cm from the outer rectal sphincter. Liversidge et al. (1986) have also described a device for delivery of suppositories that consists of a stainless steel tube (95×2.7 mm) with a plunger to eject the suppository; the use of this device allows the suppository to be maintained in the proper position.

Compounds administered vaginally are usually in the form of suppositories or creams. Creams are usually placed in the vagina by means of an applicator. The vaginal opening is then closed with a plastic alligator clip for a specified period of time. Suppositories and tablets can be inserted vaginally with a plastic tampon-like applicator (Fulper et al. 1987).

Data Collection

Observations and Physical Examinations

Clinical observations and routine physical examinations are integral parts of safety assessment studies. Daily clinical observations in dogs are usually conducted both pretreatment and postreatment and consist of a home-cage observation to record any signs indicative of poor health status or abnormal behavior (e.g., motor incoordination or reduced motor activity, tremors, salivation, abnormal feces, etc.). Before making such observations, it is important to be familiar with the spectrum of clinical signs that can be seen in a normal population of dogs. Some examples of clinical signs that are occasionally observed in untreated dogs are shown in table 8.7.

Physical examinations are conducted less frequently during a study than are clinical observations. The animal is first allowed to move around freely on the floor, and exploratory behavior, motor activity, gait, and general demeanor are evaluated. Next, the animal is moved to a table for examination of the head, including the eyes, ears, nose, mouth, teeth, gums, and tongue (for signs

Table 8.7 Occasional Clinical Observations in Normal Dogs

Vomitus:	Usually clear or brown in color
Soft stool:	Loosely formed stool
Mucoid stool:	Yellowish or dark in color
Diarrhea:	Liquid feces
Nasal discharge:	Usually slight runny nose
Injected sclera:	Slightly bloodshot eyes
	Protruding nictitating membrane (cherry eye)
Hair loss:	Slight to severe focal or generalized hair loss
Cage sores:	Usually between the digits of the paws
Signs associated with females “in heat”	
Vulval bleeding	
Vulval turgidity	
Restless behavior	

of swelling; abnormal nasal, eye, or ear discharges; tension of facial musculature; salivation; swallowing difficulties; etc.). The animal's body is then palpated for signs of masses and nodal swellings, the urogenital and anal region is examined, and rectal temperature is recorded. Pupillary light, corneal, patellar, wheelbarrowing, and hopping reflexes are usually tested, and righting and auditory responses are recorded.

Cardiovascular Parameters

Blood Pressure

Blood pressure can be determined in dogs by either indirect (noninvasive) methods or by direct determination. An example of a noninvasive method has been described by Weiser et al. (1977). The method requires the placement of an inflatable cuff over the cranial tibial artery and the use of an ultrasonic Doppler sensing device to detect arterial wall motion. Direct determination of blood pressure can be accomplished by cannulation of an artery (e.g., the femoral artery) with a 22-gauge hypodermic needle that is connected to fluid-filled polyethylene tubing that leads to a pressure transducer (Tabaru et al. 1987; Weiser et al. 1977). Transducer activity can then be monitored on an oscilloscope equipped with a paper recorder. A more invasive procedure is telemetry. For this procedure, animals are surgically instrumented with a radio transmitter, and mean, systolic, and diastolic blood pressure (as well as ECG parameters and body temperature) can be continuously monitored.

Blood pressure measurements in unanesthetized dogs can vary widely owing to variables such as exercise, anxiety, and excitement. For this reason, all recordings from conscious dogs should be performed in a quiet, environmentally controlled room with the animals placed in a comfortable position. Some investigators find it of value to condition the dogs for several days prior to recording blood pressure to lay or sit in a given position. At least three readings are usually made and the average blood pressure determined. Andersen (1970) has reported mean systolic/diastolic values of 98 ± 28/46 ± 16, 121 ± 40/65 ± 27, and 130 ± 29/67 ± 8 for beagles in the age ranges of 2 to 8, 7 to 10, and 28 to 59 months, respectively.

Electrocardiography

Procedure for Taking ECGs. Although over the last 30 years the use of serial electrocardiograms (ECGs) has become routine in toxicity studies, generally accepted standards for adequate lead systems, recording techniques, frequency of taking samples, and principles for interpretation have not been established (Detweiler 1981). A 10-lead system, consisting of the bipolar leads (I, II, III), the augmented unipolar leads (aVR, aVL, and aV6), and the unipolar precordial leads [V10, CV6LL (V2), CV6LU (V4), CV5RL (rV2)], has been recommended for dogs (Detweiler et al. 1979). There is some debate about the most suitable body posture for the dog. The right lateral recumbent position (dog restrained on right side with the head and neck held flat and in line with the long axis of the trunk) is the position of choice in many facilities. The sternal recumbent, "sphinx" position and the standing position have been also found to work well. Use of a standardized body position, however, is essential during recording, and the most critical factor is consistent positioning of the forelimbs and scapula to prevent alterations in the amplitudes of ECG waves in the various leads and resultant vectors (Detweiler 1981). Whatever the position used, complete reproducibility in any position is unattainable, probably because of the impossibility of being able to maneuver the heart into the same position each time (Eckenfels and Trieb 1979; Katz et al. 1934).

For recording purposes, animals should be kept on a warm, insulating, nonconductive surface, such as a table covered with a rubber mat or blanket. The procedure normally requires two people: one to hold and calm the dog and ensure correct positioning during the recording and one to attach the electrodes and take the ECG. Electrode clips are attached directly to the animal's skin. Stainless steel or copper alligator clips (with the teeth filed down to prevent pinching) are commonly used

as electrodes, and the total area of contact should be about 1 cm^2. Suitable electrode contact materials include electrode paste, water, and alcohol. Prior to taking an ECG, the thermal pens for each channel should be checked for correction positioning and to ensure the elimination of extraneous noise due to improperly attached electrodes. It has been recommended that ECGs be recorded for a total period of 60 sec (Detweiler 1981). For a programmed three-channel, 10-lead electrocardiograph, a rhythm strip of any three selected leads should be taken to complete the 1 min of recording.

Many cardiologists prefer to record ECGs at a chart speed of 50 mm/sec (rather than 25 mm/sec) because the faster speed will "stretch out" the electrocardiogram, and the time intervals can be measured with greater accuracy (Tilley 1985). It has been noted, however, by Detweiler (1981) that 25 mm/sec is a satisfactory chart speed for dog ECGs, and adequate accuracy can be achieved.

For toxicity studies, ECG records are usually taken by technical personnel on groups of dogs and read at a later time by a cardiologist. The quality of the tracings and decision as to whether to rerun any ECGs will be determined by evaluation of instrument performance, technician error (e.g., incorrect body positioning or electrode placement), and the presence or absence of artifacts (e.g., 50–60-Hz interference, muscle tremor, and baseline drift). An acceptable quality tracing should have no errors present, a minimum of two complexes in each lead free of artifact (except minor variations in amplitude), and artifacts present in no more than 115 or 2 of 10 leads (Detweiler 1981).

Normal ECG Pattern. Normal values for untreated dogs with aging are shown in table 8.8. The data show that by about 7 to 8 months of age, most of the ECG parameters have reached adult values.

Dog ECGs can be highly variable, and it is important to take this into account when interpretating ECG findings. Electrocardiographic variations that occur relatively frequently in dogs include changes in direction and amplitude (in the same lead) of P-waves, which appear to be related to nervousness and excitation; changes in the amplitude and reversal of direction of T-waves; and changes in the amplitude or disappearance of Q- and S-waves in all leads (Lalich et al. 1941). Positive T-waves are found more frequently in sitting dogs than those standing or lying down and are more common in lead I and less common in lead U. Variations in the direction of the cardiac vector have been shown even when the same animal position is held correctly (Eckenfels and Trieb 1979). Eckenfeld (1980) noted that in serial ECG measurements, individual variability appears to be less than that between (or among) animals.

Heart Rate. The heart rate (number of beats per minute) is usually measured from the R-R interval over a specified period of time. The heart rate has been shown to change during the growing period. At 6 months of age, the heart rate is about 150 beats/min, but it progressively falls over the next 12 months to a value of about 120 beats/min (Noel 1970). The heart rate is often extremely variable in the dog, and values can range from 45 to 220 beats/min in the conscious mature beagle (Eckenfels and Trieb 1979). For this reason, ECGs should always be measured in a quiet, unstressful setting. There is also the concern that keeping an animal in a fixed position while taking an ECG will cause stress and lead to an increase in the heart rate and an exhibition of other excitement-induced ECG anomalies. The unrestrained standing and the intermediate sitting positions have been found to induce less excitation in the animals than other positions (Eckenfels and Trieb 1979). It is also recommended that a conditioning period be established prior to study start during which the electrodes are attached and the animals positioned for ECG recording.

Frequent Anomalies in Control Animals. A frequent finding in normal dogs is respiratory-influenced sinus arrhythmia, which is an irregular sinus rhythm that originates in the sinoatrial (SA) node. It is a vagally mediated event represented by alternating periods of a slower and more rapid heart rate; the heart rate increases with inspiration and decreases with expiration (Eckenfels 1980; Tilley 1985). These cyclical changes in the heart rate are often accompanied by cyclic alterations in the amplitude of ECG waves. This type of sinus arrhythmia is well differentiated from nonrespiratory sinus arrhythmia because of the positive correlation with the phases of respiration.

Table 8.8 Effects of Aging on Electocardiographic Parameters in Beagle Dogs[a]

		Months						Years		
		0–2	**3–4**	**4–5**	**7–8**	**9–10**	**11–12**	**2–3**	**4–5**	**6**
HR (/min)	M	189.1	165.9	146.9	136.5	129.6	130.8	135.8	134.2	134.4
	SD	23.1	24.9	26.0	30.0	28.7	25.7	23.3	22.1	19.3
	Range	153–228	120–202	94–182	80–186	76–178	79–175	76–176	90–174	94–163
	N	131	272	248	118	114	95	56	71	34
PR (msec)	M	73.0	83.8	93.7	94.7	98.7	99.7	98.8	104.1	104.4
	SD	8.3	10.4	11.4	12.0	14.4	12.1	11.3	11.4	9.1
	Range	60–84	66–100	74–112	72–114	74–122	78–114	78–116	82–124	82–120
	N	131	271	248	93	114	93	56	71	34
QRS (msec)	M	41.8	43.1	44.9	46.7	46.1	47.1	48.4	49.1	50.2
	SD	3.6	4.2	3.9	4.7	3.8	4.7	4.4	3.9	5.0
	Range	36–48	36–48	36–50	36–54	38–50	38–56	40–56	42–56	42–56
	N	130	272	247	117	114	92	56	71	34
QT (msec)	M	161.6	156.4	171.1	177.3	181.5	180.1	184.4	184.9	187.6
	SD	13.5	31.2	16.6	15.7	14.9	13.7	13.5	13.6	10.7
	Range	140–190	138–178	150–194	150–202	145–204	154–202	160–202	160–206	158–204
	N	130	270	233	114	109	90	54	68	33
QTc	M	285.7	259.3	265.5	263.1	263.3	263.0	273.6	274.0	278.8
	SD	20.0	50.8	26.1	21.5	20.9	23.4	23.4	17.5	18.7
	Range	258–317	228–292	229–293	224–298	222–292	209–296	227–302	244–294	232–308
	N	130	270	233	114	109	90	54	68	33
AXIS (°)	M	66.2	74.5	74.3	68.5	70.9	69.3	64.8	68.5	64.7
	SD	36.8	29.3	22.5	27.2	34.0	24.9	32.0	15.8	21.5
	Range	6–105	28–103	32–96	16–92	36–89	33–87	4–88	41–87	3–84
	N	130	271	247	116	113	95	54	71	33

Sinus tachycardia, with a heart rate above 160 beats/min (above 180 in toy breeds and above 220 in puppies) is the most common arrhythmia in dogs. It can most often be explained by nervousness (fear or aggression). Sinus bradycardia is normally vagus dependent and often occurs in calm dogs and those accustomed to the investigator (Eckenfels and Trieb 1979).

A wandering pacemaker consists of a shift of the pacemaker from within the SA node or from the SA to the atrioventricular (AV) node (Tilley 1985). This is a variant of sinus arrhythmia and is a frequent finding in normal dogs. The shifting of the pacemaker within the SA node causes a gradual change in configuration of the P-wave without it becoming negative, whereas shifting of the pacemaker between the SA node and AV junction results in a gradual change in the configuration of the P-wave, which can become positive, biphasic, isoelectric, and negative.

Another ECG finding that can be found in normal healthy dogs is incomplete right bundle branch block (IRBBB) characterized by the presence of a right axis deviation and a large wide S-wave in leads I, II, III, aVF, CV6LL(V2), and CV6LU(V4). It has been found in the beagle as a genetically determined localized variation in right ventricular wall thickness, or just as focal hypertrophy of the right ventricle (Tilley 1985). Studies conducted in this laboratory have indicated the presence of IRBBB in about 20% to 25% of a population of young adult beagles in the absence of accompanying cardiac pathology (unpublished data).

Clinical Laboratory Sample Collection

Blood Collection

The most commonly used sites of blood collection in the dog are the cephalic and jugular veins; other veins used less frequently are the femoral, brachial, and saphenous veins. The advantage of the jugular vein is that it is large and easy to access and, therefore, suitable for serial sampling in a toxicity study. The collection site is shaved and swabbed with an ethanolic solution, and a 20- or 21-gauge needle is used to collect the sample. The vein is occluded, and the needle is inserted (bevel upward) pointing toward the animal's head; usually one person restrains the dog and occludes the vein while a second person collects the sample. Once the desired volume of blood has been collected (usually a total of 5–6 ml for clinical laboratory determinations and 1–2 ml at each pharmacokinetic collection time), the needle is withdrawn, and dry gauze applied (with pressure) to the site until the bleeding stops. Because of the potential for commercial diets to affect clinical parameters such as blood urea, glucose, and cholesterol, it is recommended that animals be fasted overnight prior to blood collection (Street et al. 1968).

Urine Collection

In many laboratories, urine collection from dogs is conducted overnight (approximately a 15-hr period) because of the difficulty in consistently obtaining sufficient volumes of urine in some dogs during shorter collection periods. There are problems, however, associated with such a protracted collection time. Urine casts can dissolve, bacterial activity can increase, and bilirubin can break down to biliverdin with exposure of the sample to light. Because only a small sample is needed for urinalysis, it is recommended that one be collected from each dog as soon as possible during the collection period. Collection containers should also be protected from light. Common urine collection systems for dogs are metabolism cages or stainless steel troughs that attach to the home cage. When collecting samples, care should be taken to avoid males being housed in cages directly above those of females because of the possibility of the males urinating along the side of the cage and contaminating the sample being collected in the lower cages.

Ad libitum access to water can also be a problem because of accidental dilution of the urine sample. Consequently, it is common for the dogs not to have access to water during the overnight

collection period. It is recommended, however, that dogs have free access to water on the following day prior to dosing. Work done to examine the effects of water rehydration on erythrocyte parameters has shown that 30 min to 60 min is a sufficient rehydration period (Guy 1989).

Common Study Protocols and Associated Considerations

Because of the large amount of background data available on the dog, in particular the beagle, it is a commonly used nonrodent species in acute, limited, repeated-dose (2- or 4-week studies), subchronic (up to 13 weeks of duration), or chronic (26 weeks or longer) toxicology studies. Examples of experimental designs and suggested timing of various study activities for 4- and 13-week toxicity studies are shown in table 8.9 and table 8.10, respectively.

Table 8.9 4-Week Toxicity Study

Experimental design	
4/sex/group; 4 dosage groups + 1 control	
Dose daily for 29 or 30 days	
Sacrifice days 29, 30, or 31	
Study activities	
Daily observations:	Pretreatment and twice daily during the study period
Physical examination:	Pretreatment and after dosing during weeks 2 and 4
ECG:	Pretreatment and after dosing during weeks 2 and 4
Ophthalmic examination:	Pretreatment and during week 4
Body weight:	Pretreatment and weekly, for moribund animals, and the day of scheduled sacrifice
Feed consumption:	Pretreatment and weekly
Clinical lab:	Twice before the first dosing day, before dosing on day 2, during week 2, and prior to sacrifice
Urine collection:	Pretreatment and during weeks 2 and 4
Pharmacokinetic sample:	Blood collected at specified times after dosing on days 1 and 28

Table 8.10 13-Week Dog Toxicity Study

Experimental design	
8/sex/group; 3 dosage groups + control	
Dose daily for 91, 92, or 93 days	
Sacrifice main group (6/sex/group), week 14	
Sacrifice reversal group (2/sex/group), week 18	
Study activities	
Daily observations:	Pretreatment, twice daily during treatment phase, and daily during reversal
Physical examination:	Pretreatment, after dosing during weeks 4, 8, and 13 of treatment, and during week 4 of reversal
ECG:	Pretreatment, after dosing during weeks 4, 8, and 13 of treatment, and during week 4 of reversal
Ophthalmic examination:	Pretreatment, during weeks 6 and 13 of treatment, and during week 4 of reversal
Body weight:	Pretreatment (three times), weekly during the treatment and reversal periods, for moribund animals, and at scheduled sacrifice
Feed consumption:	Pretreatment, weekly through first month, bimonthly during the remainder of the treatment period, and weekly during reversal
Clinical lab:	Pretreatment, during weeks 4 and 8 of treatment, day 1 of reversal, for moribund animals, and prior to scheduled sacrifice
Urine collection:	Pretreatment, monthly during treatment, and during week 4 of reversal
Pharmacokinetic sample:	Blood collected at specified times after dosing on day 1 and during weeks 6 and 12

For teratology studies, the dog does not appear to be as sensitive an indicator of teratogens as other nonrodent animal models such as primates (Earl et al. 1973) and ferrets (Gulamhusein et al. 1980). Likewise, for studies aimed at evaluating reproductive function, the dog is not the species of choice, primarily because fertility testing is virtually impossible to conduct owing to prolonged anestrus and the inability to predict the onset of proestrus. Also, there is no reliable procedure for the induction of estrus or ovulation. Although semen is relatively easy to collect from the dog, the number of sperm in an ejaculate can vary widely owing to factors such as age, testicular size, and season (Amann 1982).

The dog, however, is an appropriate nonrodent animal model for use in pediatric studies to assess the effects of various treatments on postnatal development (equivalents of human neonate, infant, child, and adolescent developmental phases).

Selection of Study Animals

Age

It has been recommended that, depending on the age of the human population projected to be exposed to a given compound, dogs should be at least 4 to 6 months and no more than 9 months of age at study start (Mosberg and Hayes 1989).

For pediatric studies, judicious selection of even younger animals for standard repeated-dose toxicology studies might obviate the need for a special pediatric study in the dog. In many laboratories, dogs are usually in the age range of 7 to 9 months at study start; however, for short-term studies (acute, 2 or 4 weeks), there is usually no problem with using older animals (up to 12 months of age).

Pretreatment Evaluation

It is imperative that dogs be properly screened prior to assignment to a study. Animals should be selected on the basis of acceptable findings from body weight, physical, ophthalmic, and electrocardiographic examinations as well as urinalysis, clinical chemistry, and hematological evaluations. Pretreatment evaluation should take place within 3 to 4 weeks of study start, and it is recommended that clinical laboratory determinations be made close to the start of the study. Additionally, it is advisable to limit the number of siblings that are assigned to a study to no more than one per sex per treatment group.

Numbers of Animals and Experimental Design Considerations

The number of animals assigned to a treatment group will depend on the duration of the study (see table 8.9 and table 8.10). In general, fewer animals are used for a 2- or 4-week study (suggested sample size is 4 animals per sex per group; no reversal group) than for a 26-week or 1-year study (suggested sample size is nine animals per sex per group; includes two to three dogs per sex per group for reversal). Whichever type of study is conducted, the number of animals should be equal across sexes for any given treatment group.

Often there is little information on repeated-dose toxicity of a test compound at the time 2- or 4-week dog toxicity studies are conducted, yet findings from these studies are often the main basis for dose selection for the longer term studies. Thus, it is recommended that four (rather than three) dosage groups be used in these studies to obtain a more complete toxicity profile of the compound.

Randomization

Dogs are usually randomly assigned to treatment groups using a blocking procedure with blocks defined from stratified body weights. Because of possible inherited susceptibility or resistance to

toxic effects induced by a test compound, the distribution of siblings needs to taken into account when reviewing the final randomization. A general rule is to limit the number of siblings to one per sex per group. This can be achieved by placing a limit on the number of siblings (i.e., not to exceed the number of groups in the study) that will be accepted from the animal supplier. Random assignment of reversal animals is usually done prior to study start; however, if any adverse findings are detected during the treatment phase, the study director might want to include some of the affected animals in the reversal group.

Study Activities

When scheduling activities for dog studies it should always be taken into consideration that most, if not all, study-related activities will be conducted in all animals. The repeated manipulation of dogs for blood collection as well as for ECG, ophthalmic, and physical examinations will likely induce some stress in the animals. For shorter term studies, this can be a concern, especially at the beginning and end of a study in which there are multiple study activities to be conducted in a relatively short period of time (see table 8.9). Efforts should be made to separate the various study activities so that no more than two activities are scheduled on the same day.

Species Peculiarities

Emesis

Dogs have a natural tendency to vomit. Although this might be easier to explain in the pet dog that has a greater chance of ingesting foreign materials and spoiled foods, emesis is often seen in the laboratory dog living in a controlled environment. For a given study, the incidence of vomitus in control animals on a single day can be as high as 40% to 50% (unpublished data). The cause of this emesis is unclear, but it is probably related to the inherent excitability of the dog. Vomiting can be a particular problem when a drug is given orally, because a portion of the dose might be lost.

Because vomiting during a study can contribute to the variability of the pharmacokinetic results for that study, it is recommended that all occurrences of vomiting be recorded and additional observations be made on days when pharmacokinetic blood sampling occurs.

Shedding and Alopecia

Shedding is frequently seen in beagles. This is in part seasonal (it occurs in both sexes primarily in the spring) and, in females, it is in part estrus related (Al-Bagdadi et al. 1977). A more serious problem arises when dogs develop pruritic alopecia, which occurs at a greater frequency in the summer and appears to be more common in females. Clinical signs include bilateral alopecia of increasing severity; pruritic ears progressing to thickening of the skin around the ears, face, and neck; and skin inflammation in severe cases.

Polysorbate-Induced Histamine Reaction

IV administration of polysorbates (Tweens) 20, 40, 60, and 80 in dogs have been found to cause a release of histamine or a histamine-like substance that results in a profound cutaneous response (includes reddening of the muzzle, inside of the ears, and sometimes of the general body as well as swollen and bloodshot eyes), scratching, vomiting, signs of weakness and postural difficulties, and hypotension (Krantz et al. 1948). These effects have been seen in this laboratory after administration of a 0.1% aqueous polysorbate 80 solution at a volume of 5 ml/kg (unpublished data). Polysorbate 80 has also been demonstrated to be a potent cardiac depressant in dogs. This allergic depressor response is not seen in the guinea pig, cat, rat, rabbit, or humans (Krantz et al. 1948).

Advantages and Disadvantages of the Dog

There are well-defined advantages and disadvantages in working with the beagle (see table 8.11). The suitability of the beagle as an experimental model arises from factors such as its medium size and even temperament. The relative ease of handling the beagle makes it possible to perform any routine study-related activities (e.g., serial collection of blood and electocardiographic examination in the conscious animal.

Table 8.11 Use of the Beagle in Safety Assessment Studies

Advantages
Medium size
Moderate length of hair coat
Eeven temperament and friendly disposition (easy to handle)
Adaptability to living in groups
Easy to work with (e.g., dosing, blood collection, ECG)
Satellite animals not needed for serial blood colletion
Disadvantages
Variation in size and body weight
Loud, penetrating bark
Cost of acquisition and maintenance
Greater test compound requirements than smaller species
Availability
Exercise and housing requirements

Disadvantages in the use of the beagle include an often wide variation in size and body weight and a loud penetrating bark, which can be controlled by partial ventriculocordectomy, a procedure that usually gives permanent results but does not affect the well-being of the dog (Andersen 1970). However, ventriculocardectomy is not a common practice in research facilities Housing and exercise requirements for the laboratory dog can be a disadvantage in terms of the large amount of laboratory space and additional personnel needed to maintain and exercise animals on study. This is particularly relevant in light of revisions in the Animal Welfare Laws of many countries (e.g., U.S. Department of Agriculture) that have resulted in increased space requirements for dogs and a specified period of daily exercise. Additionally, test compound requirements are higher for the dog (relative to the rat, guinea pig, ferret, and monkey), which can often be a major disadvantage when only limited quantities of test compound are available. As mentioned earlier, the high incidence of vomiting and the histamine release reaction to IV injection of Tweens can also be disadvantages. Finally, it should be recognized that experiments using large numbers of beagles usually cannot be set up quickly, and careful advance planning is needed to ensure that animals of the right age range and number can be obtained and housed in time to meet projected deadlines.

PATHOLOGY

This section on the pathology of the laboratory dog is not intended to be a comprehensive treatise on canine pathology, but primarily a source of information on spontaneous gross and microscopic changes seen in beagle dogs. Emphasis is placed on young adult beagles because they are most frequently used as a nonrodent species during short-term safety studies, although they are also well suited for selected longer term research studies. Commercial breeding colonies provide standardized beagles for research and safety studies. Hopefully, the information in this chapter will be useful not only to study pathologists, but to those who assist them in the postmortem laboratory and also to toxicologists and students of pathology and toxicology. The material is presented on an organ system basis, with a review of gross and microscopic anatomy, suggested necropsy

procedures, a summary of reported spontaneous pathology augmented by the writers' own experiences, and selected examples of induced or spontaneous changes useful as models of human disease. For a tabular summary with available incidence rates for gross and histopathological findings in control laboratory dogs, refer to Peckham (2002). Disease problems in laboratory dogs from random sources and conditioned for research studies were reported by Ringler and Peter (1984) and Pick and Eubanks (1965). Also, spontaneous diseases and findings in the laboratory beagle have been reported by Fritz et al. (1966, 1967), Andersen (1970), Hottendorf and Hirth (1974), Maita et al. (1977), Oghiso et al. (1982), and Glaister (1986). The pathobiology of the aging dog is described in detail in two volumes edited by Mohr et al. (2001). Additional information and more detailed descriptions of canine anatomy, physiology, parasitology, infectious and noninfectious diseases, and pathology are available in a number of veterinary medical textbooks. For example, see Aiello (1998), Bonagura (2000), Bonagura and Kirk (1995), Carter (1993), Ettinger and Feldman (1995), Jones et al. (1993), Jubb et al. (1992), Summer et al. (1995), Urquhart et al. (1996), Muller (1995), Greene (1998), and Meuten (2002).

Integumentary System

Anatomy and Histology

The entire body of the dog is covered by an organ known as the integument, or skin (integumentary system), which includes epidermis, dermis, hair, hair follicles, sweat and sebaceous glands, digital pads, claws, and mammary glands (Calhoun and Stinson 1976). The integument is bound to underlying fascia and skeletal muscles by a subcutaneous, or hypodermal, layer (subcutis, hypodermis) of loose and adipose connective tissue that is not part of the integument but that is often removed with it during dissection. The integument is continuous at all natural body openings with the mucous membranes of the digestive, respiratory, and urogenital systems. At the margins of the eyelids, the stratified squamous ectoderm of the skin becomes the mucous membrane (conjunctiva) of the eye. The outer layer of the tympanic membrane (stratum cutaneum) is very thin without hair or glands (Breazile 1976). The integument is a large organ. In the newborn puppy, the skin, hair, and subcutis represent 24% of the total body weight (Lovell and Getty 1967). At maturity, the same tissues represent 12% of the body weight. The principal function of the skin is to separate and protect the body from the environment. Additional functions include temperature regulation, sensory perception, blood pressure control, secretion, storage, and synthesis of vitamin D (Muller et al. 1983). Although the functions of the integument are similar in various laboratory animals, significant morphological differences exist between animals and significant regional differences exist within an animal. Adam et al. (1970) have illustrated many of the regional differences in the beagle. Some regional differences are subtle and are best recognized utilizing carefully collected age-, sex-, and site-matched specimens.

Epidermis, Dermis, and Hair Coat

The thickness of the skin (epidermis and dermis) and density of the hair coat (pelage) vary in a consistent way over the body. Both skin and pelage are thickest over the dorsal and lateral surfaces of the trunk and lateral surfaces of the limbs, and are thinnest on the ventral surface of the trunk and medial surfaces of the limbs (Calhoun and Stinson 1976). As a separate layer, the epidermis is generally the thinnest in well-haired areas and the thickest in hairless areas. In contrast, the dermis is usually thickest in well-haired areas and thinnest in hairless areas. In the beagle, the thinnest epidermis is found in the cheek and thinnest dermis is found in the scrotum (Warner and McFarland 1970).

The hair coat of the beagle is typically tricolored, consisting of black, white, and shades of brown (Warner and McFarland 1970). Individual variation is great and some beagles are bicolored. Beagles tend to shed hair throughout the year, although some have seasonal molts. Dogs have three

general types of hair: tactile, coarse, and fine. Tactile hairs include sinus hairs (vibrissae, whiskers) and tylotrich hairs. Sinus hairs function as slow-adapting mechanoreceptors and are located on the muzzle, upper and lower lips, chin, intermandibular space, near the oral commissures, and above the eyes (Warner and McFarland 1970). Eyelashes (cilia) are large hairs but are not sinus hairs. Sinus hairs have large follicles containing an endothelial-lined blood sinus between the two sheaths of the follicle (Calhoun and Stinson 1976). Tylotrich hairs are stout hairs scattered among ordinary hairs and function as rapid responding mechanoreceptors (Yager and Scott 1985). Tylotrich follicles are large, contain a single tylotrich hair and have a ring of neurovascular tissue at the level of the sebaceous gland. The tylotrich follicle is associated with a focal area of epidermal thickening (tylotrich pad) resting on a layer of well-vascularized and innervated connective tissue (Yager and Scott 1985). Ordinary coarse (primary, guard) and fine (secondary) hairs comprise the bulk of the pelage and grow in compound follicles in the dog with a single long coarse hair and several fine hairs emerging from a single opening in the skin. As many as 15 hairs can grow in one follicle (Calhoun and Stinson 1976).

Sebaceous and apocrine sweat glands are the two principal skin glands in the dog (and mammary gland in the female).

Sebaceous Sweat Glands. Sebaceous glands are simple alveolar glands whose oily secretion (sebum) results from complete disruption of cells (holocrine secretion). Sebaceous glands are located in the superficial dermis where each gland empties into the upper part of a hair follicle or onto the surface of the skin in hairless areas. Each primary hair has its own sebaceous gland. Secondary hairs usually share sebaceous glands. A ring of sebaceous glands opens into the follicle of tactile hairs (Muller et al. 1983). Where hair is dense, sebaceous glands tend to be long and narrow. Sebaceous glands are larger where hair is sparse and are the largest where associated with small hairs and at mucocutaneous junctions such as the lips and prepuce.

The dog has several special sebaceous glands. Two types are found in the eyelids. Meibomian glands lie within the tarsal plates of the upper and lower lids. Secretion from Meibomian glands seals the lid margins when the eyelids are closed and prevents overflow of tears when the eyelids are open (Kuwabara and Cogan 1977). Sebaceous glands associated with the eyelashes are known as the glands of Zeis.

A complex arrangement of glands is found in the anal region of the dog. The largest and most widely distributed glands in the subcutaneous perianal region are known as circumanal, or perianal, glands. Circumanal glands are bipartite glands with a typical sebaceous gland superficial portion and a larger, deeper, nonsebaceous portion. The sebaceous portion is functional and empties into hair follicles through patent ducts. The nonsebaceous portion is apparently nonfunctional and is connected to the sebaceous portion by nonpatent ducts (Calhoun and Stinson 1976). The nonfunctional portion comprises large cells that resemble hepatocytes, causing them to be referred to as hepatoid cells (Yager and Scott 1985). Circumanal glands have been found ectopically in a number of sites, including the tail, flank, back, prepuce, and chin (Yager and Scott 1985) and walls of anal sac ducts (Miller et al. 1967). Circumanal glands continue to grow throughout the life of the noncastrated male. Large, well-developed sebaceous glands are also found in a circumscribed oval area of skin on the dorsum of the tail, a short distance from its base. This gland complex is known as the tail, or supracaudal, gland (Calhoun and Stinson 1976). Large apocrine glands are also present in this area. The tail gland is marked grossly by stiff hairs and a yellow, waxy appearance to the skin surface (Lovell and Getty 1967).

Apocrine Sweat Glands. Apocrine sweat glands, as with sebaceous glands, are located throughout the skin of the dog, mainly in connection with hair follicles. The bodies of apocrine sweat glands are located deeper in the dermis than sebaceous glands; however, their ducts enter follicles above the ducts of sebaceous glands. Only one sweat gland is associated with each hair follicle complex. Apocrine sweat gland secretions are scant and rarely perceptible in the dog (Calhoun and

Stinson 1976). Specialized apocrine sweat glands are found in the eyelids, external ear canal, and anal region. In the eyelid, rudimentary sweat glands are associated with the eyelashes and are known as the glands of Moll (Getty 1967).

The apocrine sweat glands of the external ear canal are known as ceruminous glands, but are apparently only partly responsible for the waxy secretion known as cerumen (ear wax). Cerumen appears to be a product of sebaceous and apocrine sweat glands with desquamated epithelial cells as an additional ingredient (Warner and McFarland 1970). Large apocrine sweat glands are present within the walls of the anal sacs. The ducts of anal sacs also contain sebaceous glands making the anal sac contents a mixture of sebaceous and apocrine gland secretions. Apocrine sweat glands, known as anal glands, also open directly into the intermediate zone of the anal mucosa (Warner and McFarland 1970).

Eccrine Sweat Glands. Eccrine (merocrine) sweat glands are limited to the footpads of dogs. They have no thermoregulatory function (Muller et al. 1983).

Mammary Glands. Mammary glands are compound tubuloalveolar glands located in the ventral thoracic, abdominal, and inguinal skin of female dogs. The secretory product, milk, is produced by both apocrine and merocrine secretion (Calhoun and Stinson 1976). The bitch typically has 10 mammary glands arranged in two symmetrical rows. Teats indicate the location of the glands in the male and immature female. One or more glands might be missing. Nine is the usual number of functional glands in the beagle (Sekhri and Faulkin 1970). Supernumary teats are common. Mammary glands usually show a size gradient from small (thin) anterior glands to large (thick) posterior ones. The cranial two pairs of glands are the cranial and caudal thoracic glands or simply glands 1 and 2. The next two pairs are the cranial and caudal abdominal glands, or glands 3 and 4. The most caudal pair are the inguinal, or pubic, glands, or glands 5. The mammary glands are under hormonal control, which regulates development at puberty, sequential changes during the estrous cycle, and growth during pregnancy. Not all parts of active glands are necessarily in synchrony and there might be substantial variation in the size of ducts and alveoli, luminal content, and the character of lining cells. Detailed descriptions of the mammary glands during all stages of the estrous cycle have been published (Nelson and Kelly 1974; Sekhri and Faulkin 1970). The selection of mammary gland specimens for microscopic examination should be consistent not only for the purpose of group comparisons, but also for correlation of mammary gland and reproductive tract histology. Nelson and Kelly (1974) based their observations on specimens from the inguinal glands. Sixty to 65% of mammary tumors are found in the caudal abdominal and inguinal mammary glands (Crow 1980; Moulton 1990), a fact to consider in the design of long-term studies.

Necropsy and Laboratory Techniques

In anesthetized animals, a general examination of the skin should be done prior to exsanguination. All of the body surface, including the mammae, should be palpated to locate masses and to evaluate the texture, elasticity, and thickness of the skin. The luster and texture of the hair should be noted. Abnormal coloration (pallor, erythema, jaundice) should be looked for in lightly pigmented and sparsely haired regions such as the axillae and inguina. The perineal region should be examined as well as the surfaces of the pinnae, visible portions of the ear canal, interdigital spaces, and footpads. If no significant abnormality is revealed by the general examination, a single specimen of inguinal or posterior abdominal skin that includes the mammary gland can be collected as a representative sample of the epidermis, dermis, and glandular adnexa. If specimens of diseased skin are taken, adequate margins of normal skin should be included. Generally, hair should be removed from skin samples to facilitate processing and sectioning. Hair is preferably removed prior to exsanguination. Whether hair is removed by clippers or scissors, the work should be done carefully so as not to disturb or remove surface pathology or create artifacts. Skin specimens should be flattened prior to fixation. This can be accomplished by carefully stapling the specimen, with

the subcutaneous surface down, to small pieces of cardboard or corkboard. The boards can be labeled to identify multiple specimens. Fixation in a flattened position allows proper anatomical orientation and prevents artifacts associated with curling and folding. Three to 4-mm wide blocks of tissue can be excised from the fixed tissue for embedding, leaving a largely intact and labeled specimen for archival storage. Ten percent buffered formalin is the preferred fixative for skin specimens (Hargis 1988; Muller et al. 1983). Hematoxylin and eosin (H&E) is the most widely used stain but acid orcein Giemsa is also recommended as a routine stain for skin biopsies (Muller et al. 1983). The latter authors list several special stains that are useful in dermatopathology.

Special investigations, such as those utilizing the bitch as a test animal in which to study the effects of contraceptives on mammary tissue, might require a detailed examination of the mammary glands. An approach reported by El Etreby and Wrobel (1978) utilized the right mammary system as a whole mount for gross examination and the left mammary system for microscopic examination. Utilizing a modified technique of Cameron and Faulkin (1971), the entire right mammary system was fixed in Tellyesniczky's solution. After removal of the skin and additional fixation, the mammary complex was defatted in acetone, stained with hematoxylin, and cleared with methyl salicylate. The whole mount was weighed and examined by dissecting microscope and photographed utilizing transmitted light. Specimens of the left mammary system were fixed in Carnoy's, Bouin's, or paraformaldehyde solutions and stained by a variety of stains for histomorphology and histochemistry. Frozen sections were also utilized to demonstrate lipids and enzymes. Other approaches to examining the entire mammary system include slicing formalin fixed glands at 5 mm intervals and examining each slice for gross abnormalities (Nelson et al. 1973) or obtaining specimens from palpable nodules in addition to routine sampling of normal glandular tissue (Giles et al. 1978). Microscopic examination of lymph nodes draining the mammary glands is rarely indicated in short-term, routine studies. Nevertheless, knowledge of the usual lymphatic drainage pattern is desirable. The following information was obtained from Christensen (1967) and Moulton (1990). Mammary glands 1 through 3 drain to the axillary lymph nodes on the same side. Glands 4 and 5 drain to the superficial inguinal nodes on the same side. The lymphatics of glands 3 and 4 sometimes connect, allowing both anterior and posterior flow of lymph. Lymphatics can cross the midline and might pass directly through the thoracic wall to the sternal lymph nodes.

Pathology

Dermatohistopathology has developed a specialized vocabulary based largely on human disease. Some of the diagnostic criteria and terminology are not applicable to veterinary pathology and their use can be confusing if not misleading. The reader is referred to the excellent discussions and illustrations in Hargis (1988), Muller et al. (1983, 1995), and Yager and Scott (1985) for guidance in the recognition and diagnosis of conditions that might be encountered in the integument of laboratory dogs. Spontaneous diseases of the skin or the mammary gland are rarely cited, which could reflect either a true absence of pathology or a failure to sample and diagnose minor or incidental lesions.

Nonneoplastic Findings: Spontaneous

Abrasions of the skin commonly result from concrete surfaces and caging. Skin wounds also can be caused by bite wounds during fighting.

Alopecia. Oghiso et al. (1982) reported alopecia of unknown cause associated with atrophy of hair follicles, hyperkeratosis, and necrosis of the epidermis. Alopecia (the complete or partial, diffuse, or circumscribed loss of hair) without underlying skin changes is seen in laboratory dogs over bony pressure points such as the elbow and hock. Continued trauma to such areas can result in a localized hyperkeratotic lesion known as a callus (callosity). Calluses might be ulcerated and infected and become pressure point granulomas.

Circumscribed, erythematous, scaly areas of alopecia near the eyes, commissures of the mouth, or on the forelegs might be a sign of demodectic mange. Demodectic mites in the skin are estimated to occur in 80% of the beagles of some laboratory colonies (Hottendorf and Hirth 1974). *Demodex canis* is a normal resident of the skin of the dog and inhabits hair follicles and sebaceous glands (Muller et al. 1983; Yager and Scott 1985). The mites are transmitted by direct contact from dam to nursing puppies. A disease state (demodicosis) develops when the favorable equilibrium between dog and parasite is altered, allowing excessive proliferation of the mite. Microscopic examination of demodectic lesions reveals varying degrees of perifolliculitis, folliculitis, and furunculosis (penetrating or perforating folliculitis) in the presence of a large mite population (Muller et al. 1983).

Dermatosis of the Ear. Multiple irregular, soft tan nodules can be seen on the margin of the pinnae. Microscopically, the lesion consists of orthokeratotic (anuclear) or parakeratotic (nucleated) hyperkeratosis and is consistent with a condition in pendulous-eared dogs known as ear margin dermatosis (Muller et al. 1983).

Superficial necrolytic dermatitis was reported in a young laboratory beagle with diabetes mellitus. This skin lesion was restricted to the paws and interdigital areas and characterized microscopically by upper epidermal vacuolation of keratinocytes (Yoshida et al. 1996).

Mastitis. Apparently, mammary gland inflammation is rare; a single case of mastitis was found in 499 young beagle bitches (Hottendorf and Hirth 1974).

Neoplastic Findings: Spontaneous

The peak age for skin neoplasia in the dog is between 6 and 14 years (Muller et al. 1983); however, tumors do occur in young dogs including laboratory beagles. Skin tumors that have been reported in beagles under 2 years of age include one histiocytoma (0.1 %) in 647 dogs (Hottendorf and Hirth 1974) and an unspecified sarcoma and a mast cell sarcoma (Fritz et al. 1966). For a detailed description and classification of canine tumors, the reader is referred to Muller et al. (1983) and Moulton (1990). Although mammary tumors are the most common tumor in bitches, they are rarely encountered in dogs less than 2 years old (Moulton 1990). A survey of reports on the effects of oral or injectable contraceptives in the beagle revealed that mammary nodules rarely developed in nontreated (control) bitches. In a 7-year study, a total of 40 mammary nodules were palpated in 7 of 18 control bitches (Giles et al. 1978). Most of the nodules were transient and only 9 remained in 6 bitches at the time of necropsy. Histopathological examination of the nine nodules revealed five lobular or intraductal hyperplasias, two benign mixed mammary tumors, and two nonmammary nodules (benign soft tissue tumors, epidermal cysts, lymph nodes, or inflammatory nodules). In a 4-year study, nodules were palpable in 4 of 20 control beagle bitches at 21 to 24 months (Nelson et al. 1973), but all nodules had disappeared by the time of necropsy. During life span observations of 1,343 beagles, 476 (70.8%) of the 672 females and 2 (0.3%) of 671 males had one or more mammary neoplasms (Benjamin 2001; Benjamin et al. 1999). The reader is referred to Moulton (1990), Misdorp et al. (1999), Goldschmidt et al. (2001), and Misdorp et al. (2002) for descriptions and classifications of tumors and tumor-like lesions of the canine mammary gland.

Neoplastic Findings: Induced

Canine mammary glands have provided a useful model for study of effects after exposure to investigational oral contraceptive steroids. These studies strongly link combinations of progestin and mestranol with mammary neoplasia including malignant tumors in dogs (Giles et al. 1978, Kwapien et al. 1980).

Musculoskeletal System

Anatomy and Histology

Bones

The vertebrate skeleton supports the body, provides rigid attachment for muscles of locomotion and respiration, protects internal organs, stores minerals, and is the site of blood cell formation (Miller et al. 1967; Wasserman 1977). The skeleton consists of many bones varying in size, shape, and function. Long bones such as the femur, tibia, humerus, radius, ulna, metacarpals, metatarsals, and phalanges make up most of the appendicular skeleton and serve primarily as levers. The limbs also contain many short bones (carpal and tarsal) that provide flexibility to their respective joints and sesamoid bones that alter the course of tendons and protect tendons at points of greatest friction (Miller et al. 1967). Flat bones are found in both the appendicular skeleton (scapula) and axial skeleton (ribs, sternum, calvarium). The calvarium protects the brain, whereas the ribs and sternum assist in respiration and protect the thoracic organs. Irregular bones are characterized by jutting processes. Much of the axial skeleton comprises irregular bones, including the vertebrae, bones of the base of the skull, and the fused hip bones (Miller et al. 1967). The processes of irregular bones are mostly for muscular and ligamentous attachments; however, some are for articulation. The os penis is the single bone of the heterotopic skeleton of the dog and forms the skeleton of the penis. Bones are entirely of mesodermal origin and develop either by direct transformation of connective tissue to bone (intramembranous osteogenesis), by replacement of previously formed cartilage by bone (endochondral osteogenesis), or by a combination of the two processes (Wasserman 1977). Intramembranous osteogenesis is exemplified by the formation of flat bones of the skull and subperiosteal bone in the shafts of long bones. Endochondral osteogenesis is exemplified by the longitudinal growth of long bones. Bones contain many cell types: endothelial, fat, hemopoietic, chrondoblasts, chondrocytes, osteoblasts, osteocytes, and osteoclasts. Of these, osteoblasts, osteocytes, and osteoclasts have specific bone-related functions. The three types of cells are derived from a common ancestor in the bone marrow (Doige 1988).

Osteoblasts. These arise from bone marrow stromal stem cells to produce and mineralize an organic matrix called osteoid. Osteoblasts are readily observed during the formation of new bone, where they appear as epithelial-like sheets of plump, basophilic cells aligned along bone-forming surfaces. As subsequent rows of osteoblasts differentiate, new osteoblasts begin secreting and osteoblasts of the first row become embedded in bone matrix.

Osteocytes. Osteoblasts buried in mineralized matrix are known as osteocytes. Only about 10% of osteoblasts become osteocytes, and the rest apparently die (Jubb et al. 1985). Osteocytes generally appear as single, flat or oval cells in spaces called lacunae. Although surrounded by bone, osteocytes are not isolated, but maintain contact with other osteocytes and osteoblasts by means of a vast network of cytoplasmic processes contained within minute channels called canaliculi (Doige 1988). Osteocytes play a role in calcium homeostasis by demineralization and remineralization of perilacunar bone. The demineralizing process is known as osteocytic osteolysis. Osteocytes also retain some capacity for bone formation (Doige 1988).

Osteoclasts. The typical osteoclast is a large, multinuclear cell with abundant eosinophilic cytoplasm. Osteoclasts arise by fusion of mononuclear phagocytes whose precursors are derived from bone marrow hemopoietic stem cells (Doige 1988). The principal function of osteoclasts is removal of bone, both matrix and mineral. When active, osteoclasts are located in bone structure concavities known as Howship's lacunae. Osteoclasts are highly mobile, capable of migrating along surfaces

of bone, and also of entering the bloodstream (Wasserman 1977). The work of osteoclasts is the basis of the resorption phase of all bone modeling and remodeling (Jubb et al. 1985).

Bone Modeling and Remodeling

Bone modeling refers to the formation and reformation of bone required in the shaping of a growing skeleton (Doige 1988). Modeling allows the overall shape of bones to be maintained while the skeleton is growing and depends on cellular activity in all parts of the bone. Bone remodeling is the turnover of tissues in mature bones and is the process by which the skeleton renews itself throughout life (Jubb et al. 1985). Remodeling occurs on three surfaces: periosteal, endosteal, and intracortical. Because various bones stop growing at different times, the changeover from modeling to remodeling will vary from site to site in the skeleton. Modeling and remodeling occur in both cancellous (trabecular, spongy) and compact (dense, cortical, Haversian) bones. Cancellous Bone.

This is elaborated in the extremities of long bones (epiphysis and metaphysis), forms the internal substance of short and irregular bones, and is interposed between the cortices of most flat bones (Miller et al. 1967). The trabeculae of cancellous bone vary in form from densely packed plates to interconnecting rods to a delicate filigree (Jee et al. 1970). No cancellous bone is present in the middle region of long bones and the space thus formed is known as the medullary cavity. The medullary cavity of long bones in adult animals is largely filled with yellow bone marrow. The cancellous bone of ribs and vertebrae and many short and flat bones is filled with red marrow throughout life (Miller et al. 1967).

Compact Bone

This comprises mostly long, bony rods with a central vascular canal. This structure is known as the osteon, or Haversian system, and is the classic structural unit of diaphyseal bone. Osteons form between trabeculae of cancellous bone, eventually filling in the intertrabecular space and converting cancellous bone to compact bone (Jubb et al. 1985). The remaining interstices of cancellous bone are occupied by red marrow.

Joints

Joints, or articulations, are structures that join two or more bones in unions that might or might not be movable. Three main types of joints are recognized: fibrous, cartilaginous, and synovial.

Fibrous Joints. These are simple unions that provide for little movement. Examples are sutures of the skull and unions of long bones such as the tibia and fibula (tibiofibular syndesmosis).

Cartilaginous Joints. Two types of cartilaginous joints are recognized: hyaline and fibrocartilaginous. The site of endochrondral osteogenesis in long bones (physis) is a temporary hyaline cartilage joint that is eventually replaced by bone. The unions between ribs and costal cartilages are permanent hyaline cartilage joints, although they can be partially ossified with age (Miller et al. 1967). The unions between the right and left mandibular bodies (mandibular symphysis) and right and left os coxae (pelvic symphysis) are examples of fibrocartilaginous joints. The mandibular symphysis persists throughout life, but the pelvic symphysis ossifies in the adult (Miller et al. 1967). The unions of vertebrae (intervertebral disks) are special fibrocartilaginous joints consisting of a collagenous ring (annulus fibrosus) surrounding a space filled with semifluid material (nucleus pulposus).

Synovial Joints. These joints are the truly movable articulations. All synovial joints feature a capsule, cartilaginous articular surfaces, joint cavity, and a lubricating fluid. A few synovial joints also possess intra-articular ligaments, fibrocartilaginous plates (menisci), and fat pads (Miller et al.

1967). The fibrous joint capsule is continuous with the periosteum of the bone ends and encloses the joint cavity. The inner layer (intima) of the joint capsule lines the cavity except on the surfaces of the cartilage and is responsible for the production and turnover of the fluid (synovia) that lubricates the joint and nourishes the articular surfaces (Jubb et al. 1985). The articular surfaces of all principal synovial joints are covered with typical hyaline cartilage that is thickest in the young animal and at sites of maximum weight bearing. The normal capsule is strong but not rigid. Normal intima is smooth and glistening. The articular cartilage in young and healthy animals is smooth, white or somewhat bluish, semitransparent, and moist. The normal synovia is viscous, clear, colorless, or slightly yellow (Doige 1988; Jubb et al. 1985).

Muscle

Muscles are specialized collections of cells that produce directed movement through strong organized contractions (Venable and Dellmann 1976). Muscles are customarily classified as smooth, cardiac, and skeletal.

Smooth Muscle. This type of muscle is found in the walls of the digestive, respiratory, urinary, and reproductive tracts; blood vessels; spleen; and eyeball. It is also associated with glands and hair follicles (Goll et al. 1977; Miller et al. 1967). Smooth muscle has a simple appearance consisting of spindle-shaped (tapered) cells without cytoplasmic cross-striations and a single, centrally placed nucleus. Smooth muscle is commonly referred to as visceral muscle (by its location) or involuntary muscle (by its innervation), and has also been called plain, or unstriated, muscle (Miller et al. 1967).

Cardiac Muscle. This type of muscle forms the bulk of the heart and combines the features of smooth and skeletal muscles. As in smooth muscle, the nuclei are centrally placed and the fibers are under involunatry nervous control. As in skeletal muscle, the fibers are cross-striated and multinucleated. Cardiac muscle has also been called involuntary striated muscle.

Skeletal Muscle. This type of muscle is so named because of its relationship to the skeleton. Skeletal muscle comprises the single largest tissue mass in the body, at 40% of the body weight for mammals in general (Goll et al. 1977) and from 35% to 54% of the body weight of beagles (Andersen and Goldman 1970). Skeletal muscles range in size from the minute stapedius muscle of the middle ear to the massive muscles of the rump (Miller et al. 1967). Individual muscles are surrounded by and separated from other muscles by a sheet of connective tissue known as the epimysium (Greek *epi*: upon, above, beside). The epimysium can be fairly thick and tough and is the site of intermuscular fat deposits. At irregular intervals, thinner sheets of connective tissue, called perimysium (Greek *pen*: around), pass into the muscle and divide it into bundles (fasciculi). The perimysium also envelopes blood vessels and nerves and is the site of intramuscular fat deposits (Goll et al. 1977). Very delicate sheets of connective tissue, called endomysium (Greek *endo*: within), extend from the perimysium and surround individual muscle fibers. The endomysium lies immediately adjacent to the muscle cell outer membrane (sarcolemma). The endomysium carries a longitudinally oriented capillary network. Skeletal muscle fibers are striated and multinucleated, with the majority of nuclei located peripherally. Skeletal muscle is under control of the somatic or voluntary nervous system, and hence is also called somatic or voluntary muscle.

Muscle Fibers

Not all normal skeletal muscles have the same depth of color. Color variation depends on a number of factors, but is in part due to the relative percentages of type I and type II fibers. Histochemical techniques have allowed the division of skeletal muscle fibers into two major groups:

type I fibers, which are rich in oxidative enzymes, darker in color, and show a slow twitch response; and type II fibers, which are rich in glycogen, paler in color, and show a fast twitch response (Hulland 1985). The ratio of the two fiber types varies widely from muscle to muscle. In the dog, type I fibers comprise less than 15% of the extensor carpi radialis and over 90% of the vastus intermedius (Armstrong et al. 1982).

Necropsy and Laboratory Techniques

A "complete" postmortem examination rarely includes a complete examination of bones, joints, and muscles. Although a complete dissection of the musculoskeletal system is neither practical nor necessary, something more than a cursory examination is in order. Antemortem clinical findings, including clinical laboratory results, should be known. A brief visual examination and palpation of the body before exsanguination or skinning should reveal swollen or stiff joints, muscle wasting, and skeletal deformities and asymmetry. Certain bones, joints, and muscles should be routinely examined to provide completeness to the necropsy and to establish normal baseline values for color, hardness, size, volume, and so on. The prosector should be alert to general changes in the musculoskeletal system during the course of the postmortem examination. Ventral and lateral muscles of the neck and trunk are exposed during the primary ventral midline incision and reflection of the skin. Skin should also be reflected from the inner aspect of the limbs to expose the shoulder, elbow, and knee joints and major muscles of the legs. Several of the large medial muscles of the limbs must be cut to fully extend the legs and in the process large cross-sectional areas of muscle are brought to view. Several large synovial joints should be opened completely. The hindlimbs can be extended fully only by disarticulating the coxofemoral joints; however, in the process the joints are usually contaminated with blood from the femoral vessels. With practice, the shoulder, elbow, and knee joints can be opened without contamination, but the disarticulation must be done carefully to avoid slicing articular cartilage. Joints should be examined immediately on opening because articular cartilage rapidly dehydrates and discolors when exposed to air (Doige 1988). It is helpful to compare contralateral joints; however, the prosector should realize that under normal circumstances the synovial fluid might have a different appearance from joint to joint within an animal and in the same joint between animals (Jubb et al. 1985). An additional opportunity to examine a large synovial joint arises when the atlanto occipital joint is disarticulated to remove the head.

During removal of the spinal cord (as a whole or in segments) the surface of the spinal canal overlying intervertebral disks should be examined. The ventral surface of the vertebral column should be palpated following removal of the thoracic, abdominal, and pelvic viscera. Bone strength can be assessed as the ribs, calvarium, and os coxae are cut while opening the thoracic, cranial, and pelvic cavities. Jaws and teeth should be examined during removal of the tongue and larynx. At least one long bone (preferably a femur) should be examined in detail and fixed for microscopic evaluation. The femur should be cut in a standardized, midline longitudinal plane to establish a baseline for normal width of cortical bone, density of cancellous bone, width and uniformity of physes, curvature of articular surfaces, and relative proportion of red and yellow marrow. The marrow contents can be flushed away by a jet of water from one of the halves for better exposure of bony structure.

For good fixation, bone slabs should be no wider than 5 mm; therefore, additional bone will need to be cut from the proximal and distal ends of the femur. The femur is collected primarily for the study of osseous, cartilaginous, and articular tissues. An additional bone, preferably a flat bone such as a sternebra, should be collected specifically for bone marrow cytology. The sternebrae of young dogs can be cut with a stout sharp knife, thus avoiding the problem of the marrow surface being filled with bone and muscle debris from a saw.

Skeletal muscle is usually one of the last tissues collected at necropsy. Delay in fixation is not a concern, for muscle is one tissue that can appear worse histologically when fixed immediately than when fixed hours, even days, after death (McGavin 1983). Artifacts are the problem. Fresh muscle is very sensitive and will vigorously contract when pinched, crushed, cut, stretched, and placed in most

fixatives. The resulting artifacts (severe contraction bands; sarcoplasmic masses; shredded, cracked, and rounded and hyalinized fibers) will mask and even mimic pathological change (McGavin 1983).

Another problem in interpretation confronts the microscopist when the histological sections contain mostly tangentially sectioned fibers. Both transverse and longitudinal sections of muscle are required for the proper interpretation of many pathological processes. Transverse and longitudinal sections can be obtained only from muscles in which the majority of the fibers are oriented parallel to each other. Three hindlimb muscles (biceps femoris, semitendinosus, semimembranosus) have such an orientation of fibers and are also large (for ease of sampling) and easily identified. In addition, each of these muscles presents a fairly good representation of type I and type II fibers. The following percentages of type I fibers were found by Armstrong et al. (1982): biceps femoris (32 ± 8), semitendinosus (27 ±11), and semimembranosus (28 ± 5). Once selected, the same muscle should be the site from which all samples are taken. To minimize the problem of artifacts, the muscle must be handled gently; but, most important, the muscle must be prevented from contracting during removal and fixation. McGavin (1983) recommends the following relatively simple procedure: Make two parallel incisions, 3 mm to 6 mm apart, into the belly of the muscle and in the same direction as the muscle fibers, suture each end of the isolated strap of muscle to a narrow, flat, wooden stick, undercut the muscle, sever the muscle beyond the sutures, remove the stick and muscle together, and place in fixative. Paraffin-embedded, H&E-stained sections of bone and muscle meet the needs of most toxicology studies. There are, however, a number of special laboratory techniques to study both bone and muscle. Many of the bone procedures have been used extensively in the beagle and include the measurement of bone ash, volume, and specific gravity; labeling sites of mineralization by fluorescent markers; and microscopic and microradiographic examination of undemineralized sections (Anderson and Danylchuk 1978, 1979a, 1979b, 1979c; Jee et al. 1970; Jorch and Anderson 1980; Kunkle et al. 1982; Martin et al. 1981; Norrdin and Shih 1983; Saville and Krook 1969; Snow et al. 1986). Bone specimens for routine H&E staining should be well-fixed, free of debris from the bone saw, not overly decalcified, and represent both cancellous and cortical bone, an articular surface, and a physis. McGavin (1983) lists special stains and enzyme histochemical procedures to study muscle. As mentioned, H&E-stained specimens of muscle should always include transverse and longitudinal sections.

Pathology

Few spontaneous findings are described for the musculoskeletal system. Hottendorf and Hirth (1974) report a fractured rib in 1 (0.1 %) of 1,000 dogs and chondrodystrophic changes in the costochondral junctions of 1 (0.1%) of 647 dogs. Hottendorf and Hirth (1974) also report congenital hernias (digestive system). In the authors' experience, these are usually small umbilical hernias containing only omental fat. Congenital hernias were observed grossly in 5 (0.5%) of 1,000 dogs. Barron and Saunders (1966) report the fortuitous finding of *Toxocara* granulomas in skeletal muscle.

Digestive System

Anatomy and Histology

The digestive system consists of a series of connecting, mostly tubular, hollow structures that includes the mouth, pharynx, esophagus, stomach, small and large intestines; and a group of accessory organs that includes the teeth, tongue, salivary glands, liver, gallbladder and pancreas. The tubular portion from the esophagus distally is the alimentary canal.

Mouth

The mouth (oral cavity) is the most anterior opening and cavity of the digestive system. Its limits anteriorly and anterolaterally are the upper and lower lips, posterolaterally the right and left

cheeks, dorsally the palate, and ventrally the floor of the mouth and tongue. Anteriorly, the palate has a bony core, and is termed the hard palate. Posteriorly, the palate has a muscular core, and is termed the soft palate. The soft palate is unusually long in the dog and can extend to or beyond the epiglottis (Miller et al. 1967). The gingiva is the keratinized epithelial membrane that covers the alveolar processes of the jaws and attaches to the teeth. The gingival tissue adjacent to the tooth surface is known as the free gingival margin. The dog usually has 42 permanent teeth (Miller et al. 1967), whose names and placement in the upper and lower dental arcades are shown in the following formula (I = incisor, C = canine, PM = premolar, and M = molar):

$$\text{I } 3/3 \text{ C } 1/1 \text{ PM } 4/4 \text{ M } 2/3 \times 2 = 42$$

According to their location, the incisors are known as central, intermediate, or corner; the premolars as first, second, third, or fourth; and the molars as first, second, or third. Deciduous (temporary) teeth immediately replace permanent teeth in the dog (Miller et al. 1967). In the beagle, the average age of eruption (in months) for permanent teeth is shown in table 8.12 (Bartley et al. 1970).

Permanent dentition should be complete in most beagles by the age at which they are placed on study.

The tongue forms most of the floor of the mouth. The posterior third of the tongue is the root and the slender anterior two-thirds is the body. A long mucosal fold (frenulum) connects the body of the tongue to the floor of the mouth.

Pharynx

The mouth is continuous posteriorly with the pharynx, a funnel-shaped connection between the mouth and the esophagus and between the nasal cavity and the larynx. The pharynx serves both digestive and respiratory functions. An exclusively respiratory portion (nasal pharynx) lies above the soft palate. The portion below the soft palate (oral pharynx), serves a mixed digestive and respiratory function, being respiratory during panting. The nasal and oral pharynges unite just posterior to the soft palate to form the pharyngeal isthmus, where the digestive and respiratory tracts change relationships. The respiratory tract continues ventrally as the larynx and thereafter as the trachea. The digestive system continues dorsally as the laryngeal pharynx and thereafter as the esophagus. The lateral walls of the nasal pharynx are obliquely pierced by two slit-like openings, the pharyngeal ostia of the auditory tubes. The lateral walls of the nasal pharynx are indented by two crypts that contain long, thin lymphoid structures, the palatine tonsils.

Salivary Glands

Salivary glands are the first of the accessory organs to discharge secretions into the digestive tube. Salivary glands are classified by size (major, minor) and by secretion (mucous, serous, mixed),

Table 8.12 Average Age (in Months) of Beagle at Tooth Eruption

	Central	Intermediate		Corner
Incisor	3.8	4.1		4.4
	3.9	4.1		4.1
Canine	4.7			
	4.7			
	First	**Second**	**Third**	**Fourth**
Premolar	3.5	5.0	5.1	4.5
	4.3	5.0	5.2	5.2
Molar	4.4	5.2	—	—
	4.3	5.0	5.8	—

and are named by location. All major salivary glands are paired and include the parotid, mandibular, sublingual, and zygomatic glands (Miller et al. 1967). The individual and collective secretions of the salivary glands are discharged into both the dorsal and ventral parts of the mouth. Dorsally, most secretions come from the parotid and zygomatic glands and ventrally from the mandibular and sublingual glands. The saliva of the dog has no enzymatic activity of note (Harvey et al. 1983).

Parotid Gland. This is a coarsely lobulated and reddish V-shaped organ that closely embraces the base of the ear. It is predominantly serous, but occasional isolated mucous secretory units can be found (Stinson and Calhoun 1976). The parotid duct opens into the mouth lateral to the upper fourth premolar tooth.

Mandibular Gland. This is a lightly lobulated and light tan ovoid body lying just caudal and ventral to the parotid gland. It is a mixed gland (Stinson and Calhoun 1976) and its ducts open below the tongue near the frenulum.

Sublingual Gland. This gland consists of a series of two or more elongated, lobulated masses extending from the mandibular gland to the anterior margin of the frenulum. The most posterior portion of the sublingual gland is enveloped by the capsule of the mandibular gland (Miller et al. 1967). Grossly, the sublingual gland is distinguished from the mandibular gland by its darker color; however, its subcapsular location within the mandibular gland might puzzle the microscopist when first seen. The sublingual gland is a mixed gland (Stinson and Calhoun 1976) with ducts that empty onto the floor of the mouth.

Zygomatic Gland. This gland is a moderately lobulated gland located beneath the zygomatic arch, ventral and posterior to the eye. The zygomatic gland is predominantly mucous, but a few serous demilunes are present (Stinson and Calhoun 1976). Zygomatic gland secretions enter the mouth through several openings lateral to the upper molar teeth.

Minor Salivary Glands. These glands consist of clusters of serous, seromucous, or mucous elements found in various oral structures, including the tongue, lips, palate, and pharynx.

Esophagus

The esophagus is the first part of the alimentary canal and connects the laryngeal pharynx with the stomach. The esophagus can be divided into cervical, thoracic, and abdominal segments. The cervical esophagus extends from the pharynx to the thoracic inlet, the thoracic esophagus extends from the thoracic inlet to the diaphragm, and the abdominal esophagus is the short segment between the diaphragm and the stomach. The abdominal esophagus acts as a flutter valve and is important in preventing gastroesophageal reflux (Strombeck 1979). The esophagus is capable of great distention except at its beginning and end and as it passes through the thoracic inlet. The collapsed mucosa forms numerous longitudinal folds. In the dog, the entire length of the mucosa is covered by nonkeratinized stratified squamous epithelium. The submucosa contains numerous mucous glands with serous demilunes, and the tunica muscularis is comprised entirely of two oblique layers of striated muscle fibers (Miller et al. 1967).

Stomach

The stomach is the largest dilatation of the alimentary canal. A line drawn through the axis of the stomach appears somewhat like a letter C. The longer, outer convex surface of the organ is the greater curvature. The shorter, inner concave surface is the lesser curvature. The walls between the two curvatures are the dorsal and ventral walls. The inlet from the esophagus is the cardiac ostium and

the outlet into the small intestine is the pyloric ostium.* For gross, microscopic, and functional purposes, the stomach can be divided into five, three, and two regions, respectively (Strombeck 1979). Grossly, the regions consist of a narrow zone between the esophagus and body known as the cardiac region. The body is the large middle region, and the fundic region is the blind outpocketing of the body located to the side of the cardia on the greater curvature. The terminal, funnel-shaped, one-third of the stomach is the pyloric region. The pyloric region is subdivided into an initial thin-walled portion (pyloric antrum) and a terminal thick-walled portion (pyloric canal). The normal color of the cardiac, fundic, and body mucosa is pink to grayish red. The pyloric mucosa is a pale tan. The mucosa of an empty or even moderately distended stomach is thrown into numerous folds (gastric rugae).

Close examination of the gastric mucosa reveals that it is comprised of minute raised areas (areae gastricae) surrounded by narrow furrows. This block-like subdivision of the gastric mucosa can be recognized in tissue section, especially in the pyloric region. The gastric mucosa in the dog is entirely glandular.

According to its glandular makeup, the stomach is histologically divided into cardiac, gastric gland proper, and pyloric regions (there is intermixing of glands between adjacent regions). The cardiac and pyloric regions comprise primarily mucous glands. Cardiac glands are characterized by deep gland openings (foveolae) and short gland bodies. Pyloric glands have foveolae and bodies of about equal length. The body and fundic regions are populated principally by gastric glands proper (sometimes erroneously called fundic glands), which have comparatively shallow foveolae and long bodies. The gland bodies are lined by mucous neck, parietal (oxyntic), chief, and argentaffin cells. Small numbers of parietal and argentaffin cells are also found in the pyloric region and parietal cells also can be found in the cardiac region. The submucosal mucous glands of the esophagus extend into the cardiac region (Stinson and Calhoun 1976). Similarly, the submucosal glands of the duodenum (Brunner's glands) extend into the submucosa of the pyloric region.

Functionally, the proximal two-thirds of the stomach (body and fundus) adapts by expansion to serve as a storage reservoir and the caudal one-third (pyloric region) performs as a grinding mill and funnel to propel ingesta into the small intestine (Strombeck 1979). Studies of the rate at which digesta move through the GI tract of the dog reveal that at 8 hr, roughly 90% of the meal is in the stomach and roughly 10% has entered the small intestine (Stevens 1977). At 12 hr, approximately equal quantities are found in the stomach, small intestine, and colon. At 24 hr, roughly 30% remains in the stomach, 10% is in the small intestine, and 30% each in the colon and feces. The bulk of the meal (90%) has been evacuated at 38 hr.

Small Intestine

The small intestine extends from the stomach to the colon. It is the longest portion of the alimentary canal, representing about 85% of length of the entire intestine (Stevens 1977). (A length of 225–290 cm has been reported for the small intestine of the adult beagle; Andersen 1970). The small intestine represents only 23% of the total capacity of the GI tract. The stomach, cecum, colon, and rectum represent 62%, 1%, and 13%, respectively (Stevens 1977).

There are no gross features to definitely separate the three segments of the small intestine and the beginning (duodenum), middle (jejunum), and end (ileum) must be set arbitrarily.

Duodenum. This is considered to be the proximal one-tenth, or 25 cm, of the small intestine (Miller et al. 1967). The duodenum is divided into cranial, descending, caudal, and ascending portions. Brunner's glands are limited primarily to a narrow region of the pyloric duodenal junction (Titkemeyer and Calhoun 1955). Pancreatic and bile ducts discharge into the descending duodenum. The common bile and ventral pancreatic ducts share a small protuberance (major duodenal papilla) found 3 to 5 cm

* The term cardia has not been used because it has variously referred to the cardiac region, ostium, and sphincter. Similarly, the term *pylorus* was not used because it has variously referred to the pyloric region, antrum, canal, ostium, and sphincter.

caudal to the pyloric ostium. The dorsal pancreatic duct opens on a smaller protuberance (minor duodenal papilla) located about 5 cm caudal to the major papilla. To the uninitiated, the duodenal papillae might appear to be tumorous enlargements in the gut wall. Whereas the pancreatic ducts penetrate the gut wall more or less at right angles, the common bile duct courses intramurally for a distance of 1.5 cm to 2.0 cm. The microscopist should be aware of the lengthy intramural placement of the bile duct and not misinterpret its presence as an anomaly or tumor.

Jejunum and Ileum. These comprise the bulk of the small intestine. Most veterinary anatomists consider the jejunum to be substantially longer than the ileum and generally regard only the short, usually contracted, terminal part of the small intestine as ileum (Miller et al. 1967). The circularly arranged folds (plicae circulares) that characterize the small intestine of humans and some domestic animals are absent in the dog. The intestinal glands (crypts of Lieberkuhn) are long in the dog, resulting in villus-length:crypt-depth ratios of 2:1, 1:1, or even less (compare to ratios of 4:1 or greater in species such as mice, rats, pigs, and humans). Dogs lack Paneth cells (Stinson and Calhoun 1976).

Aggregated lymph follicles (Peyer's patches) are easily recognized in the dog from either the exterior or interior surface of the small intestine. Generally, the patches appear on either side of the mesenteric attachment. On the mucosal surface, Peyer's patches appear as ovoid elevations measuring about 1.5 cm by 2.0 cm. An average of 22 Peyer's patches have been reported for the dog (Titkemeyer and Calhoun 1955). They exist throughout the length of the small intestine but most are found in the duodenum and jejunum. Villi overlying Peyer's patches are generally short and oddly shaped, might even be entirely effaced, and in general are not representative of villi elsewhere in the gut. Peyer's patches, solitary lymph follicles, tonsils, and the diffuse lymphoreticular tissue of the digestive system comprise the gut-associated lymphoid tissue (GALT).

Large Intestine

The large intestine of the dog is short, representing about 13% of the entire length of the intestine (Stevens 1977), and it lacks special features such as sacculations, bands, and a verniiform appendix found in other species. The large intestine begins at the ileocolic orifice and ends at the anus, and includes the cecum, colon, rectum, and anal canal.

Colon. This is the most proximal segment of the large intestine. It is about 25 cm long and is divided into ascending, transverse, and descending portions. The cecum is a relatively short and small diverticulum of the ascending colon and communicates with the colon only through the cecocolic orifice. (Some authors erroneously refer to an ileocecal valve and orifice.) The descending colon is the longest and straightest portion of the colon. The large intestine continues as the short (about 5 cm long), entirely intrapelvic portion known as the rectum. The anal canal is the terminal 1 cm of the alimentary tube. For its length, the anal canal is very complex, with three mucosal zones, two types of glands, and the site of drainage for the anal sacs. Solitary lymph nodules are found throughout the mucosa of the entire large intestine; however, they tend to be particularly large in the rectum and often appear as raised nodules, 3 mm to 4 mm in diameter, with central crater-like depressions. The depressions are due to circular discontinuities in the muscularis mucosae, allowing intimate mixing of intestinal glands and submucosal lymphoid aggregates. Solitary lymph nodules are also found in the esophagus, stomach, small intestine, and gallbladder mucosae.

Liver

The liver is undoubtedly the digestive organ of greatest interest in toxicology studies. It is a large organ in keeping with its many functions. The liver represents about 7% of the body weight of 2- to 3-month-old pups and about 4% in adults (Andersen 1970). The liver is divided into four

lobes: left, right, quadrate, and caudate. The left lobe is the largest, comprising up to one-half of the entire liver. Both the left and right lobes are divided into sublobes, which are called lobes nevertheless. On the left, the larger sublobe is the left lateral lobe. Medial to it is the left medial lobe. The right hepatic lobe is divided into the right medial lobe and the right lateral lobe. The quadrate lobe lies between the right and left hepatic lobes. The gallbladder lies partially in a depression (fossa) on the right side of the base of the quadrate lobe. The left half of the fossa is formed by the base of the right medial lobe. The caudate lobe is the most irregularly shaped and most caudally placed lobe of the liver. Its most cranial portion is called the papillary process. The caudate process is marked by a deep impression for the right kidney. The fresh liver is reddish brown, firm to the touch, yet pliable. Normally, the borders (margins) are sharp-edged and the cut surface does not bulge. The capsular surface is patches tightly covered by a thin, transparent membrane (peritoneum). Close inspection reveals about 1-mm-sized subunits (hepatic lobules) that vary considerably in prominence according to the physiological and pathological state of the liver.

Gallbladder

The gallbladder is a pear-shaped vesicle that lies between the quadrate and right medial lobes of the liver. It has a capacity of 10 to 15 ml (Andersen 1970). When fully distended, the gallbladder is visible on the diaphragmatic surface of the liver. The emptying of the gallbladder is related to gastric digestion; during fasting it remains distended. The gallbladder has a neck, body, and a blind, rounded, cranial end known as the fundus. Bile is formed in the liver and stored and concentrated in the gallbladder. The dog has a high concentrating ability (Stevens 1977). Bile reaches the gallbladder by way of the hepatic and cystic ducts. The cystic duct extends from the neck of the gallbladder to the first hepatic duct. The common bile duct extends from the junction of cystic and hepatic ducts to the major duodenal papilla. Bile can be watery to mucoid in consistency and greenish yellow to golden brown in color. Dark green flecks are not unusual. The gallbladder mucosa is normally smooth but solitary lymph follicles might cause a slight roughness.

Pancreas

The pancreas is a V-shaped gland formed by the fusion of a slim right lobe and a shorter, but thicker and wider left lobe. The left lobe lies in the greater omentum adjacent to the stomach. The right lobe lies in the mesoduodenum adjacent to the descending duodenum. Numerous variations in the pancreatic duct system have been described in the dog (Miller et al. 1967). Most commonly, the larger ventral pancreatic duct drains the right lobe and the smaller dorsal pancreatic duct drains the left lobe. The ventral pancreatic duct terminates as a slitlike opening adjacent to the common bile duct on the major duodenal papilla. The dorsal pancreatic duct opens on the minor duodenal papilla. The pancreas is soft, coarsely lobulated, and has a pinkish gray coloration in life. The pancreatic islets of the dog usually are not visible to the naked eye.

Necropsy and Laboratory Techniques

Disease of the digestive system produces a wide variety of clinical signs. Some signs are nonspecific (anorexia, weight loss, pain, weakness, reluctance to move, generalized malaise, shivering, fever, dehydration, hemorrhage, anemia), and some signs are shared with diseases of other organs (depression, coma, polydipsia, polyuria), but many signs indicate disease somewhere within the digestive system.

The most common signs associated with problems in the upper digestive tract (mouth, pharynx, esophagus, stomach, proximal small intestine) are vomiting (forcible expulsion of ingesta into the mouth), regurgitation (passive backward flow of ingesta into the mouth or nasal cavity), and dysphagia (difficulty in swallowing; Strombeck 1979). Additional signs of upper digestive tract

disease are halitosis (offensive breath), excessive salivation, hematemesis (blood in the vomitus), melena (black feces, digested blood), abdominal pain, and bloating. Diarrhea (an increase in the frequency, fluidity, or volume of feces) is the most consistent indicator of problems in the lower digestive tract (middle and distal small intestine, large intestine; Strombeck 1979). The character of the diarrhea (and other signs) often indicates what part of the intestine is diseased.

Signs of small intestine involvement include a large quantity of bulky or watery feces, undigested food in the feces, melena, weight loss, and generalized malaise (Sherding 1983). Signs of large intestine involvement include very frequent defecation, small volume of feces, urgency, tenesmus (painful straining), mucus in the feces, red-stained feces (fresh blood), and constipation.

Liver disease is also associated with numerous clinical signs, including abdominal pain, anorexia, ascites, coma, depression, dark brown urine, dark or light-colored stools, diarrhea, fever, hemorrhage, icterus, polydipsia (excessive thirst), polyuria (abnormally large volume of urine), pruritus, weakness, weight loss, and vomiting (Strombeck 1979). Because the liver has a large functional reserve and an amazing capacity to regenerate, as much as 70% to 80% of the liver's mass must be impaired before signs of dysfunction are seen (Hardy 1983). The clinical signs of acute pancreatic disease include vomiting, pain, anorexia, depression, fever, diarrhea, abdominal distress (aside from pain, dehydration, shock, and respiratory distress; Strombeck 1979). Signs of chronic pancreatic disease (pancreatic atrophy) include diarrhea, steatorrhea (excessive amount of fat in the feces), and weight loss. With these signs in mind and all available clinical and laboratory data at hand, the prosector may undertake examination of the digestive system.

The oral cavity should always be examined, preferably after anesthesia and before exsanguination. The breath of a healthy dog is usually not unpleasant. The normal oral mucosa is pink, has a smooth and glistening surface, and shows little accumulation of saliva (Harvey et al. 1983). Normal free gingival margins might be slightly hyperemic. The oral cavity can be fully explored after removal of the tongue and pharynx. A cross-section of the body of the tongue is usually taken to represent the oral mucosa histologically. The parotid and mandibular salivary glands should be inspected. The mandibular gland is usually chosen to histologically represent the salivary gland system. The mandibular gland is a reasonable choice, for it is readily accessible and contains both mucous and serous secretory units.

The initial examination of the thoracic and abdominal viscera should be done with all organs in place and natural relationships undisturbed. This is particularly true when a dilated, flaccid esophagus is found (megaesophagus), as one possible cause is a persistent right aortic arch. The tongue, pharynx, larynx, esophagus, and trachea are usually removed intact along with the heart, great vessels, thymus, and lungs. The esophagus should be completely opened, examined, and a midsection collected for histopathology. The pancreas is excised (after removal of the spleen and liver), examined, and a specimen taken (consistently from the right or left lobe) for histopathology. The GI tract is removed. (The pelvic girdle must be cut through at the obturator foramina to excise the rectum and anal canal.) The suspensory ligaments, omenta, and mesentery can be stripped from the GI tract as it is removed. The tract should be completely opened and the entire length examined. The stomach unfolds nicely when opened along the greater curvature. Bulk digesta and feces can be picked from the surface. Brief flushing with tap water might be required to remove adherent material. Sloshing a specimen in saline or fixative might be desirable for critical examination of lesions. A complete sampling of the GI tract would include two samples of the stomach (body and pyloric regions), cranial duodenum (to include Brunner's glands), jejunum (midportion), ileum (a few centimeters from the ileocolic junction), entire cecum, and a segment of the descending colon and rectum. One of the jejunum and ileum specimens should include a Peyer's patch.

All of the specimens (except the cecum) are preferably fixed in a flattened position. This can be done by stapling the specimens (serosal surface down) to small pieces of corkboard. The boards can be labeled to identify the specimens. The gallbladder also should be fixed in a flattened position. All surfaces of the liver should be examined and multiple parallel slices made through each lobe.

Slices of tissue (no wider than 5 mm) should be taken from at least two lobes for histopathology. The right and left lateral lobes are suggested.

Pathology

Numerous observations have been reported for the digestive system of young laboratory beagles. Most of the observations are of no clinical or experimental significance. A few might complicate interpretation of apparent treatment-related effects and the pathogenesis of several have never been explained.

Mouth and Salivary Glands

Changes in the general health might be evident during examinations in the oral mucosa. Anemia results in pale mucous membranes. Icterus (jaundice) results in a yellowish appearance of the mucous membranes associated with circulating bile pigments. Icterus can be caused by excessive hemorrhage, lysis of red blood cells (hemolysis), gallbladder or bile duct obstruction, and liver diseases involving the bile ducts. Oral papillomas (papillomatosis or warts) are benign epithelial tumors caused by papovaviruses. They occur mainly in young dogs and can spontaneously disappear. The warts can be found on the lips, inside the cheeks, and on the tongue, palate, and pharynx. The gums are usually not affected. Oral papillomas were observed grossly in 2 (0.2%) of 1,000 dogs (Hottendorf and Hirth 1974).

Teeth and Gums. A number of minor dental abnormalities might be encountered, such as missing permanent teeth (usually upper and lower premolars), retained deciduous teeth (usually canine), imperfect apposition of teeth, dental plaque (soft bacterial masses), and dental calculus or tartar (mineralized plaque, usually discolored, most abundant next to orifices of salivary ducts). Inflammation of the gums (gingivitis) is often associated with plaque or tartar around the teeth.

Tongue. In addition to oral papillomatosis affecting the tongue, inflammation of the tongue (glossitis) was an infrequently observed lesion. Glossitis was reported in 1% to 2% of young dogs (Glaister 1986). Granulomatous inflammation can result from fragments of sawdust bedding embedded in the tongue.

Salivary Glands. Inflammation (sialoadenitis) is common with mild focal sialoadenitis reported in 35 (5%) of 647 dogs (Hottendorf and Hirth 1974).

Esophagus

Megaesophagus (grossly dilated and flaccid esophagus) might be seen. This is a congenital condition occasionally due to a persistent right aortic arch, but more commonly due to an apparent neuromuscular developmental disorder.

Esophageal hypertrophy is characterized by a swollen, thick-walled esophagus that suggests reflux esophagitis. Inflammation of the esophagus (reflux esophagitis) can occur as an erosive and ulcerative lesion of the esophageal wall in dogs with histories of repeated regurgitation or vomiting. Slight dilatation of esophageal gland ducts can be seen in an otherwise normal esophagus.

Stomach

Chronic inflammation (gastritis) is a common finding, with 58 instances (9%) observed in 647 dogs (Hottendorf and Hirth 1974). Granulomatous inflammation was uncommon, with 1 instance (0.1 %) in 647 dogs as reported by Hottendorf and Hirth (1974). Gastric glands can be slightly

dilated. Microscopic mineralization (microcalculi) that occur as basophilic granules in the gastric mucosa, was reported in 7% to 10% of young beagles by Glaister (1986). Spirillum-like bacteria are frequently found within the lumina of gastric glands and within the intracellular canaliculi of parietal cells (Barker and Van Dreumel 1985; Weber et al. 1958). The bacteria incite no inflammatory response, but might be associated with dilatation of the canaliculi and gland lumina and cytoplasmic vacuolation of the parietal cells. Henry et al. (1987) indicated that large numbers of gastric spirilla can induce lymphoreticular hyperplasia and atrophy of parietal cells. Lymphocytic nodules or follicles can be present within the lamina propria, particularly of the pyloric region.

Intestine

Congestion of blood vessels in the GI mucosa is common. This congestion can result from digestive processes, but can also be secondary to inflammation and restricted blood flow or occur as agonal changes.

Inflammation of the large (cecitis and colitis) and small (enteritis) intestines was very common. Catarrhal enteritis was reported in 148 (23%) of 647 dogs and mild focal cecitis or colitis was reported in 25 (4%) of the 647 dogs (Hottendorf and Hirth 1974). Parvovirus causes acute enteritis in weaned dogs, but is largely controlled by vaccination. Granulomatous inflammation was reported in both large and small intestines. Toxocara granulomas occur in the wall of the intestine (Barron and Saunders 1966). Granulomas were observed in the large intestine of 1% to 3% and in the small intestine of 24% of young beagles (Glaister 1986). Small intestine granulomas were reported in 6 (0.9%) of 647 dogs (Hottendorf and Hirth 1974).

Mild hyperplasia of individual or aggregate lymph follicles in the lamina propria might be seen. Usually these hyperplastic follicles correlate with an increased prominence of grossly observed nodules.

Intussusception was observed grossly in 1 (0.1 %) of 1,000 dogs (Hottendorf and Hirth 1974). Fritz et al. (1967) reported a congenital defect, Meckel's diverticulum, in the small intestine. Hernias in which intestines can protrude though congenital defects in the abdominal wall, were observed grossly in 5 (0.5%) of 1,000 dogs (Hottendorf and Hirth 1974). Oghiso et al. (1982) report mucoepithelial cysts of the small intestine.

Many metazoan and protozoan parasites have been reported in laboratory dogs, largely dependent on the suppler. Nematodes are common, and were observed in 23% to 28% of young beagles (Glaister 1986). Most were ascarids in the small intestines. Ascarids are the common large roundworms of dogs, *Toxocara canis*, and *Toxocara leonina*. Usually in affected laboratory dogs, a few ascarids are observed in small numbers free in small intestine lumen. Ascarids were observed grossly in 165 (17%) of 1,000 dogs (Hottendorf and Hirth 1974) and reported in 2 (5%) of 37 untreated beagle dogs (Pick and Eubanks 1965). Strongyloides (*Strongyloides sterocoralis*) are small thread-like nematodes found in the small intestines, which were reported in 9 (1%) of 647 dogs (Hottendorf and Hirth 1974). Whipworms, *Trichuris vulpis,* which occur in the cecum and colon, are uncommon in laboratory dogs. Trichuriasis was reported in 7 (19%) of 37 untreated beagle dogs (Pick and Eubanks 1965). Hookworms (*Ancylostoma spp.*) usually affected young dogs and can result in pale oral membranes, anemia, and reduced growth rate. Heavy infections can result in black tarry feces. Ancylostomiasis was reported in 1 (3%) of 37 untreated beagle dogs (Pick and Eubanks 1965). Tapeworms were observed grossly in 8 (0.8%) of 1,000 dogs (Hottendorf and Hirth 1974). The most common tapeworm is the "cucumber seed" *Dipylidium caninum,* which is usually asymptomatic, but can result in diarrhea and anal pruritus. Giardiasis is important in young dogs. The disease can result in intermittent or chronic diarrhea that might persist for several months, accompanied by malabsorption of nutrients with reduced growth rate, weight loss, dull hair coat, and other clinical signs. It can be diagnosed clinically on the basis of cysts or trophozoites found in fecal samples. Generally, there is no evidence for histopathological diagnosis of the disease. Coccidiosis in young dogs can result in diarrhea and dehydration. Infections with *Isospora bigemina* can cause intestinal hemorrhage.

Liver

Gross and microscopic findings are very frequently observed in the liver. Andersen (1970) reported subcapsular cysts filled with serous fluid. Granulomatous inflammation has several forms. Granulomas were observed in 2% to 4% of young beagles (Glaister 1986) and in 23 (4%) of 647 dogs (Hottendorf and Hirth 1974). Also, Maita et al. (1977) reported small granulomas in livers. The most common histological lesions in the liver of young beagles are small focal collections of histiocytes, lymphocytes, and an occasional neutrophil, at times accompanied by a few degenerate hepatocytes. The foci are commonly referred to as microgranulomas. Their pathogenesis and significance appear to be unknown. Granulomatous aggregations of mononuclear cells were reported by Oghiso et al. (1982). Granulomas can occur as a result of larvae of *Toxocara canis* molting or dying during their migration through the liver on the way to mature in the intestine (Barron and Saunders 1966). Eosinophilic phlebitis and periphlebitis suggestive of hypersensitivity reactions to migrating parasites were also present. Focal inflammation of veins (phlebitis) was very common and occurred in 409 (63%) of 647 dogs (Hottendorf and Hirth 1974). Leukocyte foci are very common, and occurred in 47% to 60% of young beagles (Glaister 1986).

Mild portal inflammation with and without bile duct hyperplasia was reported in 50 (8%) of 647 dogs (Hottendorf and Hirth 1974). Inflammation of bile ducts (cholangitis) occurred in association with necrosis or other inflammatory changes and was not reported as a primary finding in control beagles. Bile duct hyperplasia was observed in 2 (0.3%) of 647 dogs (Hottendorf and Hirth 1974). Hepatocyte necrosis occurs in several forms and can be associated with several degenerative and inflammatory changes. Oghiso et al. (1982) reported the presence of necrosis, whereas Maita et al. (1977) reported necrosis at the base of hepatic ligaments. Focal necrosis and inflammation were very common and reported in 423 (65%) of 647 dogs (Hottendorf and Hirth 1974). Focal subcapsular lipidosis and necrosis seen near the hilus of the liver at the attachment of hepatic ligaments is compatible with so-called tension lesions seen in other species. These changes result from tension on the liver capsule and localized anoxia of adjacent hepatocytes. Hottendorf and Hirth (1974) report small, light yellow foci in the caudate lobe near the porta hepatis. Rectangular or cubic acidophilic inclusions in the nuclei of hepatocytes (and renal tubular cells) have been frequently seen in the beagle and other dogs (as well as wolves, foxes, and jackals; Richter et al. 1965; Thompson, Cook, et al. 1959; Thompson, Wiegand, et al. 1959). These structures are commonly referred to as acidophilic intranuclear inclusions (ACN). They appear to be protein in nature. Their significance and origin remain obscure. They were described as cubic or rectangular intranuclear hyaline bodies by Maita et al. (1977), acidophilic intranuclear inclusions by Oghiso et al. (1982), and intranuclear rhomboid inclusions by Hottendorf and Hirth (1974). They were observed in 2 (0.3%) of 647 dogs (Hottendorf and Hirth 1974). Hepatocytes of beagles also contain acidophilic, globular intracytoplasmic inclusions of equally obscure origin and significance (Harleman et al. 1987; Holmes and Smith 1969; Murti and Borgmann 1965). These structures are commonly referred to as periodic acid-Schiff (PAS) positive, nonglycogenic intracytoplasmic inclusions. Studies indicate they consist of proteinaceous material and bound lipids. Andersen (1970) and Maita et al. (1977) reported these PAS positive cytoplasmic inclusions. Small brown pigment granules can be seen in both hepatocytes and Kupffer cells. In the absence of biliary stasis, the pigment is usually lipofuscin or hemosiderin or both. Lipofuscin ("wear-and-tear" pigment) is PAS- and oil red 0-positive. Hemosiderin is iron-positive. Individual hepatocytes and Kupffer cells can contain both pigments. Oghiso et al. (1982) and Maita et al. (1977) reported lipofuscin deposition. Bile pigments might be present if biliary stasis is present.

Hepatocytic fatty changes also termed vacuolar degeneration, fatty degeneration, fatty metamorphosis, or lipidosis occur in young dogs. Oghiso et al. (1982) reported vacuolar degeneration and fatty degeneration. Focal fatty change was observed grossly in 78 (8%) of 1,000 dogs (Hottendorf and Hirth 1974). Focal subcapsular lipidosis and necrosis are associated tension lesions at the base of hepatic ligaments near the hilus of the liver. Glucose is normally stored within hepatocytes as glycogen. This storage results in hepatocytic cytoplasmic vacuolation. Large amounts of glycogen are expected

after a meal; however, surprising degrees of cytoplasmic vacuolation due to glycogen can be seen in dogs fasted overnight. Severe and widespread vacuolation of hepatocytes was observed in a young laboratory beagle with diabetes mellitus. Superficial necrolytic dermatitis was also present (Yoshida et al. 1996).

Gallbladder

The early stages of cystic mucinous hyperplasia can be seen. Individual lymphoid follicles might be prominent. Fine brown or black crystalline gallstones were reported by Maita et al. (1977).

Pancreas

Ovoid, acidophic intracytoplasmic inclusions (often containing basophilic particles and surrounded by halos) may be seen in acinar cells of the pancreas. Hartman et al. (1975) have shown the inclusions to consist of whirls of rough endoplasmic reticulum, vacuoles, and cytoplasmic organelles in various stages of decomposition and to be similar to dense ribosomal autophagic vacuoles.

Oghiso et al. (1982) reported cytoplasmic alternative changes in acinar and islet cells, without specific descriptions of the changes. Edema and unspecified cellular infiltrations were also reported by Oghiso et al. (1982). Chronic focal pancreatitis was reported in 8 (1.2%) of 647 dogs (Hottendorf and Hirth 1974). Toxocara granulomas were reported by Malta et al. (1977) and Barron and Saunders (1966).

The number and size of islets were markedly reduced in a young laboratory beagle with diabetes mellitus. Only glucagon-postive cells were demonstrated. The liver had severe hepatocytic vacuolation. Also a superficial necrolytic dermatitis was present (Yoshida et al. 1996).

Respiratory System

Anatomy and Histology

The respiratory system comprises the nasal cavity, paranasal sinuses, nasopharynx, larynx, trachea, and lungs. The principal function of the respiratory system is the exchange of gases (oxygen and carbon dioxide). Other functions include the warming, humidifying, and cleansing of incoming air; regulating airflow; olfaction; phonation; and temperature control of the whole organism (Dellmann 1976c). Important immunological and metabolic functions are also attributed to the respiratory system (Yates 1988).

Nasal Cavity

The nasal cavity is the facial portion of the respiratory system, and is also known as the internal nose (Miller et al. 1967). The part of the head known as the nose consists of an internal nose (a mucosa-lined cavity) and an external nose comprising bones (incisive, maxillae, nasal) and movable cartilages (nasal cartilages). The bones give rise to an elaborate system of bony scrolls (turbinates) that are covered by mucous membranes of the nasal cavity. The nasal cartilages surround the nostrils and nasal vestibule and direct the flow of air into the various passages (meatuses) among the scrolls. On inspiration, air enters the nasal cavity through paired nostrils (flares, singular: naris) and is drawn over four types of epithelium (stratified squamous, transitional, respiratory, olfactory; Adams and Hotchkiss 1983) before it leaves via the paired choanae (internal nares) to enter the nasopharynx. The rostral (anterior) 20% to 35% of the nasal cavity is lined by thick stratified squamous epithelium. Transitional epithelium lines the next 15% to 20%. Ciliated pseudostratified columnar (respiratory) epithelium lines the next 40% to 50%, and olfactory epitheliuin lines the remainder of the cavity. The area lined by respiratory epithelium is the most vascular of the four regions. Nasal glands, scattered

throughout the nasal mucosa, are present in greatest density in the olfactory region. Plasma cells are most abundant rostrally. Lymphocytes are present throughout the nasal mucosa, but frequently occur as nodular masses in the caudal portion, forming grossly visible nodules near the choanae (Adams and Hotchkiss 1983). The nasal and oral pharynges have been described with the digestive system.

Larynx

The laryngeal ostium is guarded by a cartilaginous valve (epiglottis) that prevents inspiration of food and water and controls the volume of air entering the lower respiratory system.

Trachea

The trachea is a flexible tubular connection between the larynx and lungs. Its flexibility is derived from a skeleton of C-shaped hyaline cartilages connected longitudinally by fibroelastic tissue and closed dorsally by smooth muscle and connective tissue (Miller et al. 1967). The tracheal mucosa is covered by respiratory epithelium in which the population of individual cell types (ciliated, preciliated, basal, secretory) varies significantly anteriorly and posteriorly and even dorsally and ventrally (Schwartz 1987). Ciliated cells account for the bulk of the tracheal mucosal cells and are responsible for moving secretions. Glands of the tracheal mucosa are predominantly serous with occasional mucous acini (Dellmann 1976c).

Bronchi and Bronchioles

The trachea terminates by the formation of the right and left principal bronchi. The principal bronchi divide into lobar bronchi (secondary bronchi), which are named according to the lobe supplied. Lobar bronchi divide into segmental bronchi and the branching continues until respiratory bronchioles are formed. As bronchi penetrate into the lungs they become embedded in a sheath of loose connective tissue, which contains lymphatics, nerves, bronchial vessels, and pulmonary arteries. Bronchi are kept patent by overlapping curved cartilages. When terminal bronchioles reach a diameter of 1 mm or less, cartilages are no longer found (Miller et al. 1967). Bronchioles are lined by respiratory epithelium containing three general categories of secretory cells: serous, mucous, and nonciliated secretory bronchiolar (NBE or Clara) cells (Schwartz 1987). Clara cells are present mainly in the peripheral airways. Clara cells have been shown to be the site of cytochrome P-450 dependent mixed function oxidase activity in the lung (Gill 1982). Tubuloacinar mucous or mixed glands are located in the submucosa. Characteristically, glandular elements (and goblet cells) decrease as the bronchi decrease in size. In general, all bronchioles lack cartilage, the columnar epithelium gradually becomes cuboidal epithelium, and goblet cells are gradually replaced by Clara cells. Respiratory bronchioles give rise to alveolar ducts, alveolar sacs, and pulmonary alveoli. Alveoli are lined by three types of epithelium: type 1 alveolar epithelial cell (squamous pneumocyte), type 2 alveolar epithelial cell (secretory pneumocyte), and type 3 pneumocytes (brush cells; Schwartz 1987). Type 1 cells appear to line the alveolus and type 2 to produce pulmonary surfactant. The function of Type 3 cells is unknown. Intra-alveolar macrophages are also important components of alveoli. There appear to be permanent lymphoid structures beneath the bronchial mucosa (localized infiltrations of the lamina propria with lymphocytes have long been recognized) that are comparable to those that constitute the GALT of the intestine. The lymphoid structures in the lung have been called bronchus-associated lymphoid tissue or BALT (Gill 1982).

Lungs

The lungs (right and left) are divided into lobes. Each lung as an apical (cranial), cardiac (middle), and diaphragmatic (caudal) lobe. The right lung also has an intermediate (accessory) lobe.

The pulmonary pleura is thin and adheres tightly to the surfaces of the lung and follows all its irregularities. The pleura is covered by a pavement of flat mesothelial cells and contains elastic, collagenous, and smooth muscle fibers. The subserosa of the pleura contains a superficial lymph vessel system that drains the pleura through the interlobular septa to the hilus of the lung. A deep lymph vessel system is oriented around the bronchial tree. It too drains toward the interlobular septa. Distended portions of the superficial lymphatic system might be prominent enough to be seen grossly, usually as clear, interconnecting, threadlike tubules. Pulmonary lymphatics drain into the right, middle, and left tracheobronchial lymph nodes. Smaller, bronchopulmonary lymph nodes are occasionally seen. When present, they lie on the surface of primary bronchi.

The lung (in common with the liver) has two blood supplies: bronchial arteries that provide oxygen for the conducting airways, and pulmonary arteries that deliver blood to the alveoli for oxygen–carbon dioxide exchange. Bronchial arteries form plexuses in the bronchial wall. Pulmonary arteries branch more frequently than the airways and eventuality supply the most peripheral parts of the alveoli with blood via a capillary network. The origin and relationships of veins are not as well defined as that of the arteries.

Necropsy and Laboratory Techniques

Disease of the respiratory system produces a wide variety of clinical signs. Some of the signs might indicate pathology within a specific portion of the system, and others are less specific. Pathology of the nasal cavity can be indicated by nasal discharge, snorting, sneezing, and nasal rubbing. Disease of the larynx and trachea can be indicated by dyspnea (difficulty or distress in breathing), stridor (high-pitched, noisy respiration), gagging or retching (striving to vomit), and coughing. Coughing and dyspnea could also indicate problems in the lower respiratory tract, as does tachypnea (very rapid respiration) or hyperpnea (deep and rapid respiration). Rales (abnormal respiratory sounds heard on auscultation) might be detected. Many varieties of rales are described. Cyanosis (bluish discoloration of the skin and mucous membranes) might indicate problems in either the respiratory or cardiovascular system. Pulmonary cyanosis is the result of poor oxygenation of blood in the lungs. Many special procedures have been used in the morphological study of the respiratory system, including airway and vascular perfusion with a variety of fixatives, formalin vapor fixation of the lungs, whole lung sections (macrosections), thick and thin histological sections, vascular injection with colored latex, silicone rubber casts of airways, *in vivo* rapid freezing of the lung, stereology, and, of course, scanning and transmission electron microscopy (Dungworth et al. 1976). The complexities of the respiratory system dictate that a variety of techniques are required for adequate evaluation of toxic effects in inhalation studies. However, the basic necropsy and fixation procedures that are essential for inhalation studies are also recommended for general toxicology studies. The procedures add neither cost nor time to the postmortem examination. The critical step is perfusion of the lungs (or one or more lobes) with fixative either by the trachea or primary bronchus at a pressure that is adequate to inflate the lungs to approximately normal full expansion. After perfusion, the trachea (or bronchus) is closed (clamped or ligated) and the lung is fixed in the expanded state. Simply immersing pieces of collapsed lung in fixative is not acceptable except when the lungs are massively consolidated, edematous, or contain large solid tumors (Dungworth et al. 1976). Even in the presence of such lesions, it is usually worthwhile to attempt to fix a portion of the lung by airway perfusion. Airway perfusion should be done at 30 cm of fluid (water) pressure. (Ideally, the lungs should be supported in a bath of fixative while being perfused.) Airway perfusion not only restores normal dimensions and configurations to the lung, it also provides a large volume of fixative in intimate contact with all surfaces of respiratory tree. The disadvantages of airway perfusion are the dislocation of exudates and distension of the tissue spaces around pulmonary vessels (so-called edema artifact; Dungworth et al. 1976). The edema artifact is dealt with during the microscopic interpretation. Immersion fixation of an additional affected portion of lung can compensate for the displacement of exudates by perfusion.

The lungs should be in view as the thoracic cavity is opened. The customary approach is to catch a glimpse of the distended lungs as the diaphragm is incised close to its sternal and costal attachments. Healthy lungs collapse as air enters the thorax. Failure of the lungs to collapse should be noted by the prosector. The thoracic cavity is opened preferably by cutting the ribs just dorsal to costochondral junctions to give adequate exposure of the thoracic contents. As noted earlier, all of the thoracic organs should be examined in situ and the tongue, larynx, trachea, lungs, heart, great vessels, and thymus removed in toto. The larynx and trachea are incised along their dorsal surface (with the prosector being alert to unusual content) to the bifurcation of the trachea. The lumina of the primary and lobar bronchi are inspected. The extent to which major airways need to be opened depends on the type and amount of gross pathology. At least two lobes of the lung should be left intact for intrabronchial perfusion. A compromise must be made between thoroughness and practicality in deciding on the number of lobes to perfuse and the number of blocks to take from each lobe for microscopic examination. Perfusion of two lobes (one a cranial lobe, the other a caudal lobe) seems to be reasonable for general toxicology studies. After fixation, samples (blocks) should be taken from the dorsal (hilar) and ventral (peripheral) aspects of each lobe. The trachea is usually represented by a segment taken from the midsection. If the nasal cavity needs to be examined at necropsy, it can be split sagittally. If specimens are required, one or both halves of the nasal cavity can be fixed and tissue blocks removed after decalcification. Nasal passages should be gently flushed with fixative to remove air and any material that would prevent intimate contact between the nasal epithelium and the fixative. The frontal sinus should be opened as a routine matter when the calvarium is removed to expose the brain.

Pathology

Pathologic findings reported for the respiratory system in untreated young dogs have been largely limited to the lungs. Chronic tracheitis was observed in 11 (2%) of 647 dogs on 39 studies (Hottendorf and Hirth 1974). Inflammatory lesions of the lungs are the most frequent respiratory system findings. These lesions involve many locations and tissues, resulting in numerous diagnoses being reported including perivasculitis, peribronchiolitis, endobronchiolitis, bronchitis or bronchiolitis, bronchopneumonia, pneumonitis, focal interstitial pneumonia, fibrosing alveolitis, fibrous thickening of alveolar walls, pleuritis, subpleural fibrosis, pulmonary helminthiasis, granulomatous pneumonitis, and various pulmonary granulomas (Glaister 1986; Hottendorf and Hirth 1974; Oghiso et al. 1982; Pick and Eubanks 1965). Most of these pulmonary findings were thought to be due to infections by the lungworm, *Filaroides hirthi.* However, it should not be concluded that all pulmonary lesions are due to lungworm infection; some are believed to result from *Toxocara canis* infections, foreign body emboli from IV injection sites, inhaled sawdust bedding, and infectious agents.

Pneumonia due to *F. hirthi* infection is probably the most common nontreatment-related respiratory finding seen in the laboratory beagle. The true incidence of the disease in laboratory dogs (and the canine population at large) is unknown. Clinical signs of infection are rare. The diagnosis can usually be made at necropsy based on the finding of tan, green or gray subpleural nodules ranging from 1 mm to 5 mm (and larger) in diameter. A granulomatous response is evoked by dead or degenerating worms. Focal granulomatous interstitial pneumonitis is often seen when remnants of the parasite can no longer be identified. *F. hirthi* has a direct life cycle with infective first-stage larvae in the feces. Most pups are probably infected by their dams (Dungworth 1985). Hirth and Hottendorf (1973) first reported the pathological changes associated with *F. hirthi* infection in the laboratory beagle. The parasite has been further characterized in subsequent articles (Georgi and Anderson 1975; Georgi et al. 1975).

Granulomatous inflammation or granulomas were observed in 6% to 7% of young beagles (Glaister 1986) and in 75 (12%) of 647 dogs (Hottendorf and Hirth 1974). Granulomatous nodules were also reported by Oghiso et al. (1982). Granulomatous pneumonitis was observed in 13 (35%) of 37 untreated beagles (Pick and Eubanks 1965). Both lungworms and migrating ascarid larvae

can cause granulomas in the lungs. Living nematodes were observed in the lungs of from 2% to 6% of young beagles (Glaister 1986). Granulomas can occur as a result of larvae of *Toxocara canis* molting or dying during their migration through the lungs on the way to mature in the intestine. Barron and Saunders (1966) and Maita et al. (1977) reported Toxocara granulomas in the lungs. Vascular microgranulomas were attributed to emboli from IV injection sites in 8 (1%) of 647 dogs (Hottendorf and Hirth 1974). Cholesterinic granulomas were reported in 1 of 647 dogs (Hottendorf and Hirth 1974). Leukocyte foci are very common, and occurred in 17% to 18% of young beagles (Glaister 1986).

Bronchopneumonia is unusual in laboratory dogs because of vaccination programs against canine distemper. Canine distemper virus infections can cause viral pneumonias and predispose dogs to bacterial pneumonia. Bronchopneumonia was observed in 3 (8%) of 37 untreated beagles (Pick and Eubanks 1965). Bronchopneumonia apparently due to *Mycoplasma spp.* was reported in four laboratory beagles (Kirchner et al. 1990). Localized bronchitis and bronchiolitis occurred in 2% to 8% of young beagles (Glaister 1986). Perivasculitis and peribronchiolitis were observed in 329 (51 %) of 647 dogs (Hottendorf and Hirth 1974). Subpleural fibrosis and endobronchiolitis were reported in 139 (21%) of the 647 dogs (Hottendorf and Hirth 1974). Pneumonitis or interstitial pneumonia is common and occurred in 22% to 28% of young beagles (Glaister 1986). Focal interstitial pneumonia was observed in 111 (17%) of 647 dogs (Hottendorf and Hirth 1974). Fibrosing alveolitis, a fibrous thickening of alveolar walls, was observed in 1% to 3% of young beagles (Glaister 1986). Pleuritis occurred in 2% to 8% of young beagles (Glaister 1986).

Other findings in the lungs included atelectasis where areas of collapsed alveoli are present (Oghiso et al. 1982), as well as pulmonary hyperemia or congestion, and hemorrhage. Hyperemia or congestion occurs as an active component of inflammation or passive component of poor circulation from heart diseases and agonal events. Pulmonary hyperemia was observed in 2 (5%) and hemorrhage occurred in 1 (3%) of 37 untreated beagles (Pick and Eubanks 1965). Edema can accompany hyperemia and congestion. As noted earlier, edema must be distinguished from the artifact that can result during perfusion of the lungs. Anthracosis, pigmentation of the lungs, was reported in 19 (51 %) of 37 untreated beagles (Pick and Eubanks 1965). This finding is unusual because most laboratory dogs are not exposed to air pollution or coal dust.

Cardiovascular System

Anatomy and Histology

The cardiovascular system is a closed system of tubes (blood vessels) containing a liquid tissue (blood) that is circulated by a four-chambered, double pump (heart). Blood is a complex tissue that serves several functions, including respiratory (transport of oxygen from the air in the lungs to the tissues and carbon dioxide in the tissues to the lungs), nutritive (conveys food materials from the digestive tract to the tissues), excretory (transports waste products from tissues to organs of excretion), homeostasis (maintains a dynamic equilibrium of water, pH, and electrolyte concentration), regulation of body temperature (functions in heat transport), chemical communication, and protection (circulates hormones and antibodies; Cronkite 1973).

Blood Vessels

The blood vessels comprise an enormous network of tubes (vascular system) that serve as a distributing system (arteries), a diffusion and filtration system (microcirculation), and a collecting system (veins).

Arteries. The first arteries in the distributing system are elastic arteries (aorta, pulmonary artery, brachiocephalic trunk, etc.), which merge with muscular arteries that conduct blood to various

organs and regions of the body. Elastic arteries have a high content of elastic fibers and are characterized by large lumina and relatively thin walls. Most arteries in the body are muscular arteries, named because of their abundant smooth muscle content. Muscular arteries have smaller lumina and thicker walls than elastic arteries. In most muscular arteries, the wall thickness represents one-fourth of the vessel diameter (Simionescu and Simionescu 1977).

Microvasculature. The arterial circulation connects with the venous circulation by way of the microvasculature. The microvasculature consists of the arterioles, capillaries, and venules. Arterioles are the smallest arteries in which the media is reduced to a layer of one or two smooth muscle cells. Arteriole lumina are small (less than 300 μm), but the arteriole wall is relatively thick (about one-half the diameter of the vessel).

Capillaries. The term *capillary* is restricted to minute vessels that consist only of endothelium, basal lamina, and a few pericytes (Simionescu and Simionescu 1977). The inner diameter of blood capillaries ranges from 5 to 10 μm. Three principal types of blood capillaries have been described: continuous capillaries, fenestrated capillaries, and discontinuous capillaries (sinusoids). Continous capillaries are characterized by a continuous endothelium and are found in skeletal, cardiac, and smooth muscle; connective tissue; the central nervous system; exocrine pancreas; and gonads. Fenestrated capillaries have transcapillary openings (fenestrae) approximately 600–800 A in diameter. Fenestrated capillaries are found in the mucosa of the GI tract, endocrine glands, renal glomerular and peritubular capillaries, choroid plexus, and ciliary body.

Discontinuous capillaries are thin-walled vessels with large gaps (up to thousands of angstroms in diameter) in the endothelium and basal lamina. Discontinuous capillaries are found in the liver, spleen, and bone marrow.

Venules. These are similar to capillaries but are larger. The immediate postcapillary venules are characterized by the presence of pericytes (pericytic venules). Pericytic venules are drained by venules of increasing diameter with media that contain one or two thin layers of smooth muscle cells (muscular venules; Simionescu and Simionescu 1977).

Veins. From venules, the blood is collected in veins increasing in size, eventually becoming large veins such as the venae cavae and pulmonary vein. Blood vessels have three basic tunics: intima, media, and adventitia. The intima comprises (at most) the endothelium, basal lamina, subendothelial connective tissue, and the internal elastic lamina. The media is composed of muscular cells, elastic lamellae, and the external elastic lamina. The adventitia contains connective tissue and fine collagen and elastic fibers. As indicated earlier, the proportions and composition of the tunics vary with types of blood vessels.

Heart

The heart is basically a three-tunic segment of the vascular system with the middle layer (media) greatly developed. The outer tunic (adventitia) is called the epicardium. The media is termed the myocardium and the inner tunic (intima) the endocardium. The myocardium forms most of the mass of the heart. The heart is formed of four chambers: two atria and two ventricles.

Atria. The atria are the thin-walled, low-pressure chambers of the heart. The right atrium receives blood from the systemic circulation and contains openings for the coronary vein, posterior vena cava, anterior vena cava, and the azygos vein. The right atrium opens into the right ventricle through the right atrioventricular orifice. The left atrium receives blood from the pulmonary circulation and contains several openings for the pulmonary veins. The left atrium opens into the left ventricle through the left atrioventricular orifice. The atria are sometimes incorrectly referred to as the auricles. The auricle (L. little ear) is the blind-ending, forward-directed outpouching from each atrium.

Atrioventricular Valves. The atrioventricular valves are intake valves to the ventricles. The right atrioventricular valve (known as the tricuspid valve in humans) consists basically of two cusps in the dog (Miller et al. 1967). The cusp adjacent to the interventricular septum is the septal, or dorsal, cusp. The cusp adjacent to the outer wall is the lateral, or ventral, cusp. The left atrioventricular valve (mitral valve in humans) is basically bicuspid in the dog also, but the subdivisions are indistinct. The cusp adjacent to the interventricular septum is the dorsal cusp. The cusp adjacent to the ventricular wall is the ventral cusp.

Ventricles. The right ventricle pumps systemic blood to the lungs by way of the pulmonary orifice. The left ventricle pumps oxygenated blood to the systemic circulation by way of the aortic orifice. The valves guarding the pulmonary and aortic orifices are similar, with each consisting of three semilunar cusps. Both ventricles contain muscular projections known as papillary muscles. Papillary muscles are larger in the left ventricle. Thin fibromuscular cords (chordae tendinae) arise from the apices of the papillary muscles and attach to the atrioventricular valves. The chordae tendinae keep the atrioventricular valves from being pushed into the atria during contraction of the ventricles. The ventral portion of the thick wall of the left ventricle forms the apex of the heart. The base of the heart is the dorsal portion where atria and ventricles join and valves are inserted into narrow fibrous rings.

Functionally, there are two general types of myocardial cells: muscle cells and impulse formation and conduction cells. The cardiac muscle cells have been described with the musculoskeletal system. The impulse formation and conduction cells form the sinoatrial node, atrioventricular node, and atrioventricular bundle.

Sinoatrial Node. In the dog, the sinoatrial node is located in the right atrium in the terminal crest at the confluence of the anterior vena cava, sinus venarum cavarum, and auricular orifice (Miller et al. 1967).

Atrioventricular Node. This node is also located in the right atrium, about 5 mm cranioventral to the opening of the coronary sinus and craniodorsal to the septal cusp of the right atrioventricular valve.

Atrioventricular Bundle. This bundle runs forward and downward from the atrioventricular node. The bundle divides into right and left branches, which lie closely under the endocardium of the septal wall of the right and left ventricles. In the dog, the Purkinje fibers of the atrioventricular bundle are readily identified by their large diameter, centrally located large spherical nucleus, and scarce myofibrils (Dellmann and Venable 1976).

Pericardium

The heart is enclosed in a fibroserous envelope (pericardium, heart sac). The pericardium is divided into an outer fibrous part and an inner serous part. The serous pericardium in turn is divided into two parts or layers. The visceral layer is the epicardium and is attached firmly to the heart except in the region of coronary grooves. The parietal layer is fused with the fibrous pericardium. The pericardial cavity is located between the two layers of the serous pericardium and typically contains a small quantity of clear, light yellow fluid (pericardial fluid). The fibrous pericardium forms the strong outer part of the heart sac.

Lymphatic Circulatory System

There is also a lymphatic circulatory system that is made up of a network of channels that originate in connective tissue spaces as anastomosing capillaries. Although called a circulatory system, the lymph flows in just one direction, toward the thorax. In the dog, the thoracic duct is

the chief return channel for lymph. The thoracic duct generally joins the venous circulation near the junction of the left jugular vein with the anterior vena cava (Miller et al. 1967).

Necropsy and Laboratory Techniques

Clinical signs of cardiovascular disease include weakness, fatigue, syncope (fainting), reluctance to lie, cyanosis, ascites, subcutaneous edema, venous distension, dyspnea, polydipsia, abnormal pulse, and a wide range of abnormal auscultory sounds.

The initial examination of the heart and major vessels (pulmonary trunk, aorta, brachiocephalic trunk, left subclavian artery, and anterior and posterior venae cavae) is made with the thoracic organs in place. Abnormal location, size, shape, and proportion are looked for. The initial examination of the pericardial sac is also done in situ. The sac is grasped near the apex of the heart and a small incision is made to access the volume and character of the pericardial fluid. If indicated, a sample of the contents can be taken at this time. The sac is then fully opened and both of its surfaces are examined. The thoracic organs are removed. The aorta and posterior venae cavae are severed at the diaphragm. The remaining major vessels (or branches of them) are severed at the chest wall or thoracic inlet. In separating the heart from the lungs, the pulmonary veins are cut at the left atrium and the pulmonary trunk left attached to the heart. The pericardial sac is removed.

A routine should be followed in opening the heart. One that is easily mastered is to follow the flow of blood through the heart, beginning with the right atrium. The posterior vena cava is opened. The incision is continued into the wall of the right atrium just dorsal and parallel to the coronary groove and into the right auricle. The anterior vena cava is not incised to avoid disturbing the sinoatrial node. The right ventricle is opened by cutting along the junction of the right ventricular free wall and ventricular septum. The incision begins caudally at the right atrioventricular orifice and proceeds to the apex, conus arteriosus, and through the pulmonary orifice and pulmonary trunk. Most papillary muscles of the right ventricle arise from the apical portion of the interventricular septum. With care, the incision separating the wall from the septum will leave the papillary muscles attached to the septum.

The left atrium is opened through one of the pulmonary veins. The free atrial wall is cut just dorsal and parallel to the coronary groove with the cut continuing into the left auricle. An incision is then made caudally through the left atrioventricular orifice continuing between the posterior papillary muscle of the left ventricle and the ventricular septum. The incision is extended to the apex and into the anterior wall. Scissors are then inserted through the aortic outflow tract and an incision is made through the aortic orifice, wall of the aortic arch, and the aorta.

The brachiocephalic trunk and left subclavian arteries should also be opened. All chambers, surfaces, and valves of the heart are now exposed for detailed examination. It is suggested that the gross examination also be systematic, following the flow of blood through the heart.

The heart is usually weighed in toxicology studies. Heart weight is essentially a measure of the mass of the myocardium; therefore, as much of the nonmyocardial tissue as possible should be removed. The pericardial sac has been removed. The pulmonary trunk and aortic arch are severed just distal to their respective valves. Any great length of anterior or posterior vena cava should be removed. Fat within the coronary grooves accounts for a small fraction of heart weight and need not be removed; and, of course, the heart valves are left intact. If extracardiac tissues are removed in a consistent manner, the resulting heart weights should be representative. If the heart is opened in the manner described, it is easily subdivided into various components: right ventricular wall, left ventricular wall, interventricular septum, atrial walls, and interatrial septum.Weighing individual components was useful in documenting the cardiac hypertrophy of corpulmonale in beagle pups (Brewster et al. 1983).

The prosector should be alert to lesions in the larger systemic vessels during the course of the postmortem examination. The abdominal aorta and external iliac entries should be examined in situ after the abdominal and pelvic viscera are removed. Selection of tissues for histological study

should be as standardized as the gross examination. A complete histological examination would include samples of the conducting system, each valvular region, walls, and septa of the atria and ventricles, segments from the pulmonary trunk, and thoracic and abdominal aortae. The entire heart of the average beagle can be fixed after the right and left ventricular walls are removed and subdivided into two or three segments. The atrial and ventricular septa should be subdivided by vertical cuts that leave the sinoatrial and atrioventricular nodes intact.

Pathology

A variety of gross and histological changes have been reported for the cardiovascular system of laboratory beagles.

Major Blood Vessels, Arteries, and Veins

Collection of blood samples from the cephalic vein rarely causes sufficient injury to result in diagnosed lesions. Spontaneous gross lesions included congenital patent ductus arteriosus in 1 (0.1%) and pulmonic stenosis in 1 (0.1%) of 1,000 dogs in 39 studies (Hottendorf and Hirth 1974). Inflammation of arteries, arteritis, occurred in 2% of young beagles (Glaister 1986). Idiopathic canine polyarteritis, also termed panarteritis or periarteritis, is a spontaneous arterial disease that occurs in both immature and mature beagles and mongrels. The disease occurs in acute, subacute, and chronic forms and can be a complicating factor in toxicology studies. Incidences of 1.3% in 331 dogs to 3.3% in 668 dogs have been reported with predominance in the right coronary and epididymal arteries (Kerns et al. 2001). Extramural periarteritis affecting the coronary artery was reported in 1 (0.1%) of 647 dogs (Hottendorf and Hirth 1974). Arteritis and periarteritis were reported in 10 of the 59 dogs with detrusor myopathy (Cain et al. 2000). Calcification of the round ligament of the bladder (remnant of the umbilical artery of the fetus) was seen, but not listed as a significant finding (Hottendorf and Hirth 1974). Focal medial degeneration of the aorta was observed in 1 (0.1%) of 647 dogs (Hottendorf and Hirth 1974). Mineralization in the aortic media near the base of the heart occurred in 2% to 3% of young beagles (Glaister 1986). Aortic and cardiac mineralization was found in 21 of 3443 (0.61%) canine thoracic radiographs as incidental, non-significant findings in older dogs (Schwarz et al. 2002). Vascular microgranulomas in the lungs were attributed to emboli from IV injection sites in 8 (1%) of 647 dogs (Hottendorf and Hirth 1974). Hypertrophy of muscular arteries must be interpreted with caution, because these arteries might undergo postmortem contraction and appear to be unusually thick walled. The contracted arteries are usually devoid of blood and their internal elastic lamina has a scalloped appearance in cross-section. Postmortem inhibition of hemoglobin can produce a diffuse red staining of the intima in both the heart and blood vessels that simulates hemorrhage. Heart valvular endocardiosis is probably the most frequently observed incidental finding in the heart.

Endocardiosis chiefly affects the septal cusp of the right atrioventricular valve in young beagles. The cusp appears to be diffusely or irregularly swollen, soft, and glistening. Valvular telangiectasis or angiectasis, also termed hematocyst or hematoma, appears to be a congenital malformation (Hubben et al. 1963). The lesion usually appears as a dark red cyst, 1 mm to 5 mm in diameter that protrudes from the atrial surface of the cusp. The septal cusp of the right atrioventricular valve is also the most common site for valvular telangiectasis. Valvular telangiectasis was observed grossly in 4 (0.4%) of 1,000 dogs (Hottendorf and Hirth 1974). Neither these cysts nor the valvular endocardiosis appear to be of any functional significance. Inflammation of the myocardium, myocarditis, is infrequent. Parvovirus causes myocarditis in puppies and recently weaned dogs, but is largely controlled by vaccination. Focal myocarditis was observed in 4 (0.6%) of 647 dogs (Hottendorf and Hirth 1974). Fibrosis is a component of chronic inflammation and has been reported throughout the heart. Focal myocardial fibrosis and calcification or mineralization were observed in 2 (0.3%) of 647 dogs (Hottendorf and Hirth 1974). Chronic valvular fibrosis was observed grossly

in 27 (3%) of 1,000 dogs (Hottendorf and Hirth 1974). Toxocar granulomas have also been reported in the myocardium (Barron and Saunders 1966). Granulomas occurred in 2 (0.3%) of 647 dogs (Hottendorf and Hirth 1974). Leukocyte foci were reported in myocardium of 1% of young beagles (Glaister 1986). Fatty infiltration of the myocardium was observed in 13 (35%) of 37 untreated beagles (Pick and Eubanks 1965).

Apparent degenerative changes within the myocardium must be interpreted with caution for, as with skeletal muscle, many artifacts might result from handling and from contraction of myofibrils during fixation. Postmortem inhibition of hemoglobin produces a diffuse red staining of the intima of both the heart and blood vessels that simulates hemorrhage. Chronic focal pericarditis was observed in 1 (0.1%) of 647 dogs (Hottendorf and Hirth 1974).

Hemopoietic System

Anatomy and Histology

The hemopoietic system consists of the blood, bone marrow, and lymphoreticular tissues. Blood is a liquid tissue comprised of a fluid portion (plasma), red cells (erythron), white cells (leukon), and platelets (thrombon; Payne et al. 1976).

Erythron

The erythron consists of the erythrocytes and their precursors. The primary function of the erythrocyte is to mediate the exchange of oxygen and carbon dioxide. In the adult dog, red cell production (erythropoiesis) occurs predominantly in the bone marrow; however, other organs (spleen, liver, lymph nodes, adrenals, kidneys) retain the potential to produce erythrocytes (extramedullary hematopoiesis) in adult life (Valli 1985). The average erythrocyte life span for the dog is 110 days (Duncan and Prasse 1986).

Leukon

The leukon consists of granulocytes (neutrophils, eosinophils, basophils), monocytes, and lymphocytes. In the adult dog, virtually all granulocytes are produced in the bone marrow.

Granulocytes. Neutrophils are the most abundant of the circulating white blood cells (60%–77% in the dog (Duncan and Prasse 1986). After a brief period of circulation, neutrophils migrate to the tissues and remain there. Blood neutrophils are replaced about 2.5 times a day. The major function of neutrophils is to phagocytize and kill microbes. Like neutrophils, eosinophils are mobile phagocytes with antimicrobial properties, but they are not protective against bacterial infection. Two to 10% of the circulating white blood cells are eosinophils. Eosinophils are more numerous in tissues than in blood. Basophils are weakly motile phagocytes. Basophils and mast cells (related functionally but not by origin) contain mediators of inflammation, including histamine, heparin, eosinophil chernotactic factor of anaphylaxis, platelet activating factor, and other substances (Prasse 1983). Basophils are rarely seen in the circulating blood.

Monocytes. Produced in the bone marrow, monocytes migrate into the blood and quickly enter into tissues (lung, liver, spleen, bone marrow, pleura, peritoneum, lymph nodes) to become tissue macrophages (Valli 1985). They are the Kupffer cells of the liver and the osteoclasts of the bone. Monocytes represent 3% to 10% of circulating white blood cells. The activities of the monocyte–macrophage system include phagocytosis, myeloproliferative control, antigen processing, and production of various cytokines.

Lymphocytes. In early embryonic life, lymphoid precursors from the yolk sac colonize the bone marrow and thymus (primary lymphopoietic organs). Progeny from the primary lymphopoietic organs in turn populate lymph nodes, spleen, tonsils, gut, and lung (secondary lymphopoietic organs). The bone marrow is the larger and more active primary lymphopoietic organ. The fate of most bone marrow lymphocytes is unknown (Wintrobe et al. 1981), but some enter the circulation and migrate to the secondary lymphopoietic organs to become the source of antibody-producing cells (B lymphocytes, bone marrow-derived cells). Most thymic lymphocytes experience an intrathymic death, but some enter the blood and seed out in the thymus-dependent areas of secondary lymphopoietic organs, where they become progenitors of cells involved in cell-mediated immunity (T lymphocytes, thymus-derived cells). Compared to other leukocytes, lymphocytes are long lived (weeks to years). They also are unique because they recirculate (Duncan and Prasse 1986). The majority of permanently circulating lymphocytes are T-cells. The majority of B-cells remain in the lymphoid tissue. Lymphocytes comprise 12% to 30% of the circulating white blood cells.

Thrombon

The thrombon consists of the circulating blood platelets and the megakaryocytes and megakaryoblasts of the bone marrow (Payne et al. 1976).

Blood Platelets. Extramedullary megakaryopoiesis does occur and is most common in the lung, but is also seen in the spleen, liver, kidney, and heart (Green 1983). The normal blood platelet count in the dog is $2–9 \times 10^5$/mcl (Duncan and Prasse 1986). The platelet number can be affected by splenic contraction (increased number of platelets) and congestion (decreased number). The circulating life span of platelets is approximately 10 days. The platelets' central role is in hemostasis. The major platelet reactions are adhesion to damaged endothelium, release of biochemical substances, causing vasoconstriction, and further platelet aggregation (Green 1983).

Bone Marrow. Bone marrow maintains an embryonic function throughout adult life. Bone marrow can be red (due to the hemoglobin contact of erythrocytes and their precursors) or yellow (due to fat cells). The bone marrow has two compartments: vascular and hematopoietic. The vascular compartment is supplied by nutrient arteries that divide and gradually narrow to capillaries and then open to large sinuses lined by discontinuous endothelium. The marrow lying between the sinuses is the hematopoietic compartment. The hematopoietic compartment of red marrow contains mostly hematopoietic cells and a few fat cells. Fat cells predominate in the hematopoietic compartment in yellow marrow.

Lymphoreticular Tissues

Lymphoreticular tissues include the thymus, spleen, lymph nodes, and lymphoid tissues of the digestive and respiratory systems.

Thymus. This is a light gray, multilobulated organ that lies in the cranial ventral part of the thoracic cavity almost entirely covered by the precardial mediastinum. At birth, the thymus is large and continues to grow during the first 3 months of postnatal life in the beagle (Andersen and Goldman 1970). During the period from 6 to 23 months, the thymus undergoes progressive involution (Ploemen et al. 2003) but never completely disappears (Miller et al. 1967). The thymus is a composite of epithelial and lymphoid tissues. Each thymic lobule is comprised of a cortex that contains many lymphocytes (or cells that give rise to lymphocytes) and a medulla that contains epithelial and myoid cells (Valli 1985). The epithelial cells of the thymus provide a unique environment essential for T-lymphocyte development. Various subsets of T-cells are formed that are released and undergo final maturation in the spleen or other secondary lymphoid organs. The

medullary epithelial cells form morphologically distinct clusters of cells (thymic corpuscles, Hassall's corpuscles), with functions that are unknown. Examination of thymus from 120 healthy control beagles ages 6 to 23 months demonstrated medullary lymphoid follicles with occasional germinal centers in 70% of the dogs. The CD79 alpha marker confirmed that 97% of these dogs had B-cell-rich medullary areas. During involution, the B-cell areas and lymphoid follicles became less distinct (Ploemen et al. 2003).

Spleen. The spleens of domestic animals vary in their blood-storage capacity and the relative amount of smooth muscle found in the capsule and trabeculae. The canine spleen has abundant venous sinuses capable of storing large amounts of blood and abundant smooth muscle in the capsule capable of considerable contraction (Brown and Dellmann 1976). Splenic parenchyma is called pulp, most of which is red (red pulp) due to the presence of blood. The red pulp is almost entirely made up of splenic sinuses and thin plates of cells (splenic cords) lying between the sinuses. Scattered throughout the red pulp are lymphoid nodules (splenic nodules) and lymphoid perarterial sheaths. The lymphoid nodules and sheaths constitute the white pulp. The lymphoid nodules represent concentrations of B lymphocytes; the sheaths are predominantly T lymphocytes. The spleen has no afferent lymph supply; consequently all antigen reaches the spleen with the blood. The spleen filters unwanted elements from the blood (aged and damaged erythrocytes, particulate matter), is a major secondary lymphoreticular organ, a secondary source of hematopoiesis, and a reserve pool of erythrocytes and platelets (Contran et al. 1989).

Lymph Nodes. Lymph nodes are the most organized of the lymphatic organs. External lymph nodes are found in the protective environment of small fat pads. Internal nodes are found in the mediastinum, mesentery, near the angles of many larger vessels, and the hilus of organs. Lymph nodes are usually named according to their location. The capsules of lymph nodes are perforated at various points by afferent lymphatics that empty into subcapsular sinuses. Branches of the sinuses (medullary sinuses) extend into the node and terminate at the hilus, where the efferent lymphatics emerge. Lymph percolates into and out of the parenchyma through gaps in the sinus walls. The cortex is made up of lymphoid nodules (primary nodules) with size and morphology that vary greatly. The primary follicles represent B-cell areas. On antigenic stimulation, the primary follicles enlarge and develop pale-staining germinal centers. B-cell progeny leave the germinal centers to either migrate to medullary cords where they become plasma cells or enter the blood lymphocyte pool (Valli 1985). The paracortex (outer cortical area not occupied by germinal centers) is largely occupied by T-cells. Medullary cords are papillary-like extensions of the paracortical cells toward the hilus of the node.

Necropsy and Laboratory Techniques

Anemia is defined as an absolute decrease in the packed cell volume, hemoglobin concentration, and red blood cell count (Duncan and Prasse 1986). The clinical signs of anemia include pale mucous membranes, weakness, loss of stamina, dyspnea on exertion, tachycardia, and heart murmur. Icterus, hemoglobinuria, hemorrhage, and fever might also be seen depending on the pathogenesis of the anemia. Enlarged superficial lymph nodes might indicate disease in the region being drained: parotid, mandibular, medial retropharyngeal lymph nodes (head and neck); superficial cervical lymph nodes (head, neck, thoracic limb, thoracic wall); axillary nodes (thoracic limb, thoracic wall, cranial and middle mammary glands); superficial inguinal nodes (abdominal wall, caudal mammary glands, penis, scrotum, entire pelvic limb); and popliteal (distal pelvic limb; Miller et al. 1967). Bone marrow specimens (for histology and cytology) should be obtained as soon after death as possible (second in priority only to the eyes). Specimens can be easily obtained from the sternum.

For histology, one or two sternebrae can be cut longitudinally with a stout sharp knife. Segments are placed in fixative. One segment can be reserved for archival storage and the other decalcified and processed for paraffin embedding. Smears for marrow cytology and cell counting can be gotten

from another sternebra. After removal of muscle, fat, and an intersternabral cartilage, pressure is applied by a sturdy pair of pliers to force marrow to the cut surface, where it is removed by means of a tapered artist's brush (moistened in fetal bovine calf serum) and applied in streaks on a glass slide. The specimen is air dried. The prosector should be alert to abnormalities in individual lymph nodes during the course of the general postmortem examination. Specimens of both external and internal lymph nodes are usually taken for microscopic examination. Commonly, these are a medial retropharyngeal lymph node (the largest node in the head and neck, readily identified and easily removed) and a mesenteric lymph node. The anterior pole of the medial retropharyngeal lymph node might be discolored (tattoo ink from the ear). Mesenteric lymph nodes are often reddened (congested). Lymph nodes should be handled carefully to minimize artifacts. Searcy (1988) recommends that lymph nodes be fixed for about 1 hr prior to slicing (lymph nodes cut in the fresh state bulge through the capsule). Touch imprints can be prepared from the cut surface of one pole before the node is placed in fixative. The size of the thymus varies considerably in short-term toxicology studies. The thymic involution begun prior to sexual maturity (Andersen and Goldman 1970) might be enhanced by the direct or indirect effect (malnutrition) of test compounds. Congestion of the spleen is common at necropsy, and it especially follows euthanasia with barbiturates. Several parallel slices should be made through the spleen to examine the pulp. One or two narrow slices should be placed in fixative.

Pathology

Of incidental changes that have been reported for the hemopoietic system of laboratory beagles, most involved the spleen.

Thymus

The size of the thymus varies greatly in dogs on toxicology studies depending on the age and individual animal. Involution begins prior to sexual maturity. Atrophy or reduction in the size is an uncommon finding in untreated beagles and must be distinguished from involution. Ectopic thyroid tissue was observed in the thymus of 1% of young beagles (Glaister 1986). Thymic cysts were also reported in 1% of young beagles (Glaister 1986).

Spleen

Many vascular changes occur in the spleen of laboratory dogs. Angiectasis or telangiectasis is a localized dilatation of blood vessels that are usually observed as dark blebs around the margins of the spleen. They can arise as blood-filled sinusoids that fail to empty during the splenic contractions associated with exsanguinations. Telangiectasis was observed in 17% to 18% of young beagles (Glaister 1986). Congestion or hyperemia is diffuse dilatation of blood vessels of the spleen. Congestion can be active as when associated with inflammation or passive when associated with poor blood circulation as with heart failure. Splenic hyperemia was observed in 1 (3%) of 37 untreated beagles (Pick and Eubanks 1965). Congestion must be distinguished from hemorrhage. Hemorrhage is common in the spleen because of the large volume of blood contained in the spleen and the abdominal location. Splenic hemorrhage was reported in 1 (3%) of 37 untreated beagles (Pick and Eubanks 1965). Hematomas are large localized areas of hemorrhage, although infrequent. Hematomas were observed grossly in 2 (0.2%) of 1,000 dogs (Hottendorf and Hirth 1974). Hemosiderosis is the tissue deposition of iron pigment associated with the breakdown of red blood cells and can occur abnormally as a result of increased fragility of erythrocytes or following hemorrhage. Because of normal destruction of erythrocytes at the end their life span, the normal spleen has some degree of hemosiderosis. In dogs, hemosiderin is commonly associated with siderofibrotic nodules. Splenic hemosiderosis was reported in 17 (46%) of 37 untreated beagles (Pick and Eubanks 1965).

Although the spleen is an active site for hematopoiesis in puppies, the production of blood cells by the splenic pulp, especially the erythroid series, is uncommon in adult dogs. Splenic extramedullary hematopoiesis was reported in 3 (8%) of 37 untreated beagles (Pick and Eubanks 1965).

Small Gandy-Gamna-like bodies in the splenic capsule were reported by Maita et al. (1977). Gandy-Gamna bodies (an eponym from medical pathology) refers to firm, nodular discolorations seen on or within the spleen. In veterinary pathology, the lesions are commonly referred to as siderofibrotic nodules (Ishmael and Howell 1967). In the dog, they might appear as small, irregular, slightly raised nodules on the capsular surface to extensive irregular encrustrations covering large areas of the capsule and as nodules within the pulp. The color of the nodules varies from yellow to grayish brown. The nodules are seen most frequently on the margins of the spleen with concentrations at the extremities and also on the visceral surface with concentrations at the attachment of the gastrosplenic omenturn. Microscopically, the nodules are fibrotic foci that are commonly calcified and contain brown pigment (hemosiderin) and bright yellow pigment (hematoldin, bilirubin). They are probably the end result of hemorrhage or marginal telangiectasis, as noted by Glaister (1986). They were observed grossly in 90 (9%) of 1,000 dogs (Hottendorf and Hirth 1974).

Few lymphoid tissue changes were reported. Oghiso et al. (1982) reported lymphoid hyperplasia and reticulosis in the spleen. Reticulosis is a form of lymphoid hyperplasia consisting predominantly of reticuloendothelial or histiocytic cells. Other than those associated with siderofibrotic nodules, most inflammatory changes were limited to granulomas. Maita et al. (1977) reported Toxocara granulomas in the spleen. Splenic fibrosis was observed in 1 (3%) of 37 untreated beagles (Pick and Eubanks 1965).

One or more masses of splenic tissue might be found in the gastrosplenic omentum in addition to the spleen itself. These are known as accessory spleens. They might be congenital, but apparently many are acquired through traumatic rupture of the spleen (Valli 1985). Accessory spleens were observed grossly in 1 (0.1%) of 1,000 dogs (Hottendorf and Hirth 1974).

Fritz et al. (1966) reported lymphosarcomas in the spleen and lymph nodes of beagles under 1 year of age.

Lymph Nodes

The lymph nodes most frequently processed and examined microscopically are mandibular, mesenteric, and medial retropharyngeal. Congestion of the mesenteric lymph nodes is commonly observed. Congestion can be physiological and normal when associated with digestion, active when associated with inflammation, or passive when associated with poor blood circulation as with heart failure. Congestion must be distinguished from hemorrhage. Hottendorf and Hirth (1974) observed, but did not report as a lesion, congestion of the mesenteric lymph nodes. Granulomatous inflammation was the most commonly observed inflammatory change in lymph nodes. These lesions, granulomas, were most frequent in the mesenteric lymph nodes and usually occurred as the result of Toxocara larvae migration (Barron and Saunders 1966; Maita et al. 1977). Granulomas were reported in 21% to 27% of young beagles (Glaister 1986) and in 16 (2%) of 647 dogs from 39 studies (Hottendorf and Hirth 1974).

Inflammation or lymphadenitis can occur with generalized involvement of lymph nodes after infection with *Brucella canis* (Jubb et al. 1992). Lymphoid cell proliferation was observed in the lymph nodes of untreated dogs. Lymphoid hyperplasia in which there was a nonneoplastic increase in lymphoid cells, and plasmacytosis in which there was a nonneoplastic increase in plasma cells, were reported (Oghiso et al. 1982). Lymphoid hyperplasia is often associated with inflammation and infection in adjacent tissues. It must be distinguished from early neoplastic changes. Fritz et al. (1966) reported lymphosarcomas, in the spleen and lymph nodes of beagles under 1 year of age.

Other Lymphoid Tissues

Hottendorf and Hirth (1974) observed, but did not report as a lesion, mild hyperplasia of lymph follicles in the pylorus, small intestine, cecum, and gallbladder.

Endocrine System

Anatomy and Histology

Two systems have major coordinating responsibilities in the body. One is the nervous system, and the other is the endocrine system. Glands of the endocrine system communicate principally by way of the blood through which raw materials and special releasing and inhibiting factors (hormones) are received and into which the glands' own secretions (hormones) are discharged. Glands that are universally recognized as endocrine glands are the hypophysis (pituitary gland), thyroid, parathyroids, adrenals, pancreas (pancreatic islets, endocrine pancreas), testes, and ovaries. The endocrine function of other organs such as the pineal body, thymus, kidney, and GI tract, are beyond the scope of this chapter.

Hypophysis

The hypophysis is structurally and functionally a part of a complex hypothalamohypophyseal system (Dellman 1976a). Traditionally, only two divisions of this system (adenohypophysis and neurohypophysis) are included in a discussion of the hypophysis. The adenohypophysis consists of the pars tuberalis, pars intermedia (intermediate lobe), and pars distalis (anterior lobe). The neurohypophysis consists of the pars nervosa (posterior lobe) and the infundibulum. The hypophysis has a dual embryological origin. The adenohypophysis arises as an upward evagination of the oropharyngeal ectoderm (the lining of the future oral cavity, Rathke's pouch). The neurohypophysis is a downward evagination of the brain (diencephalon).

The hypophysis of the dog is a slightly flattened ovoid body lying in a shallow concavity of the basisphenoid bone and attached to the base of the brain by a short hollow stalk (infundibulum). The hypophysis is partly surrounded by bone (sella turcica), which is prominent caudally as a well-developed ridge with two prominent lateral projections (dorsum sellae and clinoid processes). The dura mater (outer tough membrane of the meninges) is intimately associated with both the hypophysis and the sella turcica. The close association of gland, bone, and dura mater makes it difficult to remove the hypophysis without producing artifacts. In the dog, the adenohypophysis surrounds the neurohypophysis. Dorsally, the pars tuberalis surrounds the infundibulum and more ventrally, the pars distalis and pars intermedia surround the pars nervosa. The pars distalis is by far the largest part of the hypophysis. The adenohypophysis produces a number of hormones: growth hormone (GH, somatotropin), luteotropic hormone (LTH, prolactin), luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyrotropic hormone (TSH), adrenocorticotropic hormone (ACTH), and melanocyte-stimulating hormone (MSH). Hormones produced elsewhere in the neurohypophysis but released into the bloodstream in the pars nervosa include antidiuretic hormone (ADH, vasopressin) and oxytocin (Capen 1985).

Lymphoid cell proliferation was observed in the lymph nodes of untreated dogs. Lymphoid hyperplasia in which there was a nonneoplastic increase in lymphoid cells, and plasmacytosis in which there was a nonneoplastic increase in plasma cells, were reported (Oghiso et al. 1982). Lymphoid hyperplasia is often associated with inflammation and infection in adjacent tissues. It must be distinguished from early neoplastic changes.

Fritz et al. (1966) reported lymphosarcomas in the spleen and lymph nodes of beagles under 1 year of age.

Other Lymphoid Tissues

Hottendorf and Hirth (1974) observed, but did not report as a lesion, mild hyperplasia of lymph follicles in the pylorus, small intestine, cecum, and gallbladder.

Thyroid

The thyroid is a single gland consisting of two lobes that lie lateral to the first five to eight cartilaginous rings of the trachea (Miller et al. 1967). Occasionally, a glandular isthmus is found on the ventral surface of the trachea connecting the ventral poles of the two lobes. The thyroid is fairly loosely attached to the trachea. The sizes and shapes of the lobes are not always identical. The thyroid arises from pharyngeal epithelium in close association with the aortic sac (a relationship that accounts for deposits of thyroid parenchyma anywhere from the larynx to the diaphragm). The bud of thyroid epithelium expands and the attachment to the pharynx is narrowed to a slender stalk. A vestige of this stalk might persist on the midline as the thyroglossal duct. The thyroid expands laterally and fuses with the ultimobranchial bodies that arise from the fourth branchial pouch. The inclusion of parafollicular cells in the thyroid is the result of this fusion. Parafollicular cells (calcitonin-secreting cells, C-cells) are of neural crest origin and migrate to the ultimobranchial body and from there to the thyroid.

The thyroid is unique among endocrine glands because the final assemblage of hormone occurs outside the cell, within the lumen of the thyroid follicle (Capen 1985). The manufacture and release of thyroid hormones, therefore, has an exocrine phase (synthesis and secretion of the protein, thyroglobulin, into the follicular lumen) and an endocrine phase (absorption of colloid from the lumen, release of hormones from the thyroglobulin molecule, and secretion of the hormones into the bloodstream). The thyroid hormones are triiodothyronine (TO) and thyroxine (T4).

Parathyroid Glands

The parathyroid glands are paired and usually four in number. The parathyroids are entodermal in origin and derived from the third and fourth pharyngeal pouches (Miller et al. 1967).

Parathyroid III (commonly referred to as the external parathyroid) is a flattened oval body, 2 mm to 5 mm long, and most commonly found in the connective tissue about the cranial pole of a thyroid lobe. Its location might vary, but it is always external to the capsule of the thyroid.

Parathyroid IV (commonly referred to as the internal parathyroid) is generally smaller than the external parathyroid. The internal parathyroid is usually found beneath the capsule on the tracheal surface of a lobe, but it is also found deep within the thyroid.

Accessory parathyroids can be found within the thyroid, in the region of the larynx, the carotid sheath, anterior mediastinum, and within or associated with the thymus (Miller et al. 1967).

The parathyroid glands contain a single basic type of secretory cell that secretes parathyroid hormone (parathormone, or PTH).

Adrenal Glands

The adrenal glands are composed of two separate endocrine organs that differ in embryological origin, type of secretion, and function. The adrenal glands of the dog are generally flattened, bilobed, and located cranial and medial to the kidneys on either side of the aorta and posterior vena cava (Miller et al. 1967). The left adrenal lies further caudal than the right. The right adrenal is more irregular in shape, usually with a distinct hook or comma form. On section, the adrenal cortex is usually firm and yellow, the medulla is softer and is usually light red to brown.

The adrenal cortex develops from the cells of the celomic epithelium. The chromaffin tissue and sympathetic ganglion cells of the adrenal medulla are derived from the neural crest. Neural

crest cells invade the already formed cortical primordium, frequently leaving islands of medullary cells in the cortex or carrying groups of cortical cells into the medulla (Miller et al. 1967). Some of the medullary cells might not be incorporated in the adrenal gland and develop to form paraganglia and aortic and carotid bodies. The adrenal cortex is traditionally divided into three zones: glomerulosa, fasciculata, and reticularis. The outer zona glomerulosa (zona arcuata) represents about 15% of the cortex, and is responsible for secretion of mineralocorticoid hormones. The middle zona fasciculata comprises about 70% of the cortex, and is responsible for secretion of glucocorticoid hormones. The inner zona reticularis accounts for the remaining 15% of the cortex and is responsible for the secretion of sex steroids (Capen 1985). The widths of the three zones vary and the demarcation between them might not be distinct. The adrenal medulla secretes epinephrine and norepinephrine.

Pancreas

The gross anatomy of the pancreas has been described with the digestive system. The pancreas is both an exocrine and endocrine gland. Cells responsible for the exocrine (digestive) secretions are the acinar cells. The cells of the pancreas responsible for hormone secretion (insulin and glucagon) are present within small spherical or oval islands (islets of Langerhans) dispersed throughout the organ. Islet cells are derived in common with acinar cells and are thus of entodermal origin.

Testis and Ovary

The anatomy of the testis and ovary are described with the genitourinary system. Testosterone is produced by interstitial cells (cells of Leydig) of the testis. Estridial is produced by cells of the ovarian follicle. Progesterone is produced by cells of the corpus luteum.

Necropsy and Laboratory Techniques

A presentation of clinical signs of endocrine disease is beyond the scope of this chapter. The reader is referred to Ettinger (1989) as a source of clinical information. The endocrine glands should be handled carefully during dissection and weighing to minimize the production of artifacts. The thyroid and parathyroids should be removed while the larynx, trachea, and esophagus are in place and anatomical landmarks are undisturbed. Having all structures in place is particularly helpful when the thyroid is small or outside its normal location. Parathyroid glands should be included with the thyroid. The adrenal glands should be removed before the kidneys. The hypophysis can be removed from the sella turcica with minimal damage by first freeing the dorsum sellae by cutting through its base with small bone cutters. Second, grasp a clinoid process with fine forceps to support the hypophysis as the dura mater is cut with fine curved scissors to free the hypophysis from the basisphenoid bone. Third, remove the hypophysis from the cranial cavity attached to the dorsum sellae. Fourth, weigh the hypophysis by cutting the dura mater between the hypophysis and the bone to allow the hypophysis to drop on a tared weighing boat.

Generally, a small strand of dura mater will be found on the hypophysis that can be grasped to transfer the gland to fixative or cassette. The hypophysis can be processed intact. If embedded with the dorsal (infundibular) surface down, three step sections of the gland will usually result in a good representation of the pars distalis, pars intermedia, and pars nervosa. Cells of the pars distalis are traditionally classified as eosinophils, basophils, and chromophobes on the basis of their uptake of acidic or basic dyes. Immunocytochemical methods using specific hormonal antibodies now reliably distinguish the various cells responsible for secretion of each of the various hormones.

During the trimming process, attempts should be made to include at least one parathyroid gland with each lobe of the thyroid. Both lobes of the thyroid should be represented in stained sections. Adrenal glands should be cut parasagittally. The thicker segment from each gland is embedded.

The block may be rough cut on the microtome to obtain nearly full-width representations of the cortex and medulla in stained sections.

Pathology

Of all the endocrine glands, potentially significant lesions are most likely to be seen within the thyroid gland. Cysts are frequently found (less frequently reported) in the hypophysis, parathyroid, and thyroid.

Hypophysis (Pituitary Gland)

Cysts are often observed in the hypophysis of dogs. Cystic remnants of the craniopharyngeal duct are frequently found at the periphery of the pars tuberalis and pars distalis. Most of the cysts are microscopic, but a few are visible grossly. The cysts are lined by cuboidal to columnar epithelium, often ciliated, and contain mucin. Maita et al. (1977) and Oghiso et al. (1982) reported cysts in the anterior lobe of the hypophysis. Pituitary cysts were reported in 24% to 26% of young beagles by Glaister (1986), in 44 (7%) of 647 dogs on 39 studies by Hottendorf and Hirth, (1974), and in 3 (8%) of 37 untreated beagles by Pick and Eubanks (1965). Inflammation as reported was limited to granulomas with 1 (0.1%) of 647 dogs affected (Hottendorf and Hirth 1974). Barron and Saunders (1966) reported the occurrence of Toxocara granulomas in the pituitary.

Parathyroid Glands

Small cysts (apparently from remnants of the duct connecting thymic and parathyroid primordia) can be found occasionally within or near the parathyroid. Parathyroid cysts are usually multiloculated, lined by cuboidal to columnar epithelium, often ciliated, and contain densely eosinophilic material. Parathyroid cysts occurred in 1% of young beagles (Glaister 1986). Hyperplasia of parathyroid cells was observed in 7 (1%) of 647 dogs (Hottendorf and Hirth 1974).

Thyroid

Two potentially important changes in the thyroid of laboratory dogs are idiopathic follicular atrophy and lymphocytic thyroiditis. Minor changes frequently seen within the thyroid include disassociation of follicular cells (probably artifactual), minute intrafollicular corpora amylaceae-like bodies, and the presence of brown pigment (lipofuscin) in follicular cells.

Idiopathic follicular atrophy is a progressive loss of follicular epithelium and replacement by adipose cells. One lobe might be affected more than the other. Severely affected lobes are difficult to locate at necropsy. Parafollicular cells are not affected and remain in the adipose tissue. Dogs with idiopathic thyroid atrophy might be hypothyroid. Hypothyroidism can also be seen in dogs with lymphocytic thyroiditis. Atrophy of the thyroid was observed in 10 (2%) of 647 dogs (Hottendorf and Hirth 1974). Follicular atrophy was reported by Oghiso et al. (1982).

Lymphocytic thyroiditis in dogs appears to have an immunological basis and familial occurrence in beagles. Glands with lymphocytic thyroiditis can be enlarged, but they might also be normal or even reduced in size. Histological alterations consist of multifocal to diffuse infiltrates of lymphocytes, plasma cells, and macrophages. Lymphoid nodules might be present. Thyroid follicles are usually small and can be disrupted and contain degenerate follicle cells, lymphocytes, and plasma cells. Lymphocytic thyroiditis in the beagle has been extensively investigated (Beierwaltes and Nishiyama 1968; Fritz et al. 1970; Mawdesley-Thomas 1968; Mawdesley-Thomas and Jolly 1967; Mizejewski et al. 1971; Musser and Graham 1968; Tucker 1962). Lymphocytic thyroiditis was reported by Oghiso et al. (1982). Chronic lymphocytic thyroiditis was observed in 22 (3%) of 647 dogs in 39 studies (Hottendorf and Hirth 1974).

Unspecified thyroiditis occurred in 3 (8%) of 37 untreated beagles (Pick and Eubanks 1965). Barron and Saunders (1966) reported the presence of Toxocara granulomas in the thyroid.

Ultimobranchial duct cysts are frequently seen within the thyroid. They are derived from remnants of the ultimobranchial body and have a keratinized squamous epithelial lining (Capen 1985). Ultimobranchial duct cysts were observed in 12 (2%) of 647 dogs in 39 studies (Hottendorf and Hirth 1974). Cysts occurred in the thyroids of 1% to 2% of young beagles (Glaister 1986). Young beagles have varying numbers of parafollicular or C-cells in the thyroid. Occasionally, the number or focal concentration appears adequate to justify a diagnosis of C-cell hyperplasia. C-cell hyperplasia occurred in 8% to 9% of young beagles (Glaister 1986). Interfollicular cellularity was reported by Oghiso et al. (1982). Follicular epithelial hyperplasia was observed by Maita et al. (1977). Lymphoid hyperplasia in the thyroid was observed in 2% of young beagles (Glaister 1986).

Focal squamous metaplasia was reported in 5 (0.8%) of 647 dogs (Hottendorf and Hirth 1974). Ectopic thymus was observed in 1-2% of young beagles (Glaister 1986). Additional minor changes frequently seen within the thyroid include disassociation of follicular cells (probably artifactual), minute intrafollicular corpora amylaceae-like bodies, and the presence of brown pigment (lipofuscin) in follicular cells.

Adrenal Glands

As noted earlier, the width of the various layers of the adrenal cortex can vary. The zona glomerulosa might be thin or seem to be absent for substantial distances. Cortical cells might be found in the medulla and medullary cells might be found in and on the cortex (all explainable in the embryological development of the gland. Focal cortical hyperplasia was observed in 19 (3%) of 647 dogs on 39 studies (Hottendorf and Hirth 1974). Nodular hyperplasia of the adrenal cortex was reported by Oghiso et al. (1982). Appreciable differences in cortical cell vacuolation can be observed from animal to animal. This normal vacuolation must be distinguished from fatty degeneration, which was reported by Oghiso et al. (1982). Minor focal inflammation in the adrenal glands occurred in 5 (0.8%) of 647 dogs (Hottendorf and Hirth 1974).

Urogenital System

Anatomy and Histology

The urogenital system is made up of the urinary organs (kidneys, ureters, urinary bladder, and urethra), the male genital organs (scrotum, testes, epididymides, deferent ducts, prostate, and penis) and the female genital organs (ovaries, oviducts, uterus, vagina, and vulva).

Kidneys

The kidney performs metabolic, humoral, and excretory functions. The excretory function produces a fluid (urine) that is conducted from the kidney by fibromuscular tubes (ureters) to a storage reservoir (urinary bladder), where the fluid accumulates and is periodically discharged through a single tube (urethra) to the exterior.

The kidneys are bean-shaped glands located in the sublumbar area, one on each side of the aorta and posterior vena cava. The right kidney is usually located slightly anterior to the left and is more firmly secured in its position. A whole kidney cut in a midsagittal plane has two distinct zones (cortex and medulla). The cortex is the darker outer zone lying beneath the renal capsule. The medulla is the remaining lighter zone shaped like an inverted pyramid. The apex of the pyramid is the papilla, which projects into the expanded end of the ureter (renal pelvis). The renal pelvis lies within an opening in the medial border of the kidney called the renal hilus (renal sinus). In addition to the renal pelvis, the hilus contains adipose tissue, branches of the renal artery, vein and

lymphatics, and nerves. Because the dog has only one papilla, it is classified as a unilobular or unipyramidal kidney; however, the single pyramid is actually the result of several pyramids fusing during development (Brown 1976).

The nephron is the functional unit of the kidney and has six morphologically distinct segments: renal corpuscle, convoluted and straight portions of the proximal tubule, thin segment, and straight and convoluted portions of the distal tubule. The distal tubules join collecting tubules. Collecting tubules join with other collecting tubules and finally converge into large collecting ducts (papillary ducts) that open onto the apex of the papilla. The renal corpuscles and convoluted portions of the proximal and distal tubules are located in the cortex. The thin segment and straight portions of the proximal and distal tubules are located in the medulla. The latter three segments form a loop called the loop of Henle. In unipyramidal kidneys, segments of Henle's loops are so regularly arranged that a separation of medulla into outer and inner zones is visible to the naked eye. The boundary between the zones is the junction of the ascending limb of the thin segment and the straight portion of the distal tubule. The junction of the straight portion of the proximal tubule with the descending thin limb subdivides the outer zone into inner and outer bands, which are also visible to the naked eye (Brown 1976).

Ureters

The ureters are slightly flattened tubes that begin at the renal pelvis and enter the urinary bladder by separate orifices near the neck of the bladder.

Urinary Bladder

The urinary bladder is a hollow musculomembranous organ. Its form, size, and position vary according to the volume of urine it contains. The near-full capacity of the bladder in the beagle is about 150 ml (Andersen 1970). The urinary bladder is divided into a neck region (connecting with the urethra), a blunt cranial end (fundus), and a body portion between the neck and fundus (Miller et al. 1967). Internally, a triangular area near the neck is termed the trigone. The base of the trigone is a line connecting the ureteral openings; the apex is the urethral orifice.

Urethra

The male urethra is the canal that carries urine and seminal secretions to the exterior. It is divided into prostatic, membranous, and penile portions. The female urethra extends from the urinary bladder to enter the vulva just caudal to the vaginovulvar junction.

Male Genital Organs

Scrotum. The scrotum is divided by a median septum into two cavities, each of which is occupied by a testis, its associated epididymis, and a distal part of the spermatic cord. The scrotum functions as a temperature regulator for the testes, a role made possible by its thin gland-rich skin, lack of subcutaneous fat, and an ability to contract toward the body (by means of the cremaster muscles) or relax away from the body.

Testis. The canine testis is oval and is thicker dorsoventrally than from side to side. The size (weight) of testes of young adult beagles varies so greatly as to be a significant concern in short-term toxicology studies. James and Heywood (1979) demonstrated the magnitude of the weight variation in beagles on 13-, 26-, and 52-week (and longer) studies (table 8.13). In their experience, male beagles attain full sexual maturity between 35 and 40 weeks of age (based on testicular size, semen evaluation, hormonal profiles, and quantitative histometric analysis of spermatogenesis).

Table 8.13 Testicular Weight in Beagles Under 2 Years of Age

Number of Dogs	Mean Age (Weeks)	Mean Body Weight (kg)	Mean Testicular Weight (g)	% of Dogs with Testicular Weight in Range (g)			
				< 9.9	10.0–19.9	20.0–29.9	> 30.0
42	37 ± 4	11.9 ± 1.8	20.7 ± 6.1	4.8	35.7	52.4	7.1
38	46 ± 2	15.6 ± 2.0	23.6 ± 5.0	0.0	26.3	63.2	10.5
41	73 ± 4	12.4 ± 2.0	25.0 ± 6.6	2.4	17.1	56.1	24.4

The cut surface of a normal testis bulges. The color of the testis in young beagles is light pink to tan (depth of color increases with age due to increased pigmentation of the interstitial cells). The testis is subdivided into numerous lobules by delicate connective tissue septa that extend from a core of connective tissue (mediastinum testis) to the capsule of the testis (tunica albuginea). Ducts, blood vessels, lymphatics, and nerves enter and leave the testis through the mediastinum. Each of the lobules contains one or more convoluted sperm-producing tubules (seminiferous tubules) that empty at both ends into straight tubules (tubuli recti) that connect with a series of epithelial-lined channels (rete testis) in the mediastinum. Spermatozoa are swept out of the seminiferous tubules, through the rete testis, and into the epididymis in a fluid secreted by the sustentacular cells (Sertoli cells). The interstitial cells (Leydig cells) are the endocrine (testosterone-producing) cells of the testis.

Epididymis. The epididymis consists of the ductuli efferentes and the much longer and highly tortuous ductus epididymidis. The ductuli efferentes emerge from the mediastinum to connect the rete testis with the ductus epididymidis. The epididymis lies along the dorsolateral surface of the testis. For descriptive purposes the epididymis is divided into three portions. The initial portion located near the cranial extremity of the testis and into which the ductuli efferentes empty is called the head of the epididymis (caput epididymis). The portion of the head with the ductuli efferentes is called the initial segment. The main length of the epididymis is the body (corpus epididymis), and the segment attached to the caudal extremity of the testis is the tail (cauda epididymis). The epididymis is the maturation and storage site for the spermatozoa. The ductus epididymidis is subdivided further, with each subdivision apparently fulfilling a specific function in the maturation process of the sperm.

Ductus Deferens. The ductus deferens (deferent duct) is a thick-walled tube that is continuous with the tail of the epididymis and extends to the prostatic urethra. Its initial portion is located within the spermatic cord and surrounded by the veins of the pampiniform plexus, arteries, lymph vessels, nerves, and smooth muscle.

Prostate Gland. The prostate gland (the only accessory male sex gland in the dog) is an ovoid musculoglandular organ that completely surrounds the proximal portion of the ureter. The prostate is composed of two portions: external and internal. The external portion forms most of the dog's prostate and consists of two large bilateral lobes. The internal portion consists of a few small glands scattered along the urethra. Trabeculae divide the two external lobes into lobules in which the glandular elements are most prevalent near the periphery. Secretion from prostate glands enters the urethra by way of numerous excretory ducts. The paired ductus deferens enter the dorsal surface of the prostate and run caudoventrally on either side of the median plane to open into the dorsal surface of the prostatic urethra. James and Heywood (1979) found incomplete prostate development in beagles 9 to 10 months of age and demonstrated a wide range of prostate weights in control beagles on short-term toxicity studies (table 8.14).

Penis. The male copulatory organ (penis) is composed of three principal parts: root, body, and distal free part (glans penis). The glans penis is mostly enveloped in stratified squamous epithelium and, when not erect, is entirely withdrawn into a tubular sheath of integument (prepuce). The

Table 8.14 Prostate Weight in Beagles Under 2 Years of Age

Number of Dogs	Mean Age (Weeks)	Mean Body Weight (kg)	Mean Prostate Weight (g)	% of Dogs with Prostate Weight in Range (g)			
				< 5.0	5.1–10.0	10.1–20.0	> 20.0
42	37 ± 4	11.9 ± 1.8	3.3 ± 1.6	84.4	15.6	0.0	0.0
38	46 ± 2	15.6 ± 2.0	6.5 ± 2.3	23.4	66.1	10.5	0.0
41	73 ± 4	12.4 ± 2.0	8.1 ± 2.9	9.8	63.4	26.8	0.0

mucosa of the penile urethra is lined by transitional epithelium except near the external urethral opening, where it changes to stratified squamous epithelium similar to that covering the penis.

Female Genital Organs

The female genital organs consist of the ovaries, oviducts, uterus, vagina, and vulva. The ovaries, oviducts, and uterus are attached to the walls of the abdominal and pelvic cavities by folds of peritoneum called broad ligaments. Each broad ligament contains an ovary, oviduct, and uterine horn (plus vessels, nerves, and fat).

Ovary. The ovary is an ovoid gland situated within a fossa of the peritoneum (ovarian bursa) and supported by the mesovarium (cranial portion of the broad ligament) and the suspensory ligament of the ovary. The ovary is completely enclosed by the ovarian bursa except for a narrow slit, 2 mm to 15 mm long, located on the medial side. The structure of the ovaries varies with age and the phase of the sexual cycle.

The ovary is composed of a cortex and medulla that contains a prominent rete ovarii. The cortex consists of a connective tissue stroma that contains blood vessels, lymphatics, follicles, and corpora lutea. The surface of the cortex is smooth in immature ovaries.

Oviducts. The oviducts (uterine tubes) are tortuous structures that extend from the region of the ovary to the uterine horns. In the bitch, the oviduct almost completely encircles the ovary. The large funnel-shaped ovarian end of the duct is called the infundibulum and is located near the slit in the ovarian bursa.

Uterus. The uterus is a Y-shaped tubular organ that communicates with the oviducts cranially and the vagina caudally. The uterus consists of a neck (cervix), body (corpus), and two horns (cornua). In the bitch, the uterine body is short and the horns relatively long and straight. The right horn is usually longer than the left. The size and shape of the uterus varies according to age and the stage of the sexual cycle. The uterine horns unite at the body. The body extends from the point of convergence of the uterine horns to the cervix. The wall of the uterus consists of three layers: mucosa (endometrium), muscularis (myometrium), and serosa (perimetrium). The endometrium is the thickest of the three layers and consists of three zones: crypt, intermediate, and basal (McEntee 1990). The crypt zone has numerous short, epithelial-lined recesses. The intermediate zone contains uterine glands, but it is predominantly connective tissue. The uterine glands branch, coil, and terminate in the basal zone.

Vagina. The vagina is the highly dilatable musculocutaneous canal extending from the uterus to the vulva. Cranially, the vagina is limited by the cervix, which can protrude up to 1 cm into the vagina. Caudally, the vagina ends just cranial to the urethral opening. Flat longitudinal folds extend throughout the length of the vagina. The tunica mucosa is nonglandular stratified squamous epithelium.

Vulva. The vulva is the external genitalia of the bitch and consists of the vestibule, clitoris, and labia. The vestibule is the space connecting the vagina with the external genital opening and is the

largest part of the vulva. The vestibule is the common opening for the genital and urinary tracts. The clitoris (homologue of the male penis) is located in the extreme caudal region of the vestibule near the ventral commissure of the vulva. The labia (lips) form the external boundary of the vulva. The vestibule is lined by stratified squamous epithelium and contains small, mucus-producing vestibular glands. Numerous lymph follicles are present in the vestibular mucosa and might be large enough to be seen grossly. The labia are covered with stratified squamous epithelium and are rich in sebaceous and tubular sweat glands.

The Estrous Cycle. Puberty is the age at which first estrus occurs in the bitch and varies among breeds and within breeds. The range is usually given as 6 to 12 months. The average age for first estrus in the laboratory beagle appears to be about 12 months, with a range of 10 to 14 months (Andersen 1970; Sekhri and Faulkin 1970; Sokolowski 1973). Most female beagles will be sexually immature when started on toxicology studies. At termination of short-term studies, they might still be sexually immature or be in any stage of their first estrous cycle. The sexual stage of the bitch at termination of a study impacts the gross and microscopic appearance of the sex organs (and mammary gland) and the weights of the ovaries, uterus, and conceivably the hypophysis and adrenal glands. Traditionally, the cyclical changes occurring in the reproductive system of the bitch have been divided into four phases: proestrus, estrus, metestrus, and anestrus. This classification has deemphasized the important extended luteal phase of the cycle and obscured several events that occur during the estrus phase of the cycle. Recently, several workers (Cupps et al. 1969; Holst and Phemister 1974; McDonald 1969) have reexamined the traditional classification and suggested modification. Phemister (1974) summarizes the revised view as follows.

> Proestrus remains the phase of rapid follicular growth and rising estrogen levels when there is swelling of the vulva, generalized congestion of the genital tract, and a sanguineous vaginal discharge. As a rule, proestrus lasts about 9 days. Estrus is the period of sexual receptivity, usually lasting 7 to 10 days. In most bitches the initial day of estrus coincides approximately with a surge of luteinizing hormone (LH) from the pituitary gland. The LH surge is followed by ovulation 2 days later, usually on about the 3rd day of estrus. Metestrus, as the term is used for other species, defines the brief period when the corpus luteum is being formed and becoming functional. In the bitch this phase lasts for about 4 days and occurs entirely within the period of acceptance (estrus). By 4 days after ovulation, the genital system is dominated by luteal progesterone. By definition this phase of progesterone dominance is diestrus. Its onset is signaled by an abrupt change in vaginal cytologic characteristics: from predominantly large comified, superficial squamous cells to noncomified, small intermediate and parabasal cells. On an average, diestrus begins 2 to 3 days before the end of estrus, and based on hormonal data, lasts for 2 to 3 months, or even longer if morphologic data are used. Following diestrus, the bitch enters a period of reproductive quiescence, anestrus, which lasts for 3 months or more.

Metestrus in the revised classification describes the period more in accordance with its use in other species, and emphasizes the fact that corpora lutea form and function for comparable periods (2–3 months) in a bitch whether she is pregnant or not pregnant. In other words, every nonpregnant bitch experiences a period of pseudopregnancy (pseudocyesis) to some degree. When the bitch's appearance and behavior closely mimic pregnancy, the pseudopregnancy becomes a clinical problem.

Necropsy and Laboratory Techniques

Because of the large functional reserve of the kidney, renal diseases might or might not be associated with renal dysfunction. Two-thirds to three-fourths of the renal parenchyma must be functionally impaired before clinical signs develop in chronic renal failure (Osborne et al. 1983). Urinary enzymes have been useful in dogs for detecting acute renal damage, but less helpful for chronic damage. Increases in brush border enzymes, including gamma-glutamyl transferase and

alkaline phosphatase, have been associated with proximal tubular damage, whereas increases in N-acetyl-beta-D-glucosaminidase have been observed in the early stages of renal papillary necrosis (Clemo 1998). Functional abnormalities of the kidney can be clinically manifested in a number of ways, including polyuria (formation and elimination of large quantities of urine), oliguria (decrease in the rate of formation or elimination of urine), anuria (lack of urine formation or elimination), polydipsia (excessive thirst), weight loss, anorexia, vomiting, diarrhea, dehydration, edema, stomatitis, weakness, and depression. Cloudy urine or hematuria might indicate disease of the urinary bladder. It is unlikely that signs of a reproductive system disorder would be detected in short-term toxicology studies. At postmortem, the initial examination of the urogenital system should be done with all organs of the system in place.

Kidney

The kidneys should be approximately equal in size. The normal cortex is brownish red and the medulla white to pink. The kidney should be cut into multiple transverse sections and the surfaces of each examined. One or two sections (4–5) should be taken from the midpyramidal region and placed in fixative. Proximal tubular epithelium autolyzes rapidly and kidneys should be placed into fixative as early as possible. Both the immature and mature kidney of the beagle have been studied in detail and much is known about normal morphometry of the organ (Eisenbrandt and Phemister 1977, 1978 1979, 1980; Jaenke et al. 1980; Stuart et al. 1975).

Ureters and Urinary Bladder

Usually, the ureters need only to be examined externally in situ. The urinary bladder should be removed, incised longitudinally, everted, and the mucosal surface examined. The entire bladder should be fixed. Usually, the bladder contains some urine at necropsy. Uncontaminated urine specimens can be obtained by puncturing the undisturbed bladder with a hypodermic needle (avoiding blood vessels) and withdrawing urine into a syringe.

Prostate, Scrotum, and Epididymis

The prostate should be removed and its internal surface examined by one or more transverse cuts (some prostates are so small that bisection is adequate). At least two sections should be fixed. The scrotum should be incised, the scrotal ligaments severed, the testes removed, and the vaginal tunics surrounding the spermatic cords incised to the level of the abdominal wall. After examining the structures of the spermatic cords and the surfaces of the testis and epididymis, the testis and epididymis are freed. The epididymis should be dissected from the testis before the testis is weighed. The epididymides can be fixed intact. The testis should be sliced before fixation to verify that the cut surface bulges. (Failure to bulge indicates degeneration of seminiferous tubules.) The incisions should be made with a very sharp knife. McEnree (1990) also recommends samples be taken from the head extremity, middle part, and caudal extremity. Bouin's is the recommended fixative for the testis.

Ovaries and Uterus

The ovaries and uterus are usually weighed in toxicology studies. As with the testis and prostate, the weight of the female organs varies according to sexual maturity, and, additionally, on the phase of the estrous cycle. Reproductive organs should be examined in detail in studies of compounds with known or potential effects on the reproductive system. Less attention might be paid to the reproductive tract in other studies; nevertheless, it seems appropriate that some observations be

made to establish the probable sexual state of a bitch at necropsy and to have these observations to correlate with microscopic findings and organ weight data.

Gross and Microscopic Appearance of Female Genital Organs at Necropsy

The gross and microscopic appearance of the female genital organs of the beagle during the various phases of the estrous cycle have been described (Andersen 1970; Sokolowski 1977; Sokolowski et al. 1973).

Vulva. The external genitalia swell and discharge a sanguineous fluid at the onset of proestrus. (The sanguineous discharge of proestrus and estrus is not due to mucosal hemorrhage but to extravasation of erythrocytes by diapedesis through the endometrium and into the lumen of the uterus and vagina.) The thickening of the labia and wall of the vestibule is due to congestion and edema that account for the warm feeling of the external genitalia from which the expression "in heat" is derived. The vestibular mucosa remains unwrinkled during proestrus and estrus, but lymph follicles might become prominent.

Vagina. The vaginal mucosa shows extensive wrinkling beginning at proestrus and continuing into estrus. (The normal longitudinal folds are exaggerated and in turn irregularly subdivided by numerous transverse wrinkles.) The wrinkling decreases later in estrus and by early metestrus (diestrus) the folds have lost their wrinkled appearance. The cervix protrudes prominently into the vagina during estrus, and its surface is marked by numerous folds. The cervical canal is patent throughout estrus and early diestrus. By the end of diestrus and throughout anestrus, the cervical canal is almost completely sealed.

Uterus. Enlarged blood vessels appear in the broad ligaments and in the perimetrium with proestrus and are present throughout estrus. The uterine wall thickens owing to congestion, edema, and proliferation of uterine glands. The uterus attains maximum nonpregnant size 20 to 30 days postovulation and exhibits a characteristic corkscrew appearance. At 60 days postovulation, the uterus is about the same size as in proestrus. The uterus never returns to the size seen in immature bitches.

Oviduct. During estrus, a small mass of red tissue protrudes from the slitlike opening of the bursal sac. The tissue is hyperemic and edematous fimbriae that almost completely close the slit.

Ovary. The ovaries of immature bitches are small, smooth, and have no follicles larger than 1 mm in diameter. A definite cortex and medulla are present at 6 months. On the first day of proestrus, follicles can be up to 4 mm in diameter and up to 14 mm in diameter just prior to ovulation. Ovarian weight is greatest at approximately the time of ovulation (presumably due to the size of follicles and the luteinizing that is occurring). Luteinizing begins immediately after follicles rupture, and it is not unusual to find follicles of varying size and corpora lutea in the same ovary at estrus. Corpora lutea are most numerous and fully developed about 10 days after ovulation. Corpora lutea begin to degenerate at about 20 days postovulation and by 60 days postovulation have undergone fatty degeneration and appear nonfunctional. Corpora lutea are bright salmon pink from the time of ovulation until 10 days after ovulation. They then gradually yellow and are light tan about 60 days after ovulation. No remnants of corporea lutea (corpora albicantia) are seen in ovaries during the first estrous cycle.

Mammary Gland. Gross changes are imperceptible in the mammary gland until shortly after ovulation, when a bluish plaque can be observed at the base of each teat. Thereafter the mammary glands enlarge and regress during the metestrus (diestrus) stage of the estrous cycle.

Pathology

Many changes have been reported for the kidney and male genital organs. In contrast, few observations have been made for the female genitalia.

Nonneoplastic Findings: Spontaneous

Kidney. Inflammation of the kidney is a frequent finding in dogs. The inflammation can be either primary or secondary and affects various tissue components resulting in many disease entities being reported. Inflammation of the kidney without identifying specific affected components can also be termed nephritis. Minor focal nonspecific inflammation occurred in 76 (12%) of 647 dogs (Hottendorf and Hirth 1974). Leukocytic foci were observed in 14% of young beagles (Glaister 1986). Focal embolic nephritis was reported in 1 of 647 dogs (Hottendorf and Hirth 1974).

Inflammation of the glomerulus can also be termed glomerulitis, glomerulonephritis, and glomerulosclerosis. Glomerulitis and glomerulonephritis were reported in 2 (0.3%) of 647 dogs (Hottendorf and Hirth 1974). Local or diffuse mesangial proliferation and thickened and wrinkled glomerular basement membranes are not an unusual finding in clinically healthy, nonproteinuric laboratory beagles (Stuart et al. 1975). Periglomerular sclerosis might also be present. With time, the intercapillary sclerosis generally increases in severity and could be associated with intermittent or persistent proteinuria.

A progressive alteration of the renal glomerulus called progressive intercapillary glomerulosclerosis (ISG) was described by Guttman (1970). This change is seen as early as 6 months of age and consists of thickening of basement membranes and increases in the mesangial matrix.

Inflammation of the interstitium, usually termed interstitial nephritis or chronic interstitial nephritis, is common. Interstitial nephritis was reported by Oghiso et al. (1982). Focal interstitial nephritis was recorded in 6% of young beagles (Glaister 1986). Chronic interstitial nephritis occurred in 6 (0.9%) of 647 dogs (Hottendorf and Hirth 1974).

Inflammation of the pelvis, also termed pyelitis and pyelonephritis, is another common finding. Pyelitis, usually with minor degrees of mononuclear infiltration of the lamina propria and epithelium of the renal pelvis, was observed in 4% to 7% of young beagles (Glaister 1986). Pyelonephritis is a nephritis that arises in the pelvis and spreads upward to adjacent areas of the kidney. Pyelonephritis was reported by Oghiso et al. (1982). Pyelitis or pyelonephritis occurred in 12 (2%) of 647 dogs (Hottendorf and Hirth 1974). Pyelitis and pyelonephritis were also reported by Pick and Eubanks (1965).

Granulomatous inflammation or granulomas are frequent findings. Barron and Saunders (1966) reported the kidney as a common site for Toxocara granuloma. Cortical Toxocara granulomas were reported in 4% of young beagles (Glaister 1986). Cortical granulomas were observed in 2 (5%) of 37 dogs (Pick and Eubanks 1965). Granulomas occurred in 11 (2%) of 647 dogs (Hottendorf and Hirth 1974). Toxocara granuloma were also recorded in the kidney by Maita et al. (1977).

Mineralization of the kidney is very common in dogs and is usually seen in the form of clumps of basophilic granules adjacent to and in the lumen and lining of collecting tubules. The incidence is probably higher than the reported 50%. Collecting tubule calcification was reported by Pick and Eubanks (1965) and calcification by Oghiso et al. (1982). Microcalculi of the renal medulla were found in almost 50% of the 647 males and females (Hottendorf and Hirth 1974). Mineralization of renal papilla was observed in both males and females (Glaister 1986). Degenerative changes of the glomeruli and tubules were mentioned but these changes were not described by Oghiso et al. (1982), who also reported albuminous urinary casts. Occasionally, one or more lobules of the glomerular tuft will be filled with large foam cells, which, with appropriate stains, are shown to contain fat. The condition is known as glomerular lipidosis and has no known functional significance.

Hottendorf and Hirth (1974) reported the occurrence of hydronephrosis in their 647 dogs. Acidophilic rectangular or cubic crystalline intranuclear inclusions (identical in appearance to those seen in hepatocytes) are also commonly found in nuclei of cells lining proximal and distal tubules (Oghiso et al. 1982).

Unilateral renal agenesis is occasionally found in laboratory beagles (Hottendorf and Hirth 1974; Robbins 1965; Vymetal 1965) and was observed in adult breeding colony beagles (Fritz et al. 1966, 1967). Four (0.4%) of 1,000 dogs were affected (Hottendorf and Hirth 1974). This condition is not detected during routine physical examinations. The ureter might or might not be missing. The developed kidney is usually about twice normal size (weight) and histologically of normal appearance, suggesting that the increased size is due to an absolute increase in the number of nephrons. Postnatal nephrogenesis is a characteristic of the canine kidney. Eisenbrandt and Phemister (1979) have shown that nephrogenesis continues through the first 8 to 10 days of life in the beagle, a fact that probably accounts for the normal histological appearance of the single enlarged kidney seen in unilateral renal agenesis.

Urinary Bladder. Calculi and cystitis were seen infrequently in the urinary bladder, buut more often in males (Hottendorf and Hirth 1974). Cystitis occurred in 27 (23 males and 4 females) of 647 dogs (Hottendorf and Hirth 1974). Catheterization might be the cause of some instances of prostatitis and cystitis. Detrusor myopathy is a condition characterized by degenerative lesions in the urinary bladder muscule tunics that occurred in 59 of 449 (13%) young beagles. Both sexes were affected equally. Arteritis and periarteritis were reported in 10 of the 59 dogs with detrusor myopathy (Cain et al. 2000). Calcification of the round ligament of the bladder (remnant of the umbilical artery of the fetus) was seen, but not listed as a significant finding (Hottendorf and Hirth 1974). Calculi were reported as an uncommon finding in the urinary bladder. Five animals were grossly affected in 1,000 dogs (Hottendorf and Hirth 1974).

Urethra. Calculi are an unusual finding in the urethra, with 1 animal grossly affected in 1,000 dogs (Hottendorf and Hirth 1974).

Testis. Focal atrophy of seminiferous tubules was reported in 11 (3%) of 326 dogs by Hottendorf and Hirth, (1974) and in 5% of young beagles by Glaister (1986). Testicular degeneration occurs naturally in the dog and can be focal or diffuse, unilateral or bilateral. Early degenerative lesions consist of loss of primordial germ cells that might appear within the lumen of the seminiferous tubule as individual cells or as multinucleated giant cells. As the degeneration advances, more germinal cells are lost and tubules might be lined only by sustentacular cells (Sertoli cells). Intratubular giant cells were observed in the seminiferous tubules of 9 (3%) of 326 untreated young beagles (Hottendorf and Hirth 1974). Lymphocytic orchitis has been observed in beagle colonies and occurs in laboratory beagles, usually associated with lymphocytic thyroiditis. The lymphocytic infiltration can be diffuse, aggregated, or nodular (with germinal centers) and is commonly associated with focal or diffuse degeneration and atrophy of seminiferous tubules. The epididymis might be involved (Fritz et al. 1976). Orchitis can be caused by *Brucell canis* infections (Jubb et al. 1992). Interstitial cell hyperplasia is rarely seen in untreated young beagles. It was noted in 1 (0.3%) of 326 dogs (Hottendorf and Hirth 1974).

Epididymis. Inflammation of the epididymis was reported in 1 of 326 male dogs by Hottendorf and Hirth (1974). Epididymitis can be caused by *Brucella canis* infections (Jubb et al. 1992). Lymphocytic epididymitis often associated with lymphocytic orchitis and thyroiditis is characterized by lymphocytic infiltrations (Fritz et al. 1976). Spermatic granulomas are inflammatory lesions that occur in the efferent ductules and epididymis. Intratubular granulomas were observed in the caput epididymis of three clinically normal dogs that were associated blind-ending efferent ducts. Although spermatic granulomas can be induced by trauma, infection, or toxins, spontaneous

granulomas due to blind-ending ductules should be considered in the differential (Foley et al. 1995). James and Heywood (1979) report spermatocoele granuloma (spermatic granuloma) and inflammation for the epididymis. It is normal to find intranuclear, eosinophilic, periodic acid-Schiff positive inclusions in the epididymal epithelium of the dog (McEntee 1990). The significance of the inclusions is unknown. Normal epididymal cells also contain granular, yellow to yellow-brown pigment.

Prostate. Subclinical prostatitis is common in the dog. The inflammation is usually minimal in young adult beagles. Prostatitis was reported in 124 (38%) of 326 males from 39 studies (Hottendorf and Hirth 1974). Chronic inflammation of the prostate was also reported by Maita et al. (1977) and James and Heywood (1979). Collection of urine by catheterization could be the cause of some prostatitis. Prostatitis can be caused by *Brucella canis* infections (Jubb et al. 1992). Minor accumulations of lymphocytes (leukocytic foci) to large lymphoid aggregates with germinal center formation accompanied by interstitial fibrosis and epithelial atrophy (prostatitis) were observed in 12% of young beagles (Glaister 1986). Atrophy was reported in the absence of inflammation in 7 (2%) of 326 males by Hottendorf and Hirth (1974). Focal cystic hyperplasia was reported in 15 (5%) of 326 males by Hottendorf and Hirth (1974).

Prepuce. Mild balanoposthitis (inflammation of the glans penis and prepuce) is common and can result in slight mucopurulent preputial discharge.

Ovary. Occasionally, clear cysts can be found in the vicinity of the ovaries of young adult beagles. The cysts have one of several origins (McEnree 1990). The finding of a parovarian cyst is not a specific diagnosis. Parovarian cysts were observed grossly in the ovaries of 2 (0.4%) of 499 dogs (Hottendorf and Hirth 1974).

Uterus. Distention of the uterus was observed in 11% of young beagles (Glaister 1986). Myometrial cysts have been reported in the uterus with 1 affected animal in 499 females (Hottendorf and Hirth 1974). Inflammation of the uterus or endometritis caused by *Brucella canis* infections can result in abortion during the third trimester of pregnancy (Jubb et al. 1992).

Neoplastic Findings: Spontaneous

Kidney. Spontaneous tumors are rare in the kidney of young dogs. A renal carcinoma was reported in 1 of 1,000 laboratory dogs (Hottendorf and Hirth 1974). A benign mixed mesenchymal tumor was detected in the kidney of an 11-month-old beagle (Robison et al. 1997).

Nervous System

Anatomy and Histology

The nervous system is the chief coordinating system of the body. The body's other major coordinating system, the endocrine system, is controlled by the nervous system and a major part of it (the hypothalamus) is also a major part of the nervous system. The overall function of the nervous system is to produce the proper reaction of the organism to changes in both the external and internal environment (Jenkins 1978). This function depends directly on the neuron. Neurons, along with supportive cells (neuroglia), make up the nervous system. The nervous system is divided into the central nervous system (CNS) and the peripheral nervous system (PNS), more for discussion purposes than on the basis of structure or function. The CNS consists of the brain and spinal cord (neuraxis). The PNS consists of cranial and spinal nerves, sensory and motor ganglia, sensory and motor nerve endings, and the autonomic nervous system.

The autonomic nervous system is that part of the nervous system concerned with motor innervation of smooth muscle, cardiac muscle, and glands. On anatomical, pharmacological, and functional bases, the autonomic nervous system is divided into a sympathetic portion and a parasympathetic portion. The general function of the sympathetic portion is to prepare the body for a state of emergency. The general function of the parasympathetic portion is to restore the body to a normal state of quiescence. The hypothalamus is the key to autonomic regulation. The rostral hypothalamic area controls the parasympathetic function and the caudal hypothalamic area controls the sympathetic function.

Divisions of the Nervous System: Structure and Function

All divisions of the nervous system are structurally and functionally connected. The PNS transmits impulses toward the CNS by way of sensory (afferent) neurons and away from the CNS by way of motor (efferent) neurons. The typical spinal nerve and the majority of cranial nerves have both types of functional neurons and are referred to as mixed nerves. A typical peripheral nerve consists of an outer connective tissue sheath (epineurium) enclosing bundles (fasciculi) of nerve fibers. Each fasciculus is surrounded by its own connective tissue sheath (perineurium) and is made up of individual nerve fibers (microscopic in size) surrounded by their connective tissue sheaths (endoneurium). Individual nerve fibers consist of an axon and an enclosing sheath formed by lemmocytes (Schwann cells). The axon is the elongated cytoplasmic extension (process) of the nerve cell. The surface membrane of the axon is the axolemma. Most peripheral axons are surrounded by an insulating sheath of lipoprotein (myelin). Myelin is the nonnucleated plasma membrane of the Schwann cell. The nucleated portion of the Schwann cell is the neurilemma (regeneration of axons is possible in Wallerian degeneration owing to survival of the nucleated neurilemma). The terms axolemma and neurilemma are not synonymous, and neither neurilemma nor myelin is synonymous with the total Schwann cell. The terms axon and axis cylinder are synonymous. Unfortunately, the total nerve fiber is also referred to as the axon.

Nerves of the CNS

The nerves attached to the brain are referred to as cranial nerves. There are 12 pairs, and except for the first pair (olfactory), all are attached to the brain stem. Nerves attached to the spinal cord are referred to as spinal nerves. There are usually 36 pairs of spinal nerves in the dog, derived from 36 spinal cord segments: 8 cervical, 13 thoracic, 7 lumbar, 3 sacral, and 5 coccygeal. With the exception of the first cervical nerve, all spinal nerves pass through intervertebral foramina. Because the spinal cord is shorter than the spinal column (in the embryo, the vertebral column grows more rapidly than the spinal cord, resulting in a relative caudal migration of vertebrae) there is not a one-to-one relationship between the spinal cord segments and the vertebrae. As a result, spinal nerves generally exit caudal to their segment of origin and vertebrae of one region might contain spinal cord segments of another region. The actual spinal cord segment and vertebral body correlations must be kept in mind when spinal cord segments are chosen for histological examination. The nerve distribution to the fore- and hindlimbs allows the spinal cord to be divided into functional units. The location of the units within vertebrae is shown in table 8.15 (Bailey and Morgan 1983).

The CNS is protected, supported, and nourished by three sheetlike connective tissue coverings called the meninges (singular: meninx). The outermost (and toughest) meninx is the dura mater, The dura mater of the cranial cavity serves a dual function as meningeal covering for the brain and endosteal (or periosteal) lining for the bones of the calvarium. The spinal dura mater is separated from the periosteum of the vertebrae by the epidural cavity.

The second meninx (arachnoid) is much thinner and more delicate than the dura mater. The arachnoid is separated from the dura mater by an almost nonexistent subdural cavity. The arachnoid connects with the pia mater (the third meninx) by delicate trabeculae, but it is separated from the

Table 8.15 Vertebrae and Spinal Cord Segments

Cord Segment	Nerve/Tract Distribution	Vertebrae
Cervical, C1–C5	Nerve fiber tracts ascending from forelimbs and hindlimbs and descending from brain	CI–C4
Cervicothoracic, C6–TI/T2	Origin of nerves to forelimbs	C5–C7
Thoracolumbar, T2–L3	Nerve fiber tracts ascending from hindlimbs and descending from brain	T2–L3
Caudal lumbar, L4–L7	Origin of nerves to hindlimbs except S, contribution to the sciatic nerve	L3–L4
Sacral, S1–S3	Origin of nerves to the anus, urinary bladder, and perineum. S, contribution to sciatic nerve	L5
Coccygeal, C1–C5	Origin of nerves to tail caudally	L5

pia mater by a large underlying space (subarachnoid space) filled with cerebrospinal fluid. The cerebrospinal fluid (CSF) pushes the arachnoid peripherally to contact the dura mater, forming a fluid envelope to cushion and protect the brain and spinal cord. The pia mater is closely adherent to the brain and extends deeply into the sulci of the cerebral hemispheres and between the folia of the cerebellum. The pia mater is the vascular meninx and mainly concerned with the nutrition of the CNS. The pia mater and arteries combine to form the tela choroidea of the choroid plexuses, which produce the major portion of the CSF.

The cerebrospinal fluid is a clear, colorless fluid that fills the ventricular system of the brain, the subarachnoid space of both the brain and spinal cord, and the central canal of the spinal cord. The ventricular system of the brain and the central canal of the spinal cord are lined by a layer of closely packed cuboidal or columnar epithelial cells known collectively as the ependyma. In the ventricles of the brain, the ependyma is modified to form the special secretory epithelium of the choroid plexcus. There are four ventricles in the brain: two lateral and single midline third and fourth ventricles. The lateral ventricles communicate with the third, the third with the fourth, and the fourth with a dilatation of the subarachnoid space (cisterna magna) between the cerebellum and medulla oblongata.

Necropsy and Laboratory Techniques

As noted in preceding sections, disease in another organ system can directly or indirectly involve the nervous system. Some of the more prominent signs of neurological disease include depression (dull, lethargic, inattentive), disorientation (loss of proper bearings, mental confusion), stupor (partial unconsciousness, can arouse with stimulation), coma (unconsciousness from which animal cannot be aroused with powerful stimuli), hyperexcitable (excessive response to normal stimuli), tilting of head, twisting of bod, tremors, paresis (incomplete loss of voluntary motor function) or paralysis (complete loss of voluntary motor function) of one or more limbs, ataxia (incoordination of gait), abnormal reflexes, loss of control or urination and defecation and hyperesthesia, among others (Greene and Oliver, 1983).

If in-life observations indicate that a neurological problem exists, thorough physical and neurological examinations should be done to fully characterize the clinical signs, to localize the disease process (brain, spinal cord, peripheral nervous system), and, if possible, to define more precisely where in the brain, spinal cord, or PNS the problem might be. Based on these (and clinical laboratory) findings, the pathologist can modify the postmortem procedures as needed to assure that all appropriate areas are examined and all appropriate tissues are taken.

To minimize the handling of the brain, the head should be removed from the body. First, reflect the skin from the head and neck, sever neck muscles at their attachment to the posterior aspect of the skull, and cut completely through the spinal cord at the atlanto-occipital articulation. The cut is made by passing a thin, narrow blade through the dorsal atlanto-occipital membrane into the cisterna magna and then through the spinal cord. As the cisterna magna is entered, a quick appraisal

of the cerebrospinal fluid (CSF) should be made. Expose the calvarium by removing the temporalis muscles. Remove the head by completing the disarticulation of the atlanto-occipital joint. (The disarticulation can be done quickly if the prosector is methodical in cutting the joint capsule and the various ligaments.) Three cuts are made through the calvarium. One cut is made transversely through the frontal bone at the anterior limit of the cranial cavity slightly rostral to the zygomatic processes. The incision includes the frontal sinuses. Two identical cuts are made on each side of the calvarium, just dorsal to the widest part of the brain case. The lateral cuts connect the transverse cut with the foramen magnum. The foramen magnum should be entered at its widest point slightly dorsal to the occipital condyles. The cuts through the calvarium require practice and a sense as to when the saw is about to leave bone and enter the brain. Pull the calvarium upward and backward. Generally, the dura mater will strip from the calvarium and remain with the brain.

After examining the dura mater, cut it (and the arachnoid) parallel to the bone incisions. Two large folds of the dura mater will also need to be freed from their insertions between parts of the brain. The falx cerebri is the midsagittal fold between the cerebral hemispheres. The tentorium cerebelli is the transverse fold between the occipital poles of the cerebral hemispheres and the cerebellum. Failure to completely remove these folds will interfere with removal of the brain. The brain is extremely fragile and should be removed from the skull mostly by gravitational force and a little gentle traction. This is accomplished by holding the head upside down in one hand and freeing the brain by severing the cranial nerves, posteriorly rostrally, as they come to view. In the process, the brain gradually falls into the palm of the supporting hand. The hypophyseal stalk (infundibuluni) is severed as soon as it is seen so that the hypophysis remains intact in the sella turcia. The optic nerves are cut and as much of the olfactory lobes are removed as possible. Examine the ventral surface of the brain as it lies in the palm of the hand.

Place the brain in a weighing boat and examine the dorsal surface. The brain need not be grasped, but can be slid from weighing boat to weighing boat or weighing boat to fixative. The removal of the hypophysis has been described in the endocrine system. The inner surface of the calvarium and ventral surface of the cranial cavity should be examined. The entire spinal cord, or segments of it, can be removed by either a ventral or dorsal approach. Before attempting to remove a segment, always completely transect the cord cranially and caudally to prevent stretching and twisting of the spinal cord as the spinal column is manipulated. The spinal cord should be removed by grasping the dura mater. If the entire spinal cord is to be collected, it should be fixed in a fully extended position in an adequately long container. Cervical and thoracolumbar segments of the spinal cord are easily removed from vertebrae C1–C4 and T2–L4, respectively. These segments sample ascending and descending tracts of all the limbs and the nerves of origination to the hindlimbs. Traditionally, a portion of a sciatic nerve is collected as the representative sampling of the PNS. The sciatic nerve can be removed with minimal trauma and is large enough to provide adequate samples (5 cm or longer). The sciatic nerve contains both afferent and efferent nerve fibers (mixed nerve). If a sensory nerve (afferent fibers) is required, a branch of the sciatic nerve, the caudal (lateral) cutaneous sural nerve, can be taken. The caudal cutaneous sural nerve has been studied in healthy adult beagles and morphometric and electrophysiological data exist for it (and the ulnar and saphenous nerves; Illanes et al. 1988). Artifacts in nervous tissue can be as troublesome as artifacts in muscle tissue. The makeup of nervous tissue makes it unusually sensitive to autolysis, rough handling, and the chemical effects of fixative. Primary fixation by whole body perfusion with buffered glutaraldehyde addresses most of the problems and can be performed as successfully in the dog as in smaller laboratory animals. However, whole body perfusion has practical application only in selected studies, leaving the problem of tissue artifacts in the brain, spinal cord, and peripheral nerves to be dealt with on a daily basis in routine studies. With the brain, the choice is between the artifacts of handling and distortion from slicing a brain in an unfixed state and the artifacts of autolysis in brains fixed by immersion. The choice is usually to fix the brain in toto and examine for gross internal lesions when the brain is sectioned for processing. Full transverse (coronal) sections are preferred for microscopic examination. Cross-sections and longitudinal

sections of the spinal cord should be prepared. Specimens of peripheral nerves should be at least 5 cm in length and removed with great care. Peripheral nerves should be fixed in a gently stretched state on cardboard or corkboard. Ten percent neutral buffered formalin is the traditional fixative for nervous tissue. Brains should be fixed in large volumes of fixative that should be changed frequently. The investigator might wish to treat peripheral nerve specimens as surgical specimens and follow a nerve biopsy protocol (Asbury and Johnson 1978), which provides for plastic or paraffin embedding; routine, thick, or thin sections; and special stains and nerve fiber microdissection (nerve teasing, teased specimen). Routine sections of peripheral nerves should always include transverse and longitudinal sections. H&E is the most universally used stain; however, it does not stain specific nerve components and special stains are required for myelin, Nissl substance, neurofibrils, neuroglia, and so on. The writers fully agree with Jenkins (1978): "Neurostaining is a special technique which ideally should be reserved only for the experienced histotechnician who has the time and interest to devote to the subject." Microscopists who are unfamiliar with the artifacts commonly encountered in peripheral nerves are referred to Asbury and Johnson (1978). Readers interested in contemporary neuropathological methods in toxicology are referred to Spencer and Bischoff (1982).

Pathology

Hydrocephalus is a common nervous system finding in laboratory dogs. It was observed grossly in 14 (1%) of 1,000 dogs on 39 studies (Hottendorf and Hirth 1974). Hydrocephalus (ventricular dilatation) associated with a sponge-like alteration in the surrounding brain tissue was observed by Oghiso et al. (1982). Several beagles with hydrocephalus were reported by Fritz et al. (1967).

Hydrocephalus is characterized by abnormal accumulations of CSF. It is usually manifested as dilatation of one or more ventricles of the brain (internal hydrocephalus), but ventricles might be unaffected and it is the subarachnoid space that is dilated with the excess CSF (external hydrocephalus). If excess CSF is present in both locations, the condition is referred to as communicating hydrocephalus (Sullivan 1985). Although hydrocephalus appears to be fairly common in laboratory beagles, it is likely that minor degrees of ventricular dilatation are unrecognized and the real incidence of the condition is greater than reported. Lateral ventricles of surprising size (some with thinning or rupture of the septum pellucidum) are found incidentally while trimming brains of dogs that showed no neurological signs. At necropsy, the ventral surface of the brain should be closely examined, as the piriform area will dimple under slight pressure even in mild hydrocephalus. Hydrocephalus in laboratory beagles is probably a congenital condition.

Inflammation of the nervous system was also frequently reported. Chronic focal meningitis was observed in brains from 44 (7%) of 630 dogs in 33 studies and focal encephalitis in 5 (0.8%) of these 630 dogs (Hottendorf and Hirth 1974). Focal myelitis was observed in spinal cords from 2 (0.3%) of 647 dogs (Hottendorf and Hirth 1974). Toxocara granulomas in the brain and spinal cord (cauda equina) were reported by Barron and Saunders (1966).

Hemorrhage involving the brain was reported by Pick and Eubanks (1965). They did not elaborate on the location or extent of the hemorrhage.

Hottendorf and Hirth (1974) observed, but did not report as lesions, small subependymal collections of glial cells usually seen around the anterior parts of the lateral ventricles. Spherical, eosinophilic granular structures have been observed in the medulla oblongata, pons, or anterior cervical cord. They seem to be most common in the gracilis tract and nucleus. Newberne et al. (1960) and Innes and Saunders (1962) believe the structures are degenerating axis cylinders. They apparently have no neurological or pathological significance. Spontaneous degenerative lesions of the peripheral nerves occur in the beagle as in other laboratory animals. Although commonly considered an aging change, occasionally digestion chambers and myelin bubbles are seen in the sciatic nerves of young beagles.

During examination of peripheral nerves, cylindrical, loosely textured, whorled, cell-sparse structures are occasionally observed within the nerves. These are Renaut bodies and were well known to histologists of the late 19th century, but they have gradually been forgotten (Asbury 1973; Asbury and Johnson 1978). Renaut bodies are particularly well developed in the horse and donkey, but are less conspicuous in the dog and humans. Their purpose is unknown. It is important that they be recognized as normal structures and not misdiagnosed as nerve infarction or necrotizing angiopathic neuropathy, as has been done in the past (Asbury 1973).

Eye and Ear

Anatomy and Histology

Eye

The eye is the organ of vision. It is composed of the eyeball (globe), the optic nerve, and accessory structures including the eyelids, conjunctiva, lacrimal apparatus, and ocular muscles. The canine eye is relatively large for the size of the animal. The human eye is approximately 2.5 cm in diameter (Kuwabara and Cogan 1977); the eye of an adult beagle is approximately 2.2 cm in diameter (Andersen 1970). The canine eye is also placed well forward in the head, giving the dog a large field of binocular vision. The eyeball is roughly spherical in shape, with the rostral curvature of the cornea making the anterioposterior diameter the greatest diameter of the eye. To facilitate description and orientation of the eye, special designations as to side, direction, and position are used. Oculus dexter (OD) designates the right eye; oculus sinister (OS) designates the left eye; and oculus unitus (OU) designates both eyes. The side of the globe nearest the nose is the nasal, or medial, side; the opposite side is the temporal, or lateral, side. The dorsal side is superior; the ventral side is inferior. The corneal pole is distal, or anterior; the cerebral pole is proximal, or posterior. The line connecting the two poles is the anatomical or optic axis. The equator is the greatest expansion of the eyeball perpendicular to the anatomical axis. A horizontal plane through the poles divides the eyeball into an upper half and a lower half. A vertical plane through the poles divides the eyeball into a nasal half and a temporal half.

Eyeball. The eyeball consists of an inner coat, or tunic, of neural light-sensitive tissue (retina) held in shape by surrounding coats that protect it (corneoscleral coat, outer fibrous coat) and nourish it (uvea, middle vascular coat). The outer fibrous coat is subdivided into a larger, tough, white posterior portion (sclera) that covers about 75% of the globe and a smaller, transparent anterior portion (cornea). The transition from the opaque sclera to the transparent cornea is comparatively abrupt and occurs at the corneoscleral (sclerocorneal) junction, or limbus. The limbal area of the sclera is pigmented laterally and medially but not dorsally or ventrally.

Sclera. The thickness of the sclera varies. At the equator, it is so thin as to be semitransparent and the dark color of the uvea shows through. It is thick at the ciliary region where extraocular muscles insert and around the optic nerve.

Cornea. The cornea is transparent, colorless, and nonvascular; however, it possesses dense nerve fiber plexuses and is highly sensitive. Opposite to what exists in humans, the cornea of the dog is thicker in the center than at the periphery (Getty 1967). The transparency of the cornea is due to a nonkeratinized and nonpigmented surface epithelium, lack of blood vessels and lymphatics, cell-poor stroma composed of thin collagen fibrils arranged in orderly lamellae, and a sodium-potassium pump in the cell membrane of the corneal endothelium that maintains a high degree of stromal dehydration (Wilcock 1985). The composition of the cornea is notably uniform and consists of six or seven layers (six in the dog). The outermost and often overlooked layer is the tear film

(not seen in histological sections). The outer stratified squamous epithelium is a continuum of the conjunctival epithelium. The basement membrane of the epithelium is the third layer. According to Dellmann (1976b), the dog does not have an anterior limiting membrane (Bowman's membrane). However, Prince et al. (1960) indicate that an anterior limiting membrane is present, but it is extremely thin (1.5 mcm vs. 30 mcm in humans).

Shively and Epling (1970), in their study of the fine structures of the beagle eye, found no consistent layer of randomly oriented collagen fibers that would constitute an anterior limiting membrane. The stroma constitutes the bulk of the cornea. Descemet's membrane is present (a required constituent, as it is the basement membrane of the innermost layer, the endothelium). The endothelial layer should probably be known as mesothelium based on its structural characteristics and its probable origin (Shively and Epling 1970).

Uvea. The uvea is the highly pigmented and vascular coat of the eye. It consists of the iris, ciliary body, and choroid.

Iris. This the most anterior portion of the uveal tract and is the diaphragm of the eye.

Ciliary Body. This is a ring of tissue that extends from the base of the iris to the neuroserisory retina posteriorly. The main components of the ciliary body are the ciliary processes and the ciliary muscle.

The ciliary processes are actually linear folds that appear from the rear as multiple radiating ridges. The ciliary processes are highly vascular and are thought to be the main sites for formation of the aqueous humor (an arrangement comparable to the formation of CSF by the choroid plexuses of the brain).

In accommodating for near vision, the ciliary muscle contracts, pulling the ciliary body forward, which allows the supporting fibers (zonule fibers) of the lens to relax, leading to relaxation and an anterior-posterior thickening of the lens.

Choroid. The bulk of the uvea is formed by the choroid, which consists mainly of blood vessels and melanocytes. Externally, it blends with the sclera; internally, it is bounded by a basal lamina called Bruch's membrane; and dorsally (above the optic disc) it displays a peculiar structure known as the tapetum lucidum.

Tapetum. Many animals have one of two types of tapeta: tapetum lucidum fibrosum or tapetum lucidum cellulosum. The dog has a tapetum lucidum cellulosum. The tapetum lucidum is a light-reflecting layer (responsible for the luster of eyes) that is situated in the dorsal half of the choroid. The tapetum is triangular to semicircular in shape, extending about halfway to the periphery of the choroids, and has a horizontal base that just contacts the top of the optic disc. Although the tapetum appears to be immediately adjacent to the pigment epithelium cells of the retina, there is a layer of capillaries (choriocapillaris) between the tapetum and Bruch's membrane. The tapetum is made up of flat cells varying in number from 9 to 10 layers centrally to 1 or 2 layers peripherally (Prince et al. 1960).

Retina. The innermost tunic of the eye is the retina. It is divided into a sensory portion (pars optica) that rests on the choroid and a nonsensory portion that rests on the ciliary body (pars ciliaris) and the posterior surface of the iris (pars iridica). The sensory and nonsensory portions join posterior to the ciliary body at a scalloped border known as the ora serrata. The sensory retina is firmly attached at the ora serrata and optic disc, but it is loosely attached over the choroid. Under normal circumstances, the pressure of the vitreous is sufficient to hold the retina in contact with the choroid.

The nonsensory retina consists of a simple layer of pigmented cuboidal epithelial cells that continues at the ora serrata to form the outermost of the 10 layers of the sensory retina. Not all of the pigment epithelium is pigmented; the cells overlying the tapetum lucidum are not. According to Kuwabara and Cogan (1977), two pigments can be found in the pigment epithelium: melanin and lipofuscin. The

pigment epithelium interdigitates with the overlying photoreceptors. Among other functions, the epithelial cells engulf and degrade obsolete rod and cone cell segments. The accumulation of lipofuscin appears to be a result of this phagocytic activity. In humans, the quantity of lipofuscin increases with age and the number of melanin granules decreases (Kuwabara and Cogan 1977).

The inner transparent layer of the sensory retina comprises nine layers, named from within outward: internal limiting membrane, nerve fiber layer, ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, external limiting membrane, and the layer of rods and cones. The retinal receptors in the dog are predominantly rods. That feature (and others) indicates that the dog is predominantly a nocturnal animal (Prince et al. 1960).

To the ophthalmologist, the visible portion of the posterior globe is the ocular fundus, which for descriptive purposes is commonly divided into a dorsal tapetal fundus and a ventral nontapetal fundus (tapetum nigrum). The optic disc is usually at the junction of the two. The optic disc (optic papilla) is the rounded, raised area where the optic nerve leaves the eye. Jenkins (1978) emphasizes the following important features relative to the optic nerve: Because the optic nerve is a fiber tract of the brain, it has no neurilemmal sheath of Schwann and regeneration is not possible; it contains neuroglial elements; has a meningeal investment but no epi-, peri-, or endoneurium; and the individual nerve fibers are all myelinated. Wilcock (1985) points out that, in contrast to most domestic species, the dog's fibers in the optic papilla are myelinated. Because the optic nerve is in direct contact with the eye and brain via neurons and CSF, it can be affected by diseases of both the eye and brain.

Branches of the ciliary and internal ophthalmic arteries and veins lie just along the optic nerve. When they reach the eye, some plunge into globe and others (medial and lateral long posterior ciliary arteries and veins) pass into the superficial layers of the sclera and are externally visible for a distance along the horizontal meridian of the eye. These vessels serve as landmarks in orienting the eye at trimming.

The transparent media of the eye include the cornea, aqueous humor, lens, and vitreous body (the retina, excluding the pigment epithelium, is also transparent). The aqueous humor is the clear fluid contained in a cavity bounded anteriorly by the cornea and posteriorly by the lens. The space between the cornea and the anterior surface of the iris is the anterior chamber and the space between the posterior surface of the iris and the lens is the posterior chamber. Aqueous humor is produced in the posterior chamber and drains from the anterior chamber into a meshwork of channels located at the junction of the cornea, sclera, and iris (iris angle, filtration angle).

The lens is composed entirely of epithelial cells whose basement membrane is the thick outermost capsule of the lens. The bulk of the lens is formed by layers of epithelial processes that cannot be shed, but are compacted with age to the center of the lens. The adult lens depends entirely on the aqueous humor for delivery of nutrients and removal of wastes. The vitreous body is the transparent gel that fills the inner portion of the eyeball between the lens and the retina. The vitreous shows considerable shrinkage with most fixatives (over 99% of the vitreous is water) and its normal, in-life distribution is not apparent in microscopic sections.

Third Eyelid. The dog has a third eyelid, or nictitating membrane, located at the medial angle of the palpebral aperture (medial canthus, nasal canthus). A T-shaped hyaline cartilage forms the skeleton of the third eyelid. The cross of the T supports the free margin of the lid. The lower shaft of the T is surrounded by a mixed lacrimal gland (superficial gland of the third eyelid, nictitans gland). According to Getty (1967), the dog does not have a Harder's gland (deep gland of the third eyelid). The inner conjunctival covering (bulbar conjunctiva) contains numerous lymphoid nodules.

Ear

The ear is the organ of hearing and equilibrium. It is composed of three connected divisions, each of which is referred to as an ear: external (outer) ear, middle ear, and internal (inner ear). The

inner ear is the organ for both hearing and equilibrium. The external and middle ears are sound collecting and conducting apparatuses (Getty 1967).

External Ear. This consists of the pinna (auricle) and the external auditory meatus (ear canal). The pinna is composed of a sheet of elastic cartilage (auricular cartilage) covered by skin on both sides. The auricular cartilage is pierced by many foramina that permit the passage of blood vessels. Histological sections that include foramina give the impression that the cartilaginous sheet is not continuous. The function of the pinna is to direct air vibrations into the funnel-like ear canal to the tympanic membrane (ear drum). The tympanic membrane is the partition between the external ear and the middle ear.

Middle Ear. This consists of the tympanic cavity, the auditory tube, and a chain of three small bones (auditory ossicles) that connect the tympanic membrane to the oval window of the inner ear. The tympanic cavity is filled with air and communicates with the nasal pharynx by means of the auditory tube (eustachian tube).

Inner Ear. This is contained within the temporal bone and consists of the membranous labyrinth containing the organs of hearing and equilibrium and the bony labyrinth surrounding the membranous labyrinth. The inner ear is fluid filled. The bony labyrinth, which lodges the membranous labyrinth, contains a fluid, perilymph, and communicates with the cerebrospinal space by way of the vestibular aqueduct. The membranous labyrinth is filled with a fluid called endolymph.

Necropsy and Laboratory Techniques

Eye

Many lesions of the eyelids, conjunctiva, and cornea will be clinically apparent; however, most lesions within the globe will be hidden to the pathologist without the assistance of an ophthalmoscopic examination. Ideally, the pathologist will have the notes, drawings, or photographs of an ophthalmologist to direct him or her to pathology within the eye. Minute lesions can be difficult to find without directions and lesions involving the lens could be obscured or lost because of the difficulty in obtaining complete, artifact-free sections of the lens. Autolytic changes can be detected in the canine retina within 5 min of death (Saunders and Rubin 1975). Unless there are compelling reasons to do otherwise, the eyes should be the first organs removed at necropsy and both eyes should be in fixative well within the first 5 min of the necropsy. To meet the 5-min deadline, all preliminary procedures (weighing, external examination, clipping of hair) should be done prior to exsanguination. Good exsanguination limits the degree of hemorrhage into the orbit during dissection. The enucleation can be done by a single prosector, but the assistance of a second person is required to have both eyes removed, cleaned, and in fixative within the 5-min time frame.

A single incision through the lateral palpebral angle will free the upper and lower eyelids sufficiently to give access to the eye. With an assistant stabilizing the head and stretching the skin to spread the eyelids, the prosector grasps a lateral fold of palpebral conjunctiva with tissue forceps and with curved blunt scissors (one of the authors prefers Metzenbaum scissors) makes an initial incision in the conjunctiva behind the forceps. With gentle traction and well-directed cuts with the scissors, the prosector severs the conjunctiva and extraocular muscles on the lateral, dorsal, and ventral sides of the eye, working toward the cerebral pole where the optic nerve and remaining muscles are cut. The eye is pulled gently forward and remaining extraocular tissue is cut, including the third eyelid. The position of the forceps never need be changed. The globe is placed on a wet sponge to cushion it and the extraocular muscles, fat, and fascia are gently removed close to the sclera, using the convex surface of the scissors. Although the left and right side identity of the eyes can be made using anatomical landmarks, it is easier to maintain the identity of the eyes by placing

the globes in identified containers or to tie a piece of black thread to a bit of loose extraocular tissue on one of the eyes. The eyes are gently lowered by the thread or forceps into the fixative. At least 5 mm of optic nerve should be left attached to the eye. With proper cleaning, the eye will sink in fixative. The thorough cleaning is required to permit rapid penetration of fixative and to prevent retinal detachment because of the pressure exerted by contracting muscles. For the same reason, the globe should not be grasped by the fingers. Usually, eyes are not weighed. Measuring the diameters of unfixed globes is time consuming and increases the likelihood of retinal detachment. The volume of an eye is easily determined by displacement. Choose a graduated cylinder with an inside diameter just great enough to accept an eye. Partially fill the cylinder with fixative, read the volume, lower the eye into the fixative, and read the new volume. The difference in the volume readings is the volume of the eye. Using an appropriate cylinder, the volume of a beagle eye can be measured to 0.2 ml. After measuring, the eye is poured into the primary container of fixative.

There appears to be no ideal fixative for the eye. Ten percent neutral buffered formalin generally gives good results for the cornea and lens but poor results for the retina. Rapidly penetrating fixatives such as Zenker's, Helly's, and Bouin's are generally good for the retina, but require strict attention to a fixation schedule to avoid overfixation. Many additional fixatives have been used. One of the authors has had good results with 2.5% phosphate buffered glutaraldehyde. Dog eyes placed in this fixative can be "cut in" in 1.5 hr to 2.0 hr, allowing a gross examination while the tissues retain much of their original color and the lens is still relatively clear. Fixation is complete in about 72 hr. Davidson's fixative (Humason 1972) is a simple, easy-to-use fixative that gives good results. Unless there is good reason to do otherwise, the canine eye should be trimmed perpendicular to the horizontal meridian (posterior ciliary vessels) to obtain a midsagittal block that includes the tapetal and nontapetal fundus and optic nerve. A complete sampling of the eye would include a transverse section of the optic nerve. Preparing top-quality eye sections requires special skill and (as with neurohistological technique) should be reserved for the experienced histotechnician with the time and interest to devote to the subject.

Ear

The middle and inner ears are not routinely examined in toxicology studies; however, it is not difficult to collect and prepare sections that include the tympanic membrane and middle and inner ears. The external ear is removed close to the tympanic membrane. After removal of the brain and disarticulation of the mandible, two transverse cuts are made through the base of the skull just anterior and posterior to the external acoustic meatus. One of these cuts will usually just enter the tympanic cavity of the middle ear. After ample fixation and decalcification (the temporal bone is very dense), the specimens can be trimmed and processed in a routine manner.

Pathology

Only Glaister (1986) reported ocular disease, but his observations are limited to sores and conjunctivitis without describing these lesions. The conjunctivitis was associated with sawdust bedding and dust in 4% of young beagles. Oghiso et al. (1982) described lymphoid cell infiltration of the third eyelid and gland of the third eyelid. Hottendorf and Hirth (1974) examined both eyes in 85% of their studies but reported no ocular pathology in 630 beagles. Rubin and Saunders (1965) reported Toxocara granuloma in the retina or choroid in three young beagles.

Heywood et al. (1976) followed ocular changes in 86 laboratory beagles from 6 months to 8 years of age, at which time the animals were necropsied. Prominent posterior lens sutures were the only ophthalmological change seen in beagles under 3 years of age. From 3 to 8 years of age, ophthalmological changes included corneal opacities, asteroid bodies, and tapetal pigmentation. For the lens they reported prominent posterior sutures; nuclear opacities; and anterior, posterior, and peripheral capsular opacities. Histological findings at 8 years of age included keratitis, cystoid

degeneration of the retina, thinning and absence of tapetal cells, pigment cells in rod and cone layer, scarring of the retina, and calcified body between the pigment epithelial layer and choroid.

Schiavo and Field (1974) examined 532 beagles of various ages ophthalmoscopically and biomicroscopically and reported the following in dogs between 6 and 12 months of age: superficial keratitis in 35 (6.6%); deep keratitis, posterior polar opacities, posterior cortical opacities in 54 (10.1 %); prominent posterior lens sutures, lenticular sheen, vacuoles in lens cortex, persistent hyaloid vessel remnants, zones of discontinuity (lens), vitreous floaters or filaments, atapetal fundi, tigroid fundi, tapetal aberrations/pigment clumps, peripapillary reflectivity, and increased tapetal reflectivity. Lenticular sheen is a yellowish reflection from the lens media and is generally expected as a senile change. It was observed in 120 (22.6%) of beagles sampled. The hyaloid artery is responsible for development of the vitreous and for nourishment of the fetal lens. Normally, the vessel regresses shortly after birth, but sometimes remains to be seen in the adult. The incidence of persistent hyaloid vessels was 142 (26.7%). Zones of discontinuity reflect stratification of lens fibers and are thought of as a presenile change. They occurred in 32 (6%) of beagles sampled. Vitreous floaters are asteroid bodies (small calcium bodies). Vitreous filaments are fibrous strands from vitreous hemorrhage or remnants of the posterior vascular capsule. The incidence of vitreous floaters and filaments was 53 (10%). Tigroid fundi describe the appearance of choroidal vessels through areas of nonpigmented pigment epithelium. Tapetal aberrations include areas of hyperreflectivity and old hemorrhage or scars. These aberrations occurred in 25 beagles (4.7%). Bellhorn (1974) reports similar ocular findings in 8- to 10-month-old beagles plus prolapse of the third eyelid.

The abundance of ophthalmoscopic observations and the paucity of histological findings emphasize the comparative sensitivities of the two modes of examination. The discrepancy is easily understood, for the ophthalmologist has the entire living cornea, iris, lens, and fundus to examine. On the other hand, the pathologist is limited to one or two thin sections of an organ that is both difficult to fix and difficult to section.

Peripheral retinal cystoid degeneration describes single or multiple microcysts within the retina at or near the ora serrata. This is a common change in older beagles, but it also occurs in young animals. At 8 years, the incidence rate was 85%. The lesion has no apparent functional significance. Focal retinal dysplasia manifested as retinal folds, retinal rosettes, focal absence of retinal cells, and blending of nuclear layers. Protrusion of the third eyelid can be seen in young beagles. Histological findings include inflammation of the bulbar conjunctiva and stroma of the superficial gland. The ducts of the gland might be dilated and contain leukocytes. The conjunctival lymphoid tissue is usually hyperplastic.

There is no mention of ear pathology in the articles reviewed. Cutaneous lesions of the external ear were discussed with the integumentary system.

METABOLISM

Among the nonrodent species, the dog has been the best characterized with regard to xenobiotic metabolism. This is probably because the dog has been extensively used in biomedical research for more than 100 years. Its relatively large size also provides the advantage of allowing repeat serial sampling of large amounts of biological fluids for time-course analyses. Data on the dog are a staple in numerous review articles and book chapters published on species comparisons elsewhere (see chapter 1). The focus here is not only on species differences, but also on other items (e.g., inducibility) that have not necessarily been covered in detail elsewhere. Insofar as the beagle dog is the most common breed and the most common nonrodent model used in toxicity testing, this chapter focuses on data from beagles, and presents data from other breeds where available.

At the onset, it should be noted that dog is generally believed to have a higher intestinal pH than man, leading to some differences in oral absorption of more water-soluble drugs (Dressman 1986; Lui et al. 1986). Although otherwise similar motility patterns and pH profiles prevail in the

two species for the most part, there are some differences that could affect the time profile and extent of drug absorption. These include slower gastric emptying in the fed state, faster small intestine transit, and higher and more variable intestinal pH in dogs compared with humans. An attempt is made to identify drug and dosage-form properties that would lead to differences in drug absorption in the two species (e.g., drug physiochemical properties, dosage-form size, and pH dependency of dosage-form release characteristics).

As reported by Gregus et al. (1983), the dog has a somewhat smaller liver to body weight ratio than the rat (2.3% vs. 4.0%) and somewhat less microsomal protein (20.5 vs. 25.7 mg/g liver). Identified P450 CYP isozymes are listed in table 8.16. Some of the more common parameters of hepatic xenobiotic metabolism in the dog are summarized in table 8.17. Concentrations of cytochrome P-450 range from about 0.30 to 0.80 nmol/mg protein. Using HPLC and other techniques, Amacher and Smith (1987) characterized the cytochrome P-450 isozymic "fingerprint" of naive beagle dogs. Their results suggested that cytochrome P-450 exists as three distinct isozymic groups in female dogs, and two main groups and two to three subgroups in male dogs. Further, they identified sex-related differences in chromatographic behavior between the major isozymic groups. This is an interesting finding because few, if any, sex-related differences in the activity of the microsomal mixed function oxidase (MMFO) have been identified in dogs. If table 8.17 is compared

Table 8.16 Identified P450 CYP Isoforms Active in the Dog

CYP	1A1	(Low Km; Shou et al. 2003)
CYP	1A2	
CYP	2A6	(Chauret et al. 1997)
CYP	1B11	
CYP	2C19	(Chauret et al. 1997)
CYP	2C21	(Low Km; Shou et al. 2003)
CYP	2C41	
CYP	2D6	(Chauret et al. 1997)
CYP	2D15	(Low Km; Shou et al. 2003)
CYP	2E11	
CYP	3A4	(Chauret et al. 1997)
CYP	3A12	(High Km; Shou et al. 2003)
CYP	3A26	

to similar endpoints for man or other model species (such a table being presented in other chapters), it is readily seen that the dog is frequently not a good metabolic model for man and is poorly comparable to the rat and mouse.

McKillop (1985), using SDS-PAGE techniques, identified three major cytochrome P-450 isozymes in uninduced adult male dogs. Phenobarbital increased the levels of two of these major isozymes, but primarily caused an increase in another (fourth) isozyme. β-Naphthaflavone caused increases in three other isozymes that are only present in very small amounts in naive animals. Hence, as a generality, the beagle dog has at least seven isozymic forms of cytochrome P-450, including a cytochrome P-448 with some homology with that of the rat, but that are differentiated on the basis of molecular weight, substrate specificity, and responses to inducers.

In general, the dog has less ability than the rat to form hydroxylated aromatic metabolites via the MMFO. For example, in the metabolism of amphetamine, the dog produces very little 4-hydroxyamphetamine (as reviewed by Williams 1972). Cook et al. (1982) reported that the dog, as opposed to the rat, produces no phenoxyl metabolites of disopyramide. Aniline-hydroxylating activity in dogs tends to be less than that of rats. Gregus et al. (1983) have reported benzo(a)pyrene-hydroxylating activity in the dog is much less than that of the rat. There are exceptions; for example, the dog has a much higher rate (15 times) of metabolism with 2,2',4,4',5,5-hexachlorobiphenyl than the rat (Duignan et al. 1987). Some care must be taken in interpreting data on aniline hydroxylase in the dog, as this species produces both ortho- and para-aminophenol from aniline at a ratio of 1/2 (p/o) (Williams 1972). In the

Table 8.17 CYP Specific Metabolic Activities in Beagle Dogs

Activity	Beagle Dog	References
7-Ethoxyresorufin O-dealkylation	1A1/2	Kastner and Neubert, (1994)
7-Methoxyresorufin O-dealkylation		
Caffeine 3-demethylation		
Benzphetamine N-demethylation		
7-Benzoxyresorufin O-dealkylation	2B11	
7-Pentoxyresorufin O-dealkylation	2B11	Ohmori et al. (1993)
Coumarin 7-hydroxylation		
7-Ethoxy-4-trifluoromethylcoumarin deethylation		
Ethoxycoumarin O-dealkylation		
Tolbutamide methyl-hydroxylation		
Chlorzoxazone 6-hydroxylation		
Mephenytoin N-demethylation	R enantiomers twice as fast as S	Yasumori et al. (1993)
4-Nitrophenol hydroxylation		
N-Nitrosodimethylamine N-demethylation		
Androstenedione 15α-hydroxylation		
Androstenedione 16α/β-hydroxylation	2B	Igarashi et al. (1997)
Dextromethorphan O-demethylation	2D15	
Dextromethorphan N-demthylation		
Testosterone → Androstenedione*		
Testosterone 2α-hydroxylation		
Testosterone 2β-hydroxylation		
Testosterone 6β-hydroxylation	3A12	Igarashi et al. (1997)
Testosterone 7α-hydroxylation		
Testosterone 15α-hydroxylation		
Testosterone 15β-hydroxylation		
Testosterone 16α-hydroxylation	2B11, 2C21	Ohmori et al. (1993)
Testosterone 16β-hydroxylation	3A12, 2B11[46]	Ohmori et al. (1993)
Lauric acid 11-hydroxylation		
Lauric acid 12-hydroxylation		

rat, this ratio is 6:1. As the common colormetric method (formation of a quinolinidine complex with phenol) of determining aniline hydroxylation is specific for p-aminophenol, the activities reported for aniline hydroxylase in the dog are probably low. With many other typical substrates (dealkylation rather than aromatic hydroxylation), MMFO activity in the dog is often comparable to that of the rat. Microsomal aminopyrine metabolism, for example, is about the same in dogs as in rats (Lan et al. 1983). Gregus et al. (1983) reported that the rat had about twice the activity with benzamphetamine, but four times the activity than with ethylmorphine than dogs. In contrast, dogs have a higher baseline level than rats for 7-ethoxycoumarin (primarily a substrate for P-450-dependent MMFO) and 7-ethoxyresorufin (primarily a substrate for P-448 dependent MMFO) deethylating activities (Gregus et al. 1983; McKillop 1985), but greater degrees of induction (with either phenobarbital or β-naphthaflavone) occur in the rat than in the dog (McKillop 1985). Thus, in comparing the rat and the dog (probably the two most common species in toxicity testing), one should not assume that the rat has the more rapid rates of MMFO activity but does have a more extensive range of activities.

Nelson et al. (1996) and Zuber et al. (2002) report that the dog CYP1A, 2E1, 3A12, and 3A26 actvities are not well correlated with those in humans. CYP2D15 is well correlated with human CYP2D6 activity. The dog is also the only mammalian species able to metabolize polycylic aromatic hydrocarbons through its CYP2BN enzyme.

There is considerable evidence that the MMFO of rats has stereospecificity. For example, Heimark and Trager (1985) compared the microsomal metabolism of R and S warfarin. The overall rate of oxidation (total product) was much greater with the R entaniomer. There is some evidence for MMFO stereospecificity in the dog. Cook et al. (1982) examined the disposition of (R) and (S) disopyramide *in vivo* in dogs. The (R) entaniomer had significantly longer half-life than the

(S). In addition, a much higher percentage of dose of S-disopyramide was excreted in the urine as the major metabolite. In general, one should expect stereoselective metabolism of racemic mixtures in the dog.

Dog 1A and 2E1 are somewhat different than humans and are not typically good models for humans. Only mammalian species are able to metabolize polycyclic aromatic hydrocarbons through CYP2B11 (Zuber et al. 2002).

Celecoxib, a cyclooxygenase–2 inhibitor had its pharmacokinetics in dogs evaluated as part of its nonclinical development program prior to FDA approval. Celecoxib is extensively metabolized by dogs to a hydroxymethyl metabolite, with subsequent oxidation to the carboxylic acid analog. Of the major CYP enzymes, CYP 2D15 was extensively involved in polymorphism of metabolism in dogs, probably due primarily to other CYP subfamilies than 2D (Paulson et al. 1999).

Urea synthesis of both fresh and thinned liver slices is higher than those of rat, cynomolgus monkeys, rhesus monkeys, hamsters, minipigs and rabbits. However, the dog has lower testosterone metabolism than any other species (Kanter et al. 1997).

MMFO is inducible in the dog. McKillop (1985) examined the inducing effect of phenobarbital and β-naphthaflavone in beagle dogs. Phenobarbital (in saline) was administered intraperitoneally for 7 days: 20 mg/kg for 2 days, 10 mg/kg for 2 days, and 20 mg/kg for the final 3 days. β-Naphthaflavone in arachis oil was given intraperitoneally for 6 or 7 days, 10 mg/kg. The phenobarbital treatment increased cytochrome P-450 by approximately 250%, whereas the β-naphthaflavone caused approximately a 100% increase, with a shift from cytochrome P-450 to cytochrome P-448. In contrast, β-naphthaflavone caused a greater increase (175%) in NADPH: cytochrome C reductase than phenobarbital (41%). This latter change contrasts with that of the rat, in which typical P-448 inducers do not cause increases in the reductase activity. The increases were accompanied by the expected increases in enzyme activity: aldrin epoxidase was increased by phenobarbital, 7-ethoxyresorufin deethylase was increased by 6-naphthaflavone, and 7-ethoxycoumarin was increased by both. Duignan et al. (1987) used a stepwise regimen (to avoid excessive sedation) to induce beagle dogs with phenobarbital (Na^+ salt): 10 mg/kg (po) for 2 days, followed by 30 mg/kg for 4 days, then 30 mg/kg for a final 8 days. This regimen more than doubled the microsomal concentration of cytochrome P-450 and significantly increased the activities toward 7-ethoxycoumarin, warfarin, and androstenedione. There were changes in region and site specificity that indicate that induction in the dog, as in the rat, is accompanied by shifts in isozymic character of cytochrome P-450.

Aldridge and Niems (1979) examined the effects of phenobarbital and β–naphthaflavone on the metabolism of caffeine *in vivo* in the dog. Both phenobarbital (10 mg/kg/day po for 7 days) and β-naphthaflavone (20 mg/kg/day intraperitoneally for 3 days) decreased the half-life of caffeine, but only β-naphthaflavone caused a qualitative shift in the spectrum of urinary metabolites.

Gascon-Barre et al. (1986) studied the effects of phenobarbital induction of the MMFO on vitamin D metabolism in mongrel dogs. Dogs were given approximately 80 mg/kg/day for 30 days and induction was monitored by following changes *in vivo* [^{14}C]aminopyrine metabolism $^{14}C\text{-}CO_2$ production). The paper does not mention complications due to the sedative effects of phenobarbital. As might have been expected in an outbred population, some dogs were more inducible than others. In fact, in two dogs, no induction occurred at all. Not surprisingly, induction resulted in increased hepatic catabolism of vitamin D_3. The important points to be stressed here are that variable responses of dogs to inducing agents can be a potential problem and that *in vivo* clearance of aminopyrine could provide a noninvasive probe for screening of "good" versus "poor" responders to inducing agents of the phenobarbital class.

The inducing effects of drugs and chemicals other than phenobarbital and β-naphthaflavone (typical experimental tools) have also been studied in the dog. Lan et al. (1983) compared and contrasted the inducing effect of hexahydroindazole (10–250 mg/kg/ day for a month) in three species. Increases in relative liver weights occurred in all three species, but increases in microsomal protein only in rats and monkeys (and not the dog). Increases in cytochrome P-450 and aminopyrine

metabolism occurred in all three species, but the largest increases (compared to concurrent controls) were observed in dogs.

Abramson et al. (1986) and Abramson and Lutz (1986a, 1986b) have extensively studied the relationship between enzyme induction, *in vivo* antipyrine metabolism and increases in alpha-1 acid glycoprotein. Although the relationship between the latter two parameters is not a simple one, it can be used to monitor the extent of induction by phenobarbital-type agents in the dog. Using these techniques, they demonstrated that phenytoin (Abramson and Lutz 1986a) and rifampicin (Abramson and Lutz 1986b) are effective inducing agents in the dog, whereas medroxyprogesterone is not (Abramson et al. 1986). The latter finding is of interest because medroxyprogesterone has been reported to be an inducing agent in rats. This underscores again the point that there are species-related differences in responses to inducing agents.

Epoxide hydrolase is an important enzyme in the metabolism of reactive arene oxides, but until 1980 little work had been done to characterize this enzyme in the dog. Pacifici et al. (1981) reported the activity of hepatic microsomal epoxide hydrolase (with styrene oxide) in the dog to be 9.7 ± 2.0 nmol/min/mg protein, which was intermediate between that of the mouse (1.9 nmol/min/mg) and the baboon (31.3 nmol/min/mg). Gregus et al. (1983) confirmed that the dog has relatively high epoxide hydrolase activity (approx 15 nmol/min/mg protein) compared to many other commonly used laboratory species, including the rat. Little other work has been completed to distinguish, either on structural or substrate specificity basis, epoxide hydrolase of the dog from that of other species. Given its high activity, however, it can be expected to play an important role in xenobiotic metabolism in the dog.

As in other species, conjugative metabolism (other than mercapturic acid formation) has been studied in the dog longer than oxidative metabolism. As reviewed by Hirom et al. (1977), the majority of the conjugative reactions were described in Germany during the latter part of the 19th century. The dog, like most mammals, excretes phenols as sulfates and glucuronides. In the dog, the ratio of sulfate to glucuronide (at an aromatic hydroxyl group) varies with substrate. For example, with phenolphthalein, the dog will excrete 18% as the glucuronide and 82% as the sulfate (as reviewed by Hirom et al. 1977), whereas with acetaminophen, 75% will be excreted as the glucuronide and 10% to 20% as the sulfate (Hjelle and Grauer 1986). With aryl acetates, the dog has a high tendency to form conjugates with amino acids. For example, with benzoic acid, the dog will excrete 82% as the glycine conjugate (hippuric acid) and only 18% as the glucuronide.

Relatively little work has focused on the biochemical and molecular characterization of the enzymes involved in conjugation in the dog. Gregus et al. (1983) examined the *in vitro* activity of PAPS-sulfotransferase of the dog against four substrates. Activity was noted with all four, the highest with 2-naphthol and the lowest with taurolithocholate. When compared to other species, the sulfotransferase activity of the dog tended to be lower than most.

Gregus et al. (1983) examined the activity of UDP-glucuronosyl transferase against a wide variety of substrates. The dog had somewhat higher activities than the rat with 1-naphthol, p-nitrophenol, estrone, morphine, and digitoxigenin-monodigitoxoside, whereas the opposite was true with diethylstilbestrol and bilirubin. Hence, in terms of relative activity there is little to distinguish the UDP-glucuronosyl transferases of the dog from those of other species. There is evidence, however, of species differences in stereoselectivity. Wilson and Thompson (1984) examined the stereoselectivity of dog hepatic microsomal UDP-glucuronosyl transferase by examining differences in activity toward (R)- and (S)-propranolol. When racemic mixtures were studied, the (S) entaniomer was the preferred substrate, with 3 to 4 times more (S)-propanolol glucuronide produced than that of the (R) entaniomer. Additional experiments with separate entaniomers demonstrated that the K_m and V_{max} of the dog microsomal UDP-glucuronosyl transferase are much higher with the (S) than the (R) entaniomer. In humans, plasma levels of (R)-propranolol have been shown to be higher than those of (S)-propranolol (Silber et al. 1982), whereas the opposite has been shown in dogs (Walle and Walle 1979), and this difference might be due to the differences in stereospecificity of glucuronidation in the dog versus human (Von Bahr et al. 1982). Stereospecificity of UDP-glucuronosyl

transferase with substrates other than propranolol has been reported for other species (Sisenwine et al. 1982). Thus, stereospecificity of UDP-glucuronosyl transferase is not unique to the dog or propranolol.

Schmoldt and colleagues (1987) examined the *in vitro* glucuronidation rates of dog liver microsomes toward various cardiac glycosides. In contrast to most other species examined, dog UDP-glucuronosyl transferase is capable of conjugating digitoxin; otherwise glucuronidation rates between rats and dogs were similar for all other cardiac glycosides examined. In fact, neither species has detectable activity toward digoxin. As both dogs and humans rapidly eliminate (*in vivo*) administered doses of digoxin, these results suggest that there might be greater similarity between canine and human UDPglucuronosyl transferase than between the human and rat enzyme with regard to substrate specificity. UDP-glucuronosyl transferase exists as a family of different isozymes in rats (Knapp et al. 1988). By examining a variety of model substrates and various inhibitors, Schmoldt et al. (1987) concluded that although there might be more than one canine isozyme, a single isozyme in the dog was responsible for glucuronidation of all cardiac glycosodise.

The dog has long been recognized to have less active N-acetyl transferase activity than other species (Williams 1972). This was more recently confirmed by Gregus et al. (1983), who demonstrated that the dog had almost imperceptible activity with five different substrates. This is a qualitative species difference that could result in toxicologically important species-related differences in metabolism. For example, arylamines require acetylation to be activated to mutagens and carcinogens. In an interesting paper, Neis et al. (1985) compared the cytochrome P-450 content, N-acetyltransferase activity, and mutagenic activation activity of canine and human isolated hepatocytes. The mutagenic activation of five different arylamines was examined, and it was found that human hepatocytes had much greater mutagenic activation activity; this difference was attributed to the N-acetyltransferase present in human cells. Paroxon, an inhibitor of acetyl transferase, decreased mutagenic activation by human hepatocytes. They noted that dog hepatocytes were approximately the same size as human hepatocytes, but those of the dog had higher concentrations of cytochrome P-450, 210 ± 10 versus 94 ± 2 pmol/10E6 cells. N-acetylation is thus involved in the activation of arylamines in humans, and therefore the dog might not be the appropriate model in which to study the toxicity (relative to humans) of arylamines.

Arylamines are not only substrates of the MMFO, but they can also be substrates for the flavin-based mixed function oxidase (FMFO). The FMFO of the dog has not been isolated and well characterized like that of the pig or the rat, but differential arylamine metabolism and the use of specific inhibitors has provided a probe for studying the involvement of this enzyme in dog xenobiotic metabolism. 2,[(2,4-Dichloro-6phenyl)phenoxyl-ethalamine (DPEA) is a specific cytochrome P-450 inhibitor and methimazole is a competitive inhibitor of FMFO. Using these, Hammons et al. (1985) have reported that the FMFO metabolized 2-acetylaminofluorene in pigs and humans, but not in dogs and rats. This group also studied the *in vitro* metabolism of 1-naphthylamine (1-NA) and 2-naphthylamine (2-NA) in rats, dogs, and humans. N-hydroxylation is a major pathway in all three species for 2-NA, but not for 1-NA, and this reaction is exclusively mediated by the MMFO. Ring hydroxylation, however, also occurred inall three species, and might be partially mediated by the FMFO as well as the MMFO because it was not completely inhibited by DPEA. For both chemicals, the dog has a higher rate of metabolism than the rat. Interestingly, there were greater individual human-to-human differences in the microsomal metabolism of these chemicals than the differences between rats and dogs.

Like all known and studied mammals, dogs have hepatic cytosolic glutathione S-transferase (GSH-T). Grover and Sims (1964) described this enzyme in dogs as early as 1964, and were the first to note the high activity the dog GSH-T has with 1,2-dichloro-4-nitrobenzene. Gregus et al. (1983) examined glutathione S-transferase activity against six different model 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene; otherwise rats had much higher activity than dogs with all other substrates examined.

Weiner (1986) confirmed in mongrel dogs the observation as to GSH-T activity toward 1,2-dichloro-4-nitrobenzene, and further compared the activities of rat and dog cytosolic preparations against seven other substrates and found that activity in the dog ranged from 2.5% (4-nitropyridine N-oxide) to 95% (ethacrynic acid) that of the rat. Overall, the highest activity in dogs (and rats, for that matter) was obtained with 1-chloro-2,4-dinitrobenzene. Weiner further characterized GSH-T in various cytosolic protein fractions. He found that dog GSH-T exists as four different isozymes composed of three different classes of subunits. In general, this type of isozymic pattern for this enzyme has been seen in other species. The dog enzymes are also not very different with regard to sensitivity to inhibitory ligands, such as bomcresol green or 8-anilino-1-naphthalene (Weiner 1986). Therefore, except for the expected molecular and quantitative activity differences, GSH-T in the dog is not startlingly different from that of other species. Weiner did observe that dog GSH-T has some activity in the denitrification of organic nitrates, such as isosorbide-2,5-dinitrate, and that this activity was responsible for the rapid *in vivo* conversion of 2,3-di-O-nitro-adenosine-5'-(N-ethyl-carboxamide) to the mono-nitro chemical in the dog (Wiener et al. 1983). Whether this is a pathway unique to the dog remains to be established.

In general, little in-depth work on extrahepatic metabolism in the dog has been reported, the exception being work done at the Lovelace Inhalation Toxicology Research Institute on the respiratory tract. Hadley and Dahl (1983) studied the cytochrome P-450-dependent mono-oxygenase activity in the nasal membrane of several species. The dog tended to have the lowest amount of cytochrome P-450 and the lowest MMFO activities.

Bond et al. (1988) characterized the cellular and regional distribution of xenobiotic metabolizing enzymes in the respiratory airways of beagles and found detectable cytochrome P-450-dependent activity throughout the airway, from the alar fold to the peripheral lung, but there were regional and substrate differences in specific activities. For example, the ethmoid rubinate had high activities for both benzo(a)pyrene and ethoxycoumarin metabolism, whereas higher nasal regions (alar fold, nasoturbinate, and maxilloturbinate) had much higher activity with ethoxycoumarin. In general, epoxide hydrolase and glutathione S-transferase activities were present along the entire airway, but tended to be higher in the nasal region and pulmonary airways compared to the major airways. UDP-glucuronosyl transferase tended to be evenly distributed. These detoxification enzymes were present in much greater activity than the MMFO-cytochrome P-450-dependent activation system. This work provides an excellent basis for using the dog in the study of site-specific chemical carcinogenesis of the respiratory tract.

For example, Petridou-Fischer and colleagues (1987) studied the *in vivo* disposition of nasally instilled dihydrosafrole in the dog, and recovered metabolites from the nasopharyngeal mucus, thus confirming the potential importance of the MMFO activities in the upper respiratory tract in the metabolic activation of potential carcinogens.

Other aspects of extrahepatic metabolism in the dog have been partially investigated as part of studies of various species-related differences in target organ toxicity. Garst et al. (1985) examined the pulmonary metabolism of perilla ketone by a variety of species, and concluded that the insensitivity of the dog lung to perilla ketone toxicity resulted from the relatively low amount of cytochrome P-450 with low MMFO activity toward perilla ketone.

Poupko et al. (1983) reported that the dog bladder microsomes had very low activity with both 1- and 2-naphthylamine.

Menard and colleagues (1979) examined both testicular and adrenal cytochrome P-450 in the dog, guinea pig, and rat. The dog and rat had comparable concentrations of adrenal cytochrome P-450 (1.1–1.2 nmol/mg microsome protein), whereas the guinea pig had roughly twice this concentration. In contrast, the dog had the highest concentration of testicular cytochrome P-450 (0.170 nmol/mg microsomal protein) and the rat the lowest (0.067 nmol/mg microsomal protein). Administration of spironolactone, a 7-{alpha}-thiosteroid, led to the destruction (40%–60%) of the cytochrome P-450s of both tissues in the dog. In contrast, spironolactone had no effect on adrenal cytochrome P-450 in the rat, but caused large decreases (88%) in testicular cytochrome P-450.

Pacifici et al. (1981) reported on species and tissue difference in epoxide hydrolase and glutathione S-transferase and found the epoxide hydrolase activity of the dog kidney to be comparable to that of the guinea pig and rabbit, but less than that of the hamster. Glutathione S-transferase activity of the kidney of the dog (with styrene oxide, the same substrate used to determine epoxide hydrolase activity) tended to be much lower than that of the guinea pig and rat, but higher than those of the human and monkey. There are species differences in extrahepatic cytochrome P-450 of which the investigator should be aware, but it does not appear that the dog offers advantages over any other species in the study of specific routes of extrahepatic metabolism.

Very little work has been reported on developmental or age-related changes in drug metabolism in the dog. Tavoloni (1985) examined the age-related development of the cytochrome P-450 MMFO and UDP-glucuronosyl transferase in the beagle dog. Activities were lowest at birth. They increased thereafter, tending to plateau after 6 weeks of age. Increases in the MMFO, but not UDP-glucuronosyl transferase, were induced by phenobarbital.

Baarnhielm et al. (1986) compared and contrasted the *in vivo* and *in vitro* metabolism of felodipine in the rat, dog, and human. Felodipine is a calcium channel blocker that is very lipophilic, well absorbed from the GI tract, highly protein bound, and extensively metabolized. Comparative *in vitro* rates of microsomal metabolism of felodipine were rat > dog > human. There was an excellent correlation between the V{max}S and the concentrations of cytochrome P-450. The same rank order was observed for *in vivo* plasma clearance. It would appear that with chemicals that are well absorbed and rapidly metabolized, interspecies extrapolations are possible and fairly predictive. In their studies on the disposition of etodolac, Cayen and colleagues (1981) observed that felodipine also was well absorbed, partially metabolized (different metabolites in dog and rat), and undergoes extensive intrahepatic circulation. There were differences in the serum half-life (human > dog > rat) and clearance (dog > human > rat), but there was greater similarity between the dog and human. In general, the dog appears to be a better pharmacokinetic model (more predictive of behavior in humans) for humans than the rat or other rodents for chemicals that are well absorbed and have a high hepatic clearance.

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CHAPTER 9

Primates

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TOXICOLOGY

The phylogenetic and physiological similarity between humans and nonhuman primates has resulted in an increased demand for certain species in safety and efficacy assessments of new drugs and biologics. Only a few of almost 200 primate species are utilized in toxicology studies. The most commonly used species are Old World monkeys (cynomolgus monkey, rhesus monkey, and baboon), New World monkeys (squirrel monkey and marmosets), and the great apes (chimpanzees).

Because of the genotypic and phenotypic resemblance to humans, nonhuman primates have been used in the study of induced or naturally occurring human diseases such as acquired immunodeficiency syndrome (AIDS), hepatitis, diabetes mellitus, and atherosclerosis. Our understanding of the human brain, vision, aging, reproductive function, and behavior has been enhanced by studies in primates. Safety (and sometimes efficacy) evaluations of drugs, vaccines, and biotechnology products are conducted in nonhuman primates prior to approval for general use by the public. A failure to investigate the potential teratogenic effects of thalidomide in a primate model prior to exposing pregnant women resulted in tragic consequences in the 1950s and early 1960s (Somers 1963). Unfortunately, the teratogenicity of thalidomide could not be demonstrated in rodents prior to human exposure, but was subsequently shown to cause fetal abnormalities in primates (Hendrickx 1973; Hendrickx et al. 1966). The development of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) has been a significant advancement for the treatment of hypercholesterolemia (and related hyperlipidemias). The withdrawal of a popular drug in this class, cerivastatin, highlighted the importance of preclinical toxicology in rodent and nonrodent species, as the development of life-threatening rhabdomyolysis in humans ultimately was associated with differences in animal model (and human) pharmacokinetics among drugs in the class, as well as multiple drug therapy and renal insufficiency in the target population (Evans and Rees 2002).

In spite of the obvious predictive value of primates for human responses, they must be utilized conservatively, and models in lower species should be used when appropriate. Nonhuman primates used in biomedical research are purpose-bred or ethically obtained from large feral populations. The animals are imported or domestically bred. Special handling procedures resulting from the developing body of knowledge about the zoonotic potential of known (and emerging) infectious diseases to humans create unique challenges and increase the cost associated with the use of nonhuman primates. Nevertheless, the humane use of nonhuman primates as a physiological, pharmacological, and toxicological research model is critical for safety assessment of new drugs and biotechnology products, increasing the need for prudent use of primate inventories.

The intent of this section is to define the utilization of nonhuman primates in toxicology studies and to describe the basic husbandry and technical procedures used in a primate facility. It is not intended to be an exhaustive review of the voluminous primatology literature. (See King et al. 1988, for an excellent summary of the role of nonhuman primates in research.)

Husbandry

Husbandry includes all aspects of housing and adequate care. Primates encompass a variety of species ranging from lower forms such as the tree shrews to the great apes. Much in the literature has generalized the nonhuman primate as one animal, when in reality the primate can be as small as several grams (e.g., shrews) to as large as more than 100 kg (e.g., great apes). The primary genus utilized in toxicological studies has been the Asian macaque, with lesser emphasis on the New World monkey and the chimpanzee. The primary focus of this section is on the macaque with specific reference when necessary to New World primates and apes.

Federal regulations enacted through the Animal Welfare Act, Parts 1, 2, and 3, further define the need to provide optimal caging, adequate veterinary care, protocol development through scientific and Animal Care Committee review, and programs geared toward a variety of diverse procedures in assuring the physical and psychological well-being of the animals through environmental enrichment programs. In addition to welfare regulations concerning housing and care, the nonhuman primate requires special enrichment programs to assure social and mental stimulation. Social housing of nonhuman primates, ranging from periodic commingling to group housing, has become standard in toxicology research. Special caging and compatibility assessments are needed for successful social interactions with the animals. By matching the commingled animals by dose group and scheduling the interactions to avoid interference with study procedures, successful social housing can be accomplished with almost any study. Nonhuman primates are highly intelligent,

and successful environmental enrichment requires a variety of toys, puzzles, and other devices rotated frequently enough to maintain the interest of the animals. Decreased stress and more normal physiologic responses are the rewards of a carefully designed socialization and environmental enrichment program.

Institutional Policy and Regulatory Issues

Institutional animal facilities and programs should be operated in accordance with the Animal Welfare Act (9 CFR, Parts 1, 2, and 3) and as described in the *Guide for the Care and Use of Laboratory Animals* (National Research Council 1996) and other applicable federal, state, and local laws, regulations, and policies. Nothing in the regulations intends to restrict the investigator's freedom to conduct animal experiments in accordance with scientific or humane principles. Any program of husbandry must be developed considering the proper care and humane treatment of the animals used in research or testing. Aspects of conservation, alternatives to animal use, elimination of experimental duplication, and strong scientific principles must be paramount in experimental design.

The U.S. Department of Agriculture (USDA) Animal Welfare Regulations (Federal Register 1989) and the Public Health Service through the Office for Protection from Research Risks of the National Institutes of Health (NIH/OPRR) mandate the appointing of an Institutional Animal Care and Use Committee (IACUC) to review the humane principles of research protocols and the total animal care and use program of each research institution. Included in this review are semiannual facility and program reviews; protocol review concerning euthanasia, appropriate care, and related issues; and provision of an annual written report to the responsible administrative official on the status of the institutional animal care and use program. Special consideration of restraint needs and surgical procedures come under the purview of the IACUC and must be scientifically justified.

Facilities

The Physical Plant

A key to a successful toxicological research program is the design of the animal facilities. A good design assures efficiency in animal care and personnel movements and provides managers with the facilities necessary for an economic and sound animal care program. A basic design premise is to accommodate projects involving both small and large numbers of primates.

Briefly, the following functional areas are essential to assure that diverse projects, different primate species, dosing and collection requirements, and husbandry and sanitation needs can be met (National Research Council 1996):

1. Separation of primate housing from administrative or human occupancy areas. Areas with animal procedure rooms (as distinguished from laboratories) can be adjacent to basic animal housing areas.
2. Special areas such as quarantine or receiving require isolation from primary study rooms or laboratories to assure project contiguity and disease-free studies.
3. Separate areas for surgery, necropsy, postsurgical care, clinical pathology, radiography, diet preparation, or animal treatment.
4. Office areas for administration and facility support areas, separate from animal areas.
5. Support areas for personnel, such as a break room, lockers, showers, and toilets.
6. Feed and supply storage and receiving areas.
7. Caging storage, washing facilities, and incinerator or waste disposal.
8. Central supply and receiving.

The size and complexity of these areas depends on the size of the facility. A facility less than 1,000 square feet has much different requirements from a facility housing numerous primates with

areas of thousands of square feet. Efficiency and economy in utilization of research workers' time must be carefully balanced with the need to separate animal and human facilities. Careful consideration of personnel and animal traffic patterns must be utilized in the design of animal facilities. Security has become a key issue in animal facility design. Barriers, entry locks, separate corridors, and separate floors all can enhance security.

Building Materials

Economy, maintenance, and sanitation must be considered in the selection of materials for the research facility. Key features of an animal room are durability, aesthetics, and ease of sanitation requirements while providing a humane and comfortable housing area free of pathogens and vermin hiding places.

Construction Criteria. The design of primate housing areas is unique. Basic aspects to consider are the following:

1. Corridor widths of adequate size to permit easy movement of caging to wash area and bumper guards to prevent wall or corner damage.
2. Animal room doors with viewing windows, swinging toward the corridor only when an anteroom is present. Preferable construction is solid or metal doors to eliminate vermin hiding places. The doors accessing animal areas should have self-closures.
3. Floors of durable, sanitary, nonslip construction, such as epoxy or sealed concrete.
4. Walls of sealed impervious paints or coatings using coved and sealed junctions at floor and ceiling.
5. Ceilings of nonpermeable construction or sealed with washable coatings, and light fixtures of waterproof sealed construction.

Ventilation, Temperature, and Humidity Control

Environmental control is an essential component of facility design in a toxicology laboratory. Nonhuman primates have specific requirements for ventilation, temperature, and humidity. Therefore, environmental consideration must always be given to the specific species being housed. For example, New World primates are specifically affected by low humidity (less than 50%), which can contribute to upper respiratory afflictions (National Research Council 1996). Ventilation at 10 to 15 air changes per hour is essential to reduce environmental aerosol contamination and provide odor and ammonia control. Recirculation of air is not advised unless it has been treated to remove particulate or toxic gaseous contaminants (Besch 1980).

Temperature control systems are required to assure appropriate ranges of temperature from 64°F to 84°F (18°C–29°C; National Research Council 1996). The two most important considerations in the physical environment of primates are humidity and temperature, as they closely correlate to metabolism and behavior. Humidity is recommended at between 30% and 70% for Old World species. The toxicological laboratory must be more sensitive to temperature or humidity variations because such finite measurements as cellular parameters and hormonal indexes can be affected by environmental variances (Gortan and Besch 1974).

Power and Lighting

Steady and uninterrupted power with backup emergency generators is essential to assure critical environmental controls are maintained. Lighting of sufficient intensity is necessary to provide good husbandry and sanitation practices, observation of animals, security, and safe working conditions. A biphasic or a variable intensity lighting system might be necessary in certain facilities to provide

adequate observations, yet provide soft diffused lighting for the majority of the day. Illumination of 75 to 125 foot-candles (Bellhorn 1980) has been recommended for observation periods. Time-controlled systems are essential in a research animal facility to assure regular circadian rhythms.

Lights should be sealed and preferably fluorescent for energy efficiency. Some facilities have gone to the daylight ultraviolet bulb to simulate natural lighting; either is acceptable and appropriate for the subject species.

Drainage

Adequate waste control is essential to assure that contamination, odor, and waste stoppages of drains do not occur. Research colonies are at increased risk of disease transmission from sewage overflow. All waste fixtures should be of adequate size to permit full flow of waste biscuits, feces, hair, and similar materials. Drain sizes from 4 to 6 in. are recommended (National Research Council 1996) with appropriate floor sloping to facilitate the removal of water and waste materials. Cages should be provided with flush pans and troughs to direct and keep waste off of the common pathways. Facilities using dry paper or bedding systems should have adequate disposal systems for these materials. Lockable drain covers or appropriate-sized grids might be necessary to prevent improper materials from being deposited into (or obstructing) the drain system.

Storage Areas

A variety of storage areas are necessary to permit effective husbandry procedures. These include clean food and bedding storage; temperature-controlled food storage to prevent vitamin and nutrient deterioration; refuse storage areas for dry bedding or paper; and equipment storage for clean or dirty caging, disinfectants, and personnel items (masks, gloves, etc.). Consideration should be given to the myriad of equipment (precision scales, ECG machine, blood pressure monitors, infusion pumps) required to support the research effort. Most of this equipment must be stored in a dedicated area that remains clean and dry.

External Environmental Influences

The animal facility should be free of unusual external influences, as adrenal and immune cellular function can be affected by sudden or unusual noise. Cage washing areas should be separate and apart from animal rooms. Soundproofing materials should also be utilized to prevent unwelcome noise in the animal facility (Fletcher 1976; Peterson 1980). Large primates can add significant noise pollution by shaking their cages. These animals should be housed an appropriate distance from more sensitive studies and animal species.

Special consideration is required for primate behavioral or hormonal evaluations. External influences, such as turning on a light or sudden personnel intrusion during nighttime periods, can adversely impact these studies. Fire alarm tests should be done with muffled alarms to prevent severe animal distress.

Sanitation Facilities

Critical to the husbandry of any facility are areas for washing and sterilizing animal cages, racks, water bottles, and similar accessories. The USDA/Animal Welfare Regulations mandate the sanitation and washing of cages at a minimum of every 2 weeks. Temperatures not lower than 180°F are recommended with use of appropriate disinfectants (National Research Council 1996). Key factors to consider in sanitation facilities are the following:

1. Location related to traffic, animal rooms, elevators, and waste disposal.
2. Soundproofing.
3. Utilities such as hot and cold water, steam, drainage, and power.
4. Proximity to storage and appropriate storage area to meet clean and dirty cage demands.
5. Ventilation and employee safety.
6. Access and corridor width.

The size of the facility dictates whether manual washing using brushes and portable units is satisfactory or whether large automated washers are necessary.

Animal Rooms

Animal rooms for toxicological experiments should be variably sized to house either large or small numbers of animals and to provide project segregation during (at a minimum) the dosing phase. Usually this is best accomplished by having a variety of sizes of animal rooms. Laminar flow and bubble-isolette room systems have been designed to permit multiple studies in fairly large rooms.

Generally, animal rooms should have an anteroom provided with a sink and area for outer garment storage (e.g., disposable coveralls, masks, gloves, and dedicated footwear). The animal room proper should be constructed with all of the parameters noted earlier to permit an effective sanitary and humane environment (see figure 9.1). Recent concerns about psychological enrichment might dictate play areas, group housing areas, or special cage constraints to assure animal well-being and to provide the opportunity for exercise. Social needs of the nonhuman primate influence animal room design, research, and husbandry management techniques. All programs using primates should have primate environmental enrichment and some level of socialization incorporated into the facility design.

Support Areas

Personnel support areas, including appropriate break areas, locker rooms, and shower areas, are essential to any husbandry program. These areas should be carefully designed and managed for traffic flow to prevent risks to animals and personnel from contamination and disease.

Special Areas

Most facilities will have special needs for aseptic surgery, involving pre- and postoperative care, surgeon scrub areas, and operating rooms; pathology and necropsy rooms; clinical pathology; radiology support; treatment and procedure areas; and so on. The size and complexity of the facility

Figure 9.1 Nonhuman primate animal room. Floors and walls are made of nonporous materials that are readily sanitized. Watering systems are typically automatic.

will dictate the size of each of these areas, but all are necessary parts in the support of the overall facility, husbandry, and management procedures.

Biohazard Areas

The study of drugs for AIDS, hepatitis, and similar diseases dictates the need for special facilities to work with hazardous agents whether chemical, biological, or physical. Each project will dictate the need for the level of containment. The Centers for Disease Control (CDC) has listed four levels for dealing with potential biological hazards (from BSL-1 to BSL-4), each with specific criteria. Special hoods, filters, room negative pressures, procedures, and personnel practices are involved. The reader is referred to the Center for Disease Control (1999) publication *Biosafety in Microbiological and Biomedical Laboratories* for reference to this vital area.

Caging and Equipment

The well-being and health of the nonhuman primates is critical to the success of a research project. The caging or housing system must be designed to assure that the research objectives can be met through minimizing experimental variables and assuring the "normality" of the animal via the maintenance of health and well-being. The Institute for Laboratory Animal Research (ILAR) Guide (National Research Council 1996) provides the following factors to consider in any housing system:

1. Provide adequate space to assure freedom of movement and normal postural adjustments with a resting place.
2. Comfortable environment.
3. Escape-proof caging.
4. Easy access to food and water.
5. Adequate ventilation.
6. Meets the biological needs of the animal.
7. Keeps animals dry and clean.
8. Reduces unnecessary physical restraint.
9. Protects animals from known hazards.
10. Provides options for environmental enrichment (grooming bars, commingling capability).

Individual Housing

Cages for nonhuman primates must meet the space recommendations noted in the *Guide for the Care and Use of Laboratory Animals* (National Research Council 1996). Table 9.1 lists the current space requirements provided in the *Guide*.

Institutions are encouraged to provide alternatives to individual caging. Infants and juveniles can be housed in group cages, for example. If adults are to be housed in groups, it is essential that only compatible animals be kept together. Newly grouped animals must be closely monitored to detect injuries due to fighting. Space in group cages should be enriched with structures such as resting perches and shelters. The minimum height of pens and runs used to house nonhuman primates should be 6 ft (1.8 m). For chimpanzees and brachiating species (orangutans, gibbons, spider monkeys, and woolly monkeys), the minimum cage height should be such that the animals can, when fully extended, swing from the cage ceiling without having their feet touch the floor.

Historically, cages have been constructed of a variety of materials. Caging must be constructed of sturdy, durable materials and be designed to reduce the possibility of contamination to adjacent cage units. All surfaces should be smooth and free from sharp edges or broken wires. A minimum of ledges, corners, or angles is recommended to prevent dirt or fecal retention. The squeeze device should be easily operable to avoid primate injury. The cage should facilitate animal observations

Table 9.1 Individual Housing

Animals	Weight (kg)	Type of Housing	Floor Area/Animal		Height[a]	
			ft^2	m^2	in.	cm
Nonhuman primates[b]						
Group 1	> 1	Cage	1.6	0.15	20	50.80
Group 2	1–3	Cage	3.0	0.28	30	76.20
Group 3	3–10	Cage	4.3	0.40	30	76.20
Group 4	10–15	Cage	6.0	0.56	32	81.28
Group 5	15–25	Cage	8.0	0.74	36	91.44
Group 6	> 25	Cage	25.1	2.33	84	213.36

[a] From the resting floor to the cage top.

[b'] The designated groups are based on approximate sizes of various nonhuman primate species used in biomedical research. Examples of species included in each group are as follows: Group 1: marmosets, tamarins, and infants of various species; Group 2: capuchins, squirrel monkeys, and similar species; Group 3: macaques and African species; Group 4: male macaques and large African species; Group 5: baboons and nonbrachiating species larger than 15 kg; Group 6: great apes and brachiating species.

and provide feeding access and appropriate watering devices. Cage designs have included wall-mounted, floor, rack (figure 9.2), and permanent installations, all of which are satisfactory depending on species and project needs. The cage requirement for an adult chimpanzee is much different than for the New World monkey, mostly a function of space and strength of design.

Social or Pair Housing

The benefits of social housing primates in toxicological studies are numerous. Psychological well-being programs should have written procedures for compatibility assessment, sexual maturity, gender separation, and cage security. Nonhuman primates are highly social, and interaction with other animals best satisfies this need and reduces stress. Pair housing when study procedures allow or periodic commingling of compatible animals within the same dose groups has effectively been used during toxicology studies. Special caging has been designed with sliding doors to intermittently connect cages to allow access between cages or grooming bars to allow tactile interaction if full cage access cannot be allowed. Pair housing can also be used during periods of nonobservation or during nighttime hours for some studies. Animals can be housed socially in larger groups when not on study using larger pens at both indoor and outdoor facilities.

Figure 9.2 Hanging cage rack system for mature macaques. Pair housing is accomplished through commingling doors on the side of adjacent cages.

Group caging has its own set of requirements that by necessity must consider group dynamics, behavioral needs, usable surface areas, volume versus square footage, escape areas, and management practices.

Equipment Ancillary to the Cage

Equipment necessary to move the cage, handle the animal, and maintain safety of the handler and animal is essential to the husbandry operation. This equipment can include cage lifts, transfer cages, and restraint apparatus (e.g., pole and collar, leather gloves, capture gun, nets).

Systems of Removing Waste

The two primary methods of waste handling are dry and wet methods. Dry methods involve the use of bedding or plastic or paper liners under the cage. This system permits aerosol-free waste removal and cleaning of animal rooms, yet has the disadvantage of excessive waste disposal and high labor. This procedure for waste removal is sometimes employed where there are heightened biosafety considerations (biological radioactive waste). The alternative system is the wet procedure using water under high pressure to move the waste down the trough to the floor drains. The advantage of this system is fast cleanup, but care must be taken to prevent aerosolization and potential contamination of adjacent animals and personnel. A modification of this method using a low-pressure manual or automatic flush system is another approach. The use of wall-mounted proportionators allowing dual disinfectant and rinse flushing reduces contamination possibilities.

Cages and Housing for the Great Apes

The most important considerations for housing the ape, specifically the chimpanzee, are strength, environmental and behavioral enrichment, space for adequate movement and exercise, and design simplicity. Group housing of chimps might become the rule. New rules for individual housing could require at least two times the current space recommendations for adult chimpanzees of 25 ft^2. This would certainly have a direct impact on housing requirements and protocol design.

Environmental Enrichment and Special Concerns

Congress has coined the term "psychological enrichment" as a housing requirement for nonhuman primates. Essentially this has been meant to include environmental enrichment, social housing, behavioral well-being, and similar terms. Any primate research facility must take measures to assure that programs are in place utilizing and developing techniques to enrich the environment of primates on or off study. The USDA requires a written program for environmental enrichment. A variety of toys, puzzles, mirrors, and so on can be purchased or constructed for use in environmental enrichment. Care must be taken to assure that the equipment can be sanitized no parts of the equipment are hazardous. Use of occasional treats with human interaction or forage boards or as part of a feeder puzzle can be used. Such treats should not be of a quantity to adversely affect intake of a balanced diet, and certain research studies might limit the type or nature of the treats.

Nutrition and Water

Fortunately, there are adequate diets available commercially for nonhuman primates most commonly used in research. Most important, concern must be shown with the feeding techniques, feeding frequency, and feeding receptacles used with primates. Careful observation of feeding behavior is essential. An empty feed cup does not signify that a primate is eating because most of

the biscuits might be on the floor. Nor does a half-empty feed cup indicate an animal is not eating adequately, as each animal's metabolic rate is different. Regular monitoring of body weights and body condition are the best measures for determining the adequacy of the diet.

Physical Form and Presentation

The majority of commercial primate feed is produced through an extrusion process resulting, after baking, in a very hard product. A young primate might find the product to be unpalatable unless softened by soaking. (The liquid used for moistening must be provided to the primate, as the vitamin C sprayed on the biscuit will be rinsed off into the liquid if discarded.)

Some toxicology studies require oral dosing using a specific vehicle. Careful attention to amount of vehicle, particularly on twice or three times a day dosing regimens, is required to avoid "filling" the primate up with resultant reductions in food intake and potential nutritional deficiency.

Careful attention should be given to dates of manufacture and rotation of feed to prevent deterioration of vitamin C. There are now diets containing stabilized forms of vitamin C, which allows the shelf life of commercial primate diets to be extended up to 180 days. Check with the feed manufacturer to make sure they will guarantee this shelf life and set up a rotation schedule appropriate to the manufacturer's recommendations. The food must be kept palatable to the primate. Feed presented on the bottom of the cage without a feed cup can be contaminated by urine or feces, causing disease or an iatrogenic anorexia.

Available Diets and Analysis

Commercial diets are usually adequate and can be followed with pre- or poststudy analysis. Certified diets are also available and are required by GLP regulations. Analysis of noncertified commercial and noncommercial diets should be made to avoid potential contamination. Special diets for special studies, such as low-sodium or high-fat diets might be available commercially or might require custom preparation.

Food Restriction

It is common to fast the primate overnight prior to collection of clinical chemistry samples that might be affected by feeding or for preparation for anesthesia and surgery. The animals should not be fasted for excessive periods to prevent hypoglycemia. Some level of food restriction might be necessary to facilitate dose level when bioavailability is affected by the presence of food in the stomach or proximal intestine. Longer term food restriction during studies requires careful analysis to assure that animal well-being is not compromised (when balanced against potential scientific gains). Such modification to routine feeding practices must be justified to the IACUC.

Water

Clean, fresh water should be available at all times and should meet or exceed the appropriate regulatory standards for drinking water. Modern husbandry systems provide water through filtered automatic water systems. Clean water bottles are also an acceptable method to provide water but require more monitoring and maintenance to assure sanitation and proper function of the bottle. Water can be a source of potential contaminants that can interfere with a variety of study results. Careful analysis for chemical and microbiological contaminants should be conducted routinely. Chemical testing includes analysis for contaminants (i.e., pesticides, heavy metals, and specific organic compounds). Special testing can be conducted if required for certain test articles or studies.

Prevention of Disease and Injury

Procurement, Quarantine, and Conditioning

The assurance that the animal model used is of high quality and healthy is essential to any research program. This is particularly critical for nonhuman primates, which for the most part are obtained from breeding facilities in China, Indonesia, or the Philippines. Relatively few primates are bred in the United States at this time.

To prevent introduction of contagious conditions or zoonotic hazards into the animal colony, new animals should be quarantined a set period of time. The CDC requires a 31-day quarantine for imported primates, but a 7- to 14-day quarantine is more appropriate for animals introduced from domestic sources. The primary reason for the quarantine length is to detect potentially latent diseases and prevent the introduction of these agents into a larger (and likely susceptible) laboratory animal colony.

Procurement Sources. Most nonhuman primates used for research today are foreign or domestic purpose-bred animals. Although some primates are feral caught, this represents a small proportion of the animals used in preclinical research. Nonhuman primates that are island-bred or raised in a controlled environment are typically of a superior health status, and they readily adapt to the laboratory environment. Cesarean-delivered or specific pathogen-free monkeys might be required on occasion, based on the study objectives and the known properties of the test article.

Quarantine and Conditioning Procedures. Because most of the monkeys used for research are transported from breeding centers or quarantine facilities in other countries to the laboratories, quarantine and conditioning procedures are vital to assuring the health of the animals and protecting the laboratory colony from infectious diseases. A quarantine and conditioning program that pertains to the macaque is described as an example. It should be understood, however, that the following procedural descriptions might not be applicable to all institutions that utilize nonhuman primates because of differences in research needs, staffing, and facilities.

The quarantine facility should consist of standard animal rooms equipped with squeeze-back cages. Prior to receipt of newly arrived monkeys, each cage should be washed in a cage washer and sanitized, and the room should be washed, disinfected, and rinsed thoroughly. It is recommended that iodophores and phenolics should be used for their tuberculocidal properties.

The monkeys are delivered in crates containing individually segregated animals. Shipment lots can contain up to 120 animals. Immediately after receipt into the institutional quarantine facility, the monkeys are transferred from their shipping containers to quarantine cages directly and without handling. Drinking water and fruits are offered to assist in adaptation.

The animals are fed a commercial 15% to 20% protein monkey diet supplemented with fruit on the afternoon of arrival and are allowed to acclimatize for a few days. After 3 to 5 days acclimatization, the monkeys are sedated with ketamine hydrochloride (10 mg/kg, intramuscularly) and examined. Stress to the animals and hazards associated with physical restraint are contraindicated; therefore, mild chemical restraint is better for the animal's welfare and the safety of personnel. An electronic thermometer with an acrylic, rectal probe is used to obtain temperatures. The probe is shielded with a disposable plastic cover, which is changed and discarded between animals. Elevated temperature in the sedated primate is a more accurate disease indicator than rectal temperature measured in the physically restrained animal. If indicated by clinical signs of diarrhea or blood in the stool, fecal cultures can be obtained prior to temperature measurement. The animals are then examined for respiratory problems, suspicious enlargements, oral herpetic lesions, lacerations, dermatitis, and ectoparasitism. Minor health problems, including skin wounds and dermatitis, are treated at this time and supportive therapy is administered to monkeys with diarrhea or dehydration. Consideration of severe disease problems in individual monkeys that might require extensive workup is reserved until

all animals have been examined. Additional fecal cultures and hematological evaluations are performed as indicated. During physical examination, monkeys are tested for tuberculosis (TB) using 0.1 mL of veterinary mammalian tuberculin, full strength, administered intradermally in the eyelid (National Academy of Sciences [NAS] 1986). Each monkey is given an injection of Ivermectin the first week of the conditioning program. Additional parenteral anthelmintics (e.g., Droncit for tapeworms and levamisole for roundworms) are administered the third week of the conditioning program. A second injection of levamisole is administered the fifth week of the conditioning program.

Prophylactic injections of amoxicillin (20–40 mg/kg subcutaneously) or enrofloxacin (5 mg/kg, intramuscularly) might be indicated for respiratory or GI diseases, depending on the suspected infectious agent. Vitamins C and B complex (0.25–0.50 mL, intramuscularly) can also be given to improve the overall nutritional status of the animal.

Monkeys are tattooed consecutively, typically using a combination of letters and numbers per institution policy. Information such as weight, abnormalities, and clinical signs is entered on the animal's individual record.

The quarantine period procedures are based on requirements of the CDC.

In summary, monkeys are quarantined for at least 31 days to diagnose and eradicate TB. A total of three consecutive negative TB tests on each animal (after receipt) in the quarantine room must be achieved prior to releasing the group from quarantine. All monkeys that test positive for TB (reactors) are examined, possibly radiographed, euthanized, and necropsied. Tissues (typically the mediastinal lymph nodes) are evaluated microscopically (acid fast stain) for the presence of the TB organism. If deemed to be positive for TB, the quarantine procedures for the remaining animals need to be reevaluated to determine if depopulation or a repeat of the quarantine period is appropriate.

All monkeys in the facility are observed daily by a technician or veterinarian. If needed, the veterinarian prescribes treatments and institutes diagnostic protocols for sick or debilitated animals. Urgency of treatment is paramount in any primate facility. For example, a debilitated animal exhibiting anorexia, nasal discharge, or diarrhea requires prompt attention. Treatment is initiated immediately to alleviate clinical signs. Diagnostic tests will only be performed if absolutely necessary during the CDC quarantine period.

Diarrhea is one of the most common clinical signs in monkeys, particularly in new or stressful environments. Diarrhea is initially treated with pink bismuth (5 mL/kg/day, per os). Rectal cultures are collected when blood or mucus is seen in the feces, and a broad-spectrum antibiotic is given immediately. This original treatment is continued or changed depending on the results of culture and sensitivity testing. Other antidiarrheal agents that have been used successfully are amino pentamide hydrogen sulfate (0.1 mg/4.5 kg/day, intramuscularly), kanamycin sulfate (10 mg/kg/day, intramuscularly), and sulfa combinations. Because specific animal groups usually harbor bacteria with similar antibiotic sensitivities, one drug can usually be employed for treatment. Antibiotics are continued for at least 5 days after cessation of clinical signs. Fluid therapy (lactated Ringer's solution intravenously or subcutaneously or a commercially available oral electrolyte solution) is often used for monkeys with diarrhea or dysentery.

Pneumonia is a problem associated with the stress of shipment. Supportive therapy, including fluids, nutritional support, a temperature-controlled environment, and administration of antibiotics (e.g., penicillin or gentamicin) is recommended to treat pneumonia caused by secondary bacterial invaders. Severe epizootics of pneumonia have occurred in rhesus or cynomolgus monkeys that were associated with measles (rubeola) outbreaks in the first few weeks of quarantine (Potkay et al. 1971). Vaccination with modified live measles vaccine is recommended after the third TB test has been evaluated.

Other disease problems include amebiasis, dermatophytosis, and miscellaneous traumatic injuries. Most of these entities manifest clinically during the first 14 to 21 days after arrival. Thereafter, the health status of most animals stabilizes considerably.

Blood is obtained following the quarantine period from selected animals to further evaluate their health status and for reference serology. Fecal examinations for parasites are conducted.

Specific antihelmintics, antiprotozoal agents, and antibiotics are prescribed as indicated to individual monkeys or to groups.

The preceding description of quarantine and conditioning procedures is applicable to cynomolgus and other macaques imported from purpose breeding farms (NAS 1986; Renquist 1975). Domestically bred macaques that have been tested and found to be free of infectious diseases communicable to humans (e.g., shigellosis herpes B virus infection and TB) do not require such rigid quarantine measures. It is necessary, however, to provide a sufficient period of time for such monkeys to become adapted to a laboratory cage environment, as the majority are born and raised in large harem-type cages or corrals. During the adaptation period, valuable baseline data (hematological, serological, microbiological, and physiological) can be compiled for individual monkeys.

Occupational Health Program

Transmission of disease between primates and humans has been described. Recently, cases of herpes B, latent in the macaque but fatal in humans, have been incriminated in several deaths of animal handlers (CDC 1987a, 1987b). An occupational health program is essential, including preemployment screens for TB and physical examination with complete medical history. Banked reference serum samples and continuous monitoring are necessary with evaluation by the physician to assure employee health. The reader is referred to the NAS (1986) standards for specific information on evaluation criteria (CDC 1987a, 1987b).

There are three central components to a sound occupational health program in a primate facility. The first component is the staff. They must be well trained in the biohazards of working with primates. The institution must provide adequate personal protective equipment (PPE). Procedures and policies requiring the use of PPE should be incorporated into the training program.

The second component is to require written programs and procedures to be followed in the event that there is an exposure (scratch, bite, bodily fluid) from a primate or primate-soiled equipment. These procedures can include postexposure disinfecting of the wound or area, as well as biological samples from the animal and human for surveillance and medical care where appropriate.

The third component is the need to educate the medical community. Occupational health providers, emergency room staff, and medical specialists (e.g., virologists, trauma surgeons) should be a part of your medical support structure in the event that an exposure results in human disease. This will facilitate better care for your employees.

Common Diseases

The diseases and descriptions noted herein are those most common to the primate toxicology laboratory. A more comprehensive overview of the pathology associated with infectious diseases in presented in the pathology portion of this chapter.

Respiratory Diseases

Pneumonia is seen in primate colonies and is most often due to changes in environment related to stress of shipment, or sudden changes in humidity or temperature. In macaques, this is often a complication of rubeola (measles) and specific bacterial pathogens (e.g., *Branhamella cattarhalis* or streptococcus). Appropriate antibiotics and measles vaccination is recommended (Good and May 1971).

Enteric Diseases

Diarrhea and dysentery are probably the most common diseases in nonhuman primates. The major causative organisms are gram-negative enteric bacteria (e.g., *Salmonella, Shigella, Campylobacter,*

Yersinia, and *E-coli*; refer to Renquist 1987a; Weil et al. 1971). Unfortunately, latent infection without clinical signs is common and can be exacerbated by the rigor of the experimental protocol. Clearing protocols might be necessary to reduce the latent carrier state; however, these antibiotic regimens are often costly and should not be performed without culture and sensitivity testing. When evidence of clinical disease is seen, appropriate therapy is often the best approach.

Tuberculosis

The bane of the research laboratory is TB caused by a variety of mycobacterial organisms (Renquist 1987a). Appropriate tuberculin testing coupled with rigorous quarantine procedures can decrease the likelihood that it will enter the colony. A diligent intradermal mammalian tuberculin (MOT) TB testing program, a health surveillance program to include radiology where appropriate, and specialized assays such as the Gamma interferon test can be employed to achieve a comprehensive approach to TB prevention.

Viral Diseases

Measles (rubeola) is a common viral disease in humans and nonhuman primates. Certainly, however, other viral pathogens (e.g., poxviruses, hepatitis, simian hemorrhagic fever, and rabies) have also caused problems in a variety of research facilities. Most recently, serious concerns have been caused by herpes B (Renquist 1987b). Unfortunately, the incidence of herpes B antibody approaches 60% in most colonies. Herpes B is latent in macaques, causing a perioral blister-like lesion that is typically not associated with other abnormalities. When an individual is scratched or bitten by a positive primate, fatal consequences can result. The CDC has developed a set of guidelines for herpes B exposure and Ebola-like disease that should be carefully followed for both liability and employee health reasons (CDC 1987b, 1990).

Simian retroviruses (SRVs) have been associated with morbidity that has confounded the results of toxicological studies. SRV is prevalent in many macaque populations, and therefore should be part of a prestudy viral testing protocol. It is prudent to eliminate SRV-positive animals from assignment to long-term and pivotal toxicological studies.

Licensing and Records

Licensing

The Animal Welfare Act (Public Law 89-544), as amended most recently in 1991 (Federal Register 1991), requires the registration and inspection of primate facilities. The USDA Animal Plant Health Inspection Service is involved with enforcement. Direct importers of primates must also be registered with the CDC.

Other regulations and agencies covering primates and their care and use are the Department of the Interior, Federal Wildlife Permit Regulations, Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and the National Academy of Sciences, Institute of Laboratory Animal Resources.

Records

Accurate recordkeeping is required by law and is essential from the source to the completion of a project. This involves source country, date of receipt, importer, age, sex, body weight, health histories, and tattoo numbers. This is particularly critical for animals for which a federal wildlife permit is required or when animals are on the threatened or endangered CITES list (e.g., chimpanzees).

Study Design

General Considerations

The design of a preclinical toxicology study protocol (also referred to as a study plan) is dependent on the intended therapeutic indication of the investigational drug, the human (or animal) population for which it is intended (Hobson and Fuller 1987), or the potential for environmental exposure for population risk assessment. Guidance documents issued by worldwide regulatory agencies, including the U.S. Food and Drug Administration (FDA), the U.S. Environmental Protection Agency (EPA), the European Union Organisation for Economic Cooperation and Development (OECD), the Japanese Guidelines for Nonclinical Studies of Drugs Manual, and the International Conference on Harmonization (ICH) Tripartite Guidelines, typically provide information to assist the researcher in the development of appropriate study designs intended for safety assessment. Study duration ranges from single-dose acute studies to chronic multiple-dose studies that could exceed 1 year of test article exposure. The number of animals required for each study design varies substantially, and is directly impacted by the objectives of the study, duration of exposure, toxicologic endpoints, and potential need for assessment of posttreatment recovery. Acute (single-dose) and short-term repeated-dose toxicity studies are typically used to determine appropriate dose levels for subsequent studies of longer duration.

Drugs that are intended for the reproductively active population sometimes require a three-segment testing plan (not to be confused with the standard three-segment reproductive evaluation in rodents; Hendrickx and Binkerd 1990; Hendrickx and Cukierski 1987). In segment I, menstrual cycles and hormone levels (estrogen, progesterone, luteinizing hormone, and follicle-stimulating hormone) are monitored for 90 to 180 days, depending on protocol design, and spermatogenesis and testosterone levels are evaluated in males during a minimum 60-day treatment period. In segment II studies, pregnant females are treated during the period of organogenesis (gestation days 21–89) and a cesarean section is usually performed on day 100 for assessment of fetal abnormalities. The late gestational effects of a test material are examined in segment III studies. Pregnant females are administered the drug from gestation days 90 to 150 for evaluation of any abnormal neonatal neurological or behavioral responses.

Selection of Primate Species for Toxicology Studies

As discussed earlier in this chapter, there are many different species of primates used in research and determining the best nonhuman primate species for a particular research study is challenging. In toxicological studies in which many compounds are novel to primates, animal availability and industry trends have had a significant impact on the choice of species used for these studies. From the early research into polio vaccine development (during the 1940s) and continuing until approximately 20 years ago, the rhesus macaque was the monkey of choice for most research programs. Today the cynomolgus macaque is the most commonly used monkey in toxicological studies, although the rhesus monkey still remains a suitable surrogate from a scientific standpoint. The investigator should be aware that there could be more than one species suitable for toxicity studies. When choosing a primate for a toxicological program, it is often preferable to use one particular species from the same country of origin throughout the drug development program to minimize variability in the clinical parameters evaluated. Other considerations when choosing primates for studies include study design, dose administration techniques, health specifications (e.g., specific pathogen-free animals) weight of the animal, degree of maturity, and gender.

Humane Endpoints

Humane endpoints are the physical, clinical, or other in-life observations that mark or describe adverse toxicity that results in an animal welfare concern. Humane endpoints in primates can be a

valuable tool to quantitatively communicate morbidity to investigators, study directors, sponsors, and the IACUC. Humane endpoints are intended to describe or quantify the course and progression of adverse toxicities; they should be determined by active communication among all members of the study team. It is critical to any humane endpoint determination that individuals performing the assessment are qualified in the evaluation of these parameters and consistent methodology is employed. Close evaluation of the in-life data could serve as an early predictive indicator of present or impending adverse effects. If excessive pain, distress, or an unnecessary level of illness will result if the procedures of a study continue, humane endpoints are determined for the study to effectively modify the procedures or humanely euthanize the animals (if necessary).

Dose Levels

Dose levels tested are usually based on a proposed human dose or exposure. The lowest dosage in a safety evaluation study is usually greater than the expected human dose and should define the highest no-effect level. The highest dose is some multiple (e.g., 10–100 times) of the proposed clinical human dose and should induce toxicity. The middose is an intermediate level usually with minimal toxicity to provide characterization of a dose response to the test article.

Dosing Techniques

Dosing procedures used in research protocols will vary depending on the experimental objectives or the biochemical characteristics of the compound. The dosing technique utilized in safety studies with nonhuman primates approximates as closely as possible the expected route and frequency of drug exposure in humans. Typically, pharmaceuticals are administered via nasogastric intubation, orally by gavage, or via capsule or masking in dietary supplements. Biotechnology-derived materials are given either intravenously (bolus or IV infusion), intramuscularly, or subcutaneously. Other less common routes of dose administration are intranasal, intraperitoneal, intravitreal, or site-directed administration (i.e., intrajejunal, hepatic artery, or intra-articular). On occasion test article formulations are administered using alternate oral routes such as in fruit slices, sugar cubes, or a fruit juice drink. Methods of dosing can also be dictated by volume and characteristics of test article, duration of administration, and intended therapeutic use.

Dose Volumes

Guidelines for maximum dose volume administration will vary from facility to facility, and there is a paucity of information available in the literature. In general, the dosing volumes for nonhuman primates are described in table 9.2.

Table 9.2 Dosing Volumes for Nonhuman Primates

Route	Maximum Volume	Comments
Capsule	Single “0” capsule	Capsule size limitation due to anatomic limitations
Nasogastric/oral	10 mL/kg	Per dose; twice daily dosing possible, minimum 4 hr apart
IV bolus (≤ 3 min)	10 mL/kg	Also described as “slow push”
IV infusion (≤ 1hr)	15 mL/kg	Maximum volume might be exceeded depending on formulation
IV infusion (1–6 hr)	10 mL/kg/hr	Animal should be closely monitored for urine output
IV infusion (6–24 hr)	5 mL/kg/hr	Single dose; monitor urine output
IV infusion (> 24 hr)	4 mL/kg/hr	Continuous infusion, chronic administration possible

Oral Administration

Oral administration of drugs in safety evaluation studies has generally been conducted in squirrel monkeys or young macaques, and in limited numbers of immature baboons. The reasons for the size limitations in nonhuman primate models are the expense of special handling equipment and the size and aggressive nature of mature monkeys and great apes. Also, an adequate amount of historical data is available only in a limited number of primate species. Procedures must be employed that minimize stress to the animal, yet protect personnel and animals from injury. Infectious agents that might be carried by some of the primate models (i.e., herpes B virus) could be transmitted to animal handlers with potentially fatal consequences. The fecal or oral transmission of bacterial pathogens (salmonella, shigella, campylobacter, and *E. coli* species, to name a few) can potentially occur, leading to disease in the animal handler. As a result, the zoonotic potential of these (and other) infectious agents must be considered for all handling and dosing procedures. However, with proper training of personnel in the handling of nonhuman primates, oral toxicity studies can be successfully conducted and the potential risks minimized.

Techniques for Oral Administration

Techniques for oral administration of a test article include oral gavage, nasogastric gavage, capsule (using a modified oral gavage tube), or (when palatable) on a piece of fruit or in a drink such as fruit juice.

Equipment

The nasogastric route of administration is the most common and preferable means of gavage administration for macaques. Commercially available infant feeding tubes are best suited for young adult to adult cynomolgus and rhesus monkeys. When oral gavage is preferred (either due to the size of the animal, restraint method used, or test article properties) gavage tubes are available and range in size from 8 to 12 French (ID), and are utilized for the oral gavage of liquid formulations. When specialized tubing is required (e.g., for capsules), tygon or polyethylene tubing of appropriate size is utilized. The end of the tube is beveled, then blunted by heat to prevent esophageal damage or stomach injury. Capsule administration is not recommended for studies in nonhuman primates of longer duration (greater than 14 days of daily administration), due to the increasing chance of injury to the animal over time.

For both nasogastric and orogastric administration, a syringe is attached to the tube for delivery of the test article and the flushing solution. An 8 French feeding tube is generally used for nasogastric intubation (figure 9.3) when small volumes or low-viscosity liquids are presented or for stomach tubing of small primates (e.g., squirrel monkeys or infant macaques).

Although large monkeys can be manually restrained for dosing procedures, the risk of injury to personnel is high, as the head of the animal must be held securely by animal technicians.

A chair restraint system can be utilized for larger monkeys. This method restrains the animal's head and limbs during the dose administration procedure, minimizing the risk to technical personnel. In addition, this restraint system can be less stressful to the animal than manual restraint, if the animal is properly acclimated to the procedure. When using this system an aluminum or hard plastic collar is placed around the neck of an anesthetized animal and remains in place for the duration of the study. Aluminum poles with snap-type hooks are inserted into the cage and attached to the collars. The animal is led from the cage and placed in a chair (figure 9.3) and the collar secured to the chair. In this manner, minimal handling of the animal's head is required for dosing. It is important that proper positioning of the animal is achieved to reduce the risk of test article aspiration in the event vomition occurs. After dosing, the animal can be led back to the cage and released. Animals typically adapt to this handling procedure within a short period of time (typically 1–2 weeks).

Figure 9.3 Nasogastric dose administration.

Chimpanzees are utilized on a very limited basis for acute nonterminal toxicity studies, where the characteristics of the test article preclude the use of other nonhuman primate species. Animals of this size are usually maintained anesthetized for serial bleeds over a maximum of 6 hr, after which they can be immobilized with titrated doses of ketamine HCl for subsequent collections. Some chimpanzees can be trained for alert bleeding, but only experienced personnel should be allowed to handle the animals under these circumstances.

Test Article Preparation

The test article formulation is typically prepared on the day of dosing, although there might be instances where the known stability of material allows for preparation at an earlier date. A number of vehicles are used for test article formulation, depending on the aqueous solubility of the compound. True solutions (where aqueous solubility is high) commonly use distilled water, buffered saline, or aqueous admixtures that increase stability and solubility. Polyethylene glycol (PEG400 or similar) is commonly used when the aqueous solubility of the test article is poor; however, dose volumes should not exceed 1 mL/kg due to the increased incidence of diarrhea with this vehicle. Suspensions of the test article are commonly prepared in carboxymethylcellulose (typically 1%), and this material is well tolerated in all nonhuman primates. Capsules can be prepared prior to the day of use, if a powder; if liquid, it is necessary to fill the capsule shortly before administration.

Control animals should be administered a concentration and volume of the vehicle equivalent to that received by the high-dose animals.

Dose Administration

For oral or IV administration, the individual doses are drawn up into graduated syringes in the formulation laboratory or in the animal room. If prepared in the formulation laboratory, syringes are labeled with the animal number, the study, and group color code. A research technician not involved in the dose preparation verifies this information. Syringes are placed in a rack, also color coded, and

transported to the animal room. If dose withdrawal occurs in the animal room, the source container is appropriately identified with the study and group information. The test article formulation, dose volume for each animal, and dose administration is verified and documented by the technical staff. Depending on the test article, monkeys can be fasted overnight prior to a morning dosing.

Manual restraint methods include cageside dosing, removal from the cage and immobilization by the technician, tube restraint, and chair restraint. Cageside dosing is achieved by use of the squeeze-back mechanism of the animal's cage. Once the animal is appropriately restrained, the dose is administered. One technician can accomplish this procedure. For dose administration outside of the cage, the animal is caught (using protective gloves) after partial closing of the squeeze-back mechanism. The animal is removed from the cage and restrained (typically cradled on the side of the animal handler). A second technician administers the dose, which is confirmed by another individual in the room. For tube restraint (most appropriate for IV dose administration and femoral venous blood collection), the same method of extracting the animal from the cage is used, and the animal is placed (headfirst) into an appropriately sized clear Lucite tube. The animal's legs are restrained and the dose is administered into the saphenous vein. For chair restraint dosing, two handlers, each with a pole, are required to capture an animal and place it in the primate chair. The door to the animal's cage is opened slightly, a pole inserted, and the pole attached to a ring on the collar. The animal is restrained within the cage, the door is opened, and the other pole is attached to the ring on the opposite side of the collar. The animal is then led out of the cage and into the chair for restraint. The third technician, meanwhile, has the animal's dosing syringe and tube (nasogastric or oral) available for dose administration. A technician, wearing appropriate gloves and arm covers, restrains the animal's head. The tube is inserted and slight negative pressure is applied to the syringe to check for air bubbles, indicating a possible lung intubation. The dose is administered, a flushing syringe containing a small amount of water or vehicle is attached, and the remaining contents of the tube are flushed into the stomach. The tube is removed and the animal is observed for a moment to check for vomiting. The poles are reattached to the collar and the animal is returned to the cage.

Risks to the animals are minimal if the proper procedure is followed; however, if the dose is inadvertently placed in the lung rather than the stomach chances for animal survival are minimal.

Caustic or irritating compounds or large gavage volumes might induce an emetic response that, if aspirated in the lungs, can result in the death of the animal. Consideration must be given to reducing the dose or reformulating the test article to reduce the possibility of aspiration.

Capsule

Plain gelatin or enteric-coated capsules are administered when testing a potential new drug delivery system or when a slower absorption rate is required. The technique for dosing with capsules is similar to oral dosing with a liquid. The plain or coated capsule is slightly wedged into the end of an 8- to 14-in. length of tygon tubing of slightly larger inside diameter than the diameter of the capsule. A syringe, usually containing 10 to 15 mL of tap water, is attached to the end of the tube. The capsule-plugged end of the tube is intubated and the capsule flushed into the stomach. The administration of multiple capsules simultaneously is possible, but this is generally used only in single-dose studies. Several capsules can, in this way, be administered to one animal without repeated intubations. A stylet can also be used to expel capsules out of the tube once properly placed in the stomach. In general, several hundred milligrams of test article can be administered in this manner. Prestudy acclimation to the dosing procedures will minimize the possibility of dosing errors and animal injury.

Other Oral Dose Administrations

Nonhuman primates have a propensity for playing with their food and expelling portions of their dietary rations from their cage or breaking it up and dropping it through the cage floor, thus

making it essentially impossible to quantify dietary consumption (or assure a specific dose of test article, if added to the diet). On a limited basis, small quantities of test article have been placed on sugar cubes, in small pieces of fruit, or dissolved in a commercially available fruit drink. Animal technicians present the test-article-treated food item and observe to verify that the animal consumes it. Liquid can be administered from a syringe such that the animal voluntarily drinks the dosing solution. Expected problems encountered with these methods include animals removing portions of the sugar cube or fruit from their mouth and refusal to complete the dosing, or losing some of the fruit drink through spillage and refusal to swallow all of their dose solution, making quantification of the total dose administered difficult. These problems are minimal or nonexistent if the test article or food mixture is highly palatable to the animal. Additionally, the effects of the content of such vehicles on metabolism of the compound must be ascertained as part of the study design.

Intravenous Administration

The IV route of drug administration is a commonly employed technique in preclinical toxicology studies. This route is preferred for test articles with poor bioavailability or limited GI absorption, and for biotechnology-derived proteins and monoclonal antibodies. The cephalic and saphenous veins are the most accessible for peripheral venipuncture, in both single-dose and repeat-dose studies. Peripheral veins, including the femoral and the jugular, can be catheterized for acute or subchronic IV infusions, although many veins require surgically invasive procedures for catheter placement. In addition, specialized restraint devices are required for protection of the catheters from the animals (e.g., primate chairs, vascular ports, jacket-tether infusion, or backpack-port ambulatory infusion systems).

Peripheral Venous Administration (Bolus)

The two largest, most accessible superficial veins for IV dose administration are the cephalic and saphenous veins. The cephalic vein is located on the dorsal arm near the bend of the elbow of the nonhuman primate. The saphenous vein can be found on the ventral side of the leg from the knee to the ankle. Toxicology studies requiring a single daily injection for as long as 12 months can be performed successfully if proper techniques are utilized to minimize vascular and perivascular tissue damage.

Alert-Capture Techniques

Study animals are housed singly in squeeze-back cages for the study duration. Wearing appropriate protective apparel (lab-specific clothing, gloves, mask, arm guards), trained technicians capture the animals and administer the test article. Small and medium-sized squirrel monkeys (weight 5–6 kg) are caught by technicians wearing heavy leather gloves with long gauntlets by reaching into the cage, placing a thumb and forefinger firmly around the animal's body, and gently removing it from the cage. The animal is restrained on a table, stomach down, by the technician, exposing the back of the leg for a saphenous injection administered by a second technician.

Large macaques and small baboons are usually not removed from their cages for treatment. The animal is pulled to the front of the cage by the squeeze mechanism, which is locked in position. The technician carefully maneuvers an arm or leg near the feeding hole in the lower front of the cage. Once the foot or hand is gripped tightly, the squeeze mechanism is allowed to return approximately one-third of the width of the cage, permitting the animal to lean toward the rear of the cage. The limb can now be extended through the hole for accessibility to the vein (figure 9.4). Prior to injection, a portable clipper is used to remove the hair from 2 to 4 in. along the length of

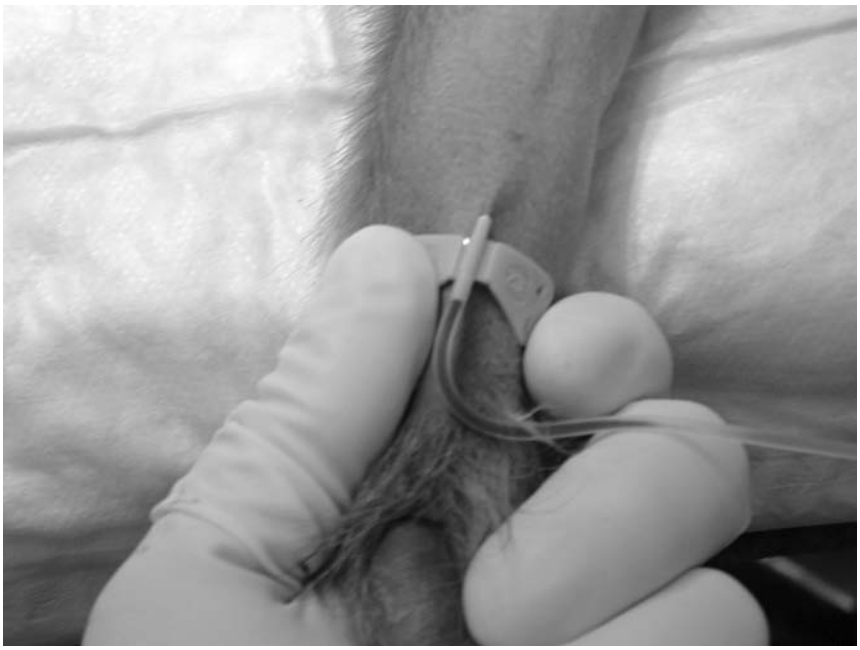

Figure 9.4 Intravenous (saphenous) injection of rhesus monkey.

the vein. The exposed area is cleaned with an alcohol pad and pressure is applied to the vein with the thumb while holding the limb with the hand. Pressure is applied proximal to the intended injection site. As blood flow is impeded, the vein becomes visible to the technician. The needle, attached to the dosing syringe, is placed bevel up against the skin. Pressure is exerted until there is a slight give, usually indicating entry into the vein. Slight back pressure on the syringe will draw blood into the hub of the needle, verifying venous placement, at which point the test compound can be administered.

Syringe and needle sizes vary with the volume and characteristics of the compound to be injected. Needle sizes usually range from 21 to 25 gauge and syringes from 1 to 35 mL. Injection volumes greater than 10 mL are more successfully administered when a small butterfly catheter is placed into the vein for dosing and the animal is removed from the cage. This can be facilitated by the use of two technicians: one to restrain the limb and ensure that the butterfly is not dislodged and the second to administer the dose through the distal end of the catheter and subsequently flush the remaining dose with appropriate vehicle (e.g., saline).

Tube Restraint Technique

The tube restraint technique is used for femoral venous blood collection techniques and for IV bolus (or slow push) administration of dosing formulations into the saphenous vein. The tube is constructed of clear Lucite plastic and varies in wall thickness, diameter, and length.

A variety of tube sizes should be available to accommodate differences in age, species, and size of monkey restrained. Animals acclimate to the tube restraint technique quickly, as the barrier between the handler and animal not only provides a greater level of safety, but with repeated use it also has a calming effect on the animals. This restraint technique should be considered if frequent blood sampling is required, as it minimizes the potential for inadvertent cageside trauma (i.e., bruising and abrasions) and has been found to decrease errors related to strict time collection requirements.

Intravenous Infusion Techniques

A variety of techniques are utilized to administer dosing solutions via slow IV infusion. The following section provides a brief overview of these techniques; however, a more comprehensive discussion of IV infusion in nonhuman primates can be found in Healing and Smith (2000).

Chair Restraint Technique. Studies requiring an IV infusion for periods of time ranging from 30 min to a suggested maximum of 4 hr are conducted using primate restraint chairs. Studies requiring a longer duration of infusion, or repeated daily doses similar to the maximum infusion duration, should be conducted using the jacket-tether or ambulatory infusion model (see later).

Animals should normally be adapted to chairs prior to study initiation. Several chairing sessions beginning 2 weeks prior to study start reduces stress related to the chairing procedure. Animals are first anesthetized with ketamine HCl (approximately 10 mg/kg) and an indwelling catheter introduced into the cephalic or saphenous vein (Flynn and Guilloud 1988). The animal is placed in a restraint chair and allowed to recover from the anesthesia prior to dosing. If the cephalic vein is used for dosing, some provision must be made to secure the opposite hand and arm to prevent the catheter from being pulled from the vein by the animal's free hand. The prepared dose is drawn up in an appropriate-sized syringe and placed on an infusion pump set to deliver the designated dose concentration at a preset rate. An extension tube (typically 10 in. in length) is attached from the syringe to the catheter for delivery of the dosing solution. The volume of the extension tube (and other external components, such as in-line filters) must be factored into the dose preparation calculations to assure sufficient volumes to dose all animals. At the termination of dosing, the catheter is removed, hemostasis is induced, and the animal is removed from the chair and returned to the home cage.

Jacket and Tether Infusion Technique. The femoral and jugular veins are ideal for chronic catheterization and the conduct of chronic infusion studies. When the catheter is externalized, the placement of an indwelling catheter requires a surgical procedure, but this technique offers the advantage of limited animal inaccessibility to the catheter. Using the jacket and tether infusion technique, the catheter is channeled subcutaneously to an exit site located on the dorsal thoracic region of the animal and it is protected by a jacket and tether system (Bryant 1980). The dosing formulation is delivered via an infusion pump external to the cage, and doses can be administered over extended periods of time (i.e., several hours or days). These procedures are applicable for New and Old World monkeys but are not feasible for animals as large as adult chimpanzees.

Animals are acclimated to the tethers and jackets for 2 to 3 days prior to placement of the catheter. During this period, the jacket is worn by the animal while the tether (flexible steel tube) hangs freely in the cage unattached to the animal. After the acclimatization period, the animal is sedated and taken to the surgery suite. The animal is prepared for surgery and an incision is made in the skin above the vein or artery to be catheterized. The vessel is located and separated from connective tissue by blunt dissection. The vessel is elevated with sutures to impede blood flow and a small cut is made in the vessel with iris scissors. A catheter introducer is inserted into the incision to facilitate introduction of the catheter.

The catheter is inserted into the vein or artery for 5 in. to 8 in., depending on the size of the animal. The most desirable placement of the catheter is in the femoral artery or vein so that the catheter tip extends to either the vena cava or aorta, facilitating infusion and blood sampling procedures. The catheter is held in place by ligatures placed around the vessel and on both sides of an elevation (or "donut") located several inches up the catheter. This prevents the catheter from being pulled from the vein or artery by pressure exerted from normal body movement. As an additional precaution, a stress loop is made in the catheter and anchored with a suture or sutures just under the skin. A trocar is used to tunnel the catheter under the skin to the middle of the back

where it is exited. The catheter is then passed through the mounting plate attached to the jacket and through the tether to the appropriate channel on the underside of the swivel. Another length of catheter is attached to the same channel on the top of the swivel and then to an infusion pump. Aseptic techniques in handling the exposed end of the catheter are extremely important to the success of this system. Frequent observations for potential problems with jackets and the tethering system are important if study objectives are to be met. Primates are particularly adept at manipulating this system, and thus require frequent monitoring to correct animal attempts to remove the jacket.

Vascular Access Port Administration

The vascular access port (VAP) is a subcutaneously implanted device that provides chronic vascular access and eliminates the need for multiple venipunctures in repeat-dose IV bolus or IV infusion studies (Dalton 1985). The VAP is a silicone rubber septum housed in a titanium metal casing that can be anchored with sutures under the skin. The placement of the vascular port is usually dorsolateral thoracic region, from which the catheter (typically polyurethane or silicone) is tunneled under the skin to the femoral vein (or another vein or artery) for catheterization.

VAPs have proven to be quite durable and reliable, as long as local and systemic infection is rigorously monitored and treated proactively. Repeat-dose studies in excess of 9 months in duration have been successfully conducted; however, it should be recognized that continuous use of these devices results in an increasing rate of catheter failure after 13 weeks of dosing. Catheter failure is typically preceded by repeated dermal or subcutaneous infections, formation of adhesions, and necrosis of the skin overlying the VAP, as well as infections along the catheter tract characterized by swelling and occasional necrosis or drainage. Although there is no clear explanation for the increased incidence of catheter failure in chronic studies, gross and histopathologic findings from animals chronically instrumented with VAPs indicate that changes occur at the catheterization site. At gross necropsy, tissue adjacent and distal to the tip of the catheter thickens, becomes more brittle, and strictures form as fibrous connective tissue replaces the nonviable regional vasculature over time. Histopathologic findings include fibrosis of the vascular wall, fibrous capsule formation, chronic active inflammation, thrombosis, and hypertrophy of vascular and perivascular tissues.

Dosing is performed by injecting the test article into the port and the catheterized vein. Location of the port and septum is easily accomplished by palpation. The area of the skin above the port is prepared using aseptic techniques to minimize the chance of infection. To prevent a coring effect, a 21-gauge Huber point (noncoring) needle is used for port access. If possible, locking solutions containing other materials should be aspirated prior to flushing the port. When flushing the port and administering a dosing solution, the Huber needle is inserted through the septum, and the dose is administered. A saline solution (or saline containing heparin up to 40 IU/mL) is used to flush the remaining test article from the catheter, and the appropriate locking solution is instilled to maintain catheter patency.

Ambulatory Intravenous Infusion. Ambulatory infusion employs the use of a VAP for dose administration, and offers some advantages over the jacket and tether technique. Animals are first instrumented with a VAP, and sufficient healing time (approximately 7–10 days) is allowed prior to initiation of acclimation procedures. Each animal is fitted with an appropriately sized infusion jacket, which contains a dorsal opaque (black canvas) pocket. A battery-powered ambulatory infusion pump (CADD Legacy PLUS, or equivalent, SIMS Deltec, Inc., Minneapolis, MN) and self-contained dosing reservoir (50 or 100 mL cassette fill volume), along with a winged infusion set (i.e., infusion line connecting the pump and reservoir to the VAP via Huber-point needle) is contained within the dorsal pocket (figure 9.5 and figure 9.6).

Figure 9.5 Cynomolgus monkey fitted with ambulatory infusion jacket. The winged infusion set is used to access the VAP, which is implanted in the dorsolateral thoracic region under the jacket.

Figure 9.6 Cynomolgus monkey moving freely in the home cage using the ambulatory infusion system.

The ambulatory infusion system allows the investigator to implant a number of animals prior to initiation of dosing, thus providing a readily available pool of monkeys for a series of studies. If intermittent infusion is required (i.e., multiple daily doses or a defined dosing intervals per week), this system can result in decreased animal handling due to the programmable nature of the infusion pump. In addition, the jacket and infusion system can be removed between doses (e.g., once or twice weekly), allowing the animal more normal activities and minimizing infusion-system-related injuries (i.e., pressure necrosis to the skin, obscured infections, etc.).

Ambulatory infusion is not appropriate for test articles of limited room temperature stability, or for those formulations that require very small or very large volumes of administration (i.e., less than 5 mL/dose or more than 250 mL/dose for the designated infusion period). In addition, this system is not optimal for monkeys larger than 5 kg, due to handling and safety concerns. Additional information concerning the use of ambulatory infusion in nonhuman primates can be found in the earlier referenced literature, as well as the bibliography for this chapter.

Intramuscular Injection

Intramuscular administration of a test article formulation provides for a reasonably consistent and rapid absorption and distribution profile, depending on the test article characteristics and the properties of the vehicle and excipient formulation. Biologics delivered in oil are released much slower than those from an aqueous carrier; in addition, test article suspensions, where the particle size is larger than for true solutions, might have a longer time to maximal plasma concentration (C_{max}) and a concomitant longer half-life ($t_{1/2}$). In nonhuman primates, the injection site is usually the outer thigh (quadriceps femoris mm), although the triceps are also utilized in larger animals.

Monkeys and small baboons can be dosed alert, whereas large baboons and chimpanzees should be sedated. The cage squeeze mechanism allows the monkey to be immobilized to allow access for injection to a leg or arm. Hair is clipped from the injection site and the site is either cleansed with alcohol or aseptically prepared using a Betadine solution. Using an appropriate-sized needle (we suggest no larger than 21 gauge) and syringe, the skin and muscle are penetrated. A slight vacuum is applied to the syringe to check for possible venous puncture. If blood is drawn into the syringe, the needle should be removed and the process repeated. If no blood appears, the plunger is depressed slowly until the dose is administered. The needle is then removed and pressure applied to the injection site for approximately 30 sec with a gauze pad to complete the dosing procedure.

Dosing volume should be limited to less than 0.5 mL/kg to minimize pain and the incidence of skeletal muscle irritation. If larger dosing volumes are required, two injection sites should be utilized.

Subcutaneous Injection

Subcutaneous administration of a test compound is frequently utilized in toxicity studies when a slower absorption rate is required, or if the characteristics of the formulation (i.e., low pH, large-particle suspension, large volume, etc.) preclude the use of the intramuscular route. The dose is delivered between the dermal and muscle layers, usually in the dorsal midscapular area. The loose skin at this site allows for volumes up to 5 mL/dose to be administered. Other subcutaneous injection sites to be considered are the inner thigh and limbs for smaller volumes of dosing material (less than 0.5 ml). The relatively large potential subcutaneous space at each dose site should be demarcated with either a tattoo or indelible pen, depending on the duration of dosing and need to evaluate the dose site(s) for adverse reactions.

Capture procedures for the animals are as previously described. The injection site is shaved and cleansed with alcohol, or prepared aseptically with a Betadine solution. The skin is grasped between the thumb and forefinger and retracted from the underlying muscle. The skin is penetrated with the needle at approximately a 15° angle to the injection site. As the plunger is depressed, a small bubble should appear as the dosing progresses. There should be no resistance to the injection. If resistance is noted and the bubble is firm, close to the surface, and appears white in color, the injection is being administered intradermally.

The needle should be inserted to the deepest point into the subcutaneous space initially, and slowly removed as dosing is completed to assure a broad distribution of the dosing solution. The needle exit site should be digitally compressed for approximately 30 sec after dosing to prevent leakage of the formulation. One animal technician should restrain the animal while another

administers the dose to ensure complete delivery of the test compound and prevent possible injury to the animal or handler.

Miscellaneous Routes of Exposure

Intranasal

The development of aerosol drug delivery systems for inhalation therapy has resulted in increased intranasal toxicity assessments. Test articles are administered into one (or both) nostrils with the aid of a calibrated and metered aerosolizer device. Discharge from the reservoir can be accurately calibrated, but total delivery into the nasal cavity is not always successful (or consistent) in an alert animal. Depending on the frequency of dosing and size of the animal, dosing can be accomplished via hand restraint (i.e., one animal handler for restraint, and one technician for dose administration), or via the use of chair restraint (for multiple doses over a short period of time, or for larger monkeys). This technique allows for better control of the head while the delivery tube is placed a short distance into the nostril for dose administration.

Intraperitoneal

Although not a common route for exposure to a test compound in nonhuman primates, intraperitoneal injections or infusions permit rapid absorption into the portal circulation. In humans, this route would place therapeutic agents in close proximity to tumors of the abdominal organs; thus nonhuman primate testing by this route can occur on a limited acute basis.

The jacket and tether system is ideal for continuous intraperitoneal infusions for extended periods of time. This method has been utilized successfully to deliver tumor-specific monoclonal antibodies in safety-evaluation studies. Serial interperitoneal injections (needle or catheter) of nonhuman primates are not recommended because of the high probability of infection as well as the potential for bladder or intestinal puncture. Single injections are usually performed on anesthetized animals. The entire abdomen and inner upper thigh is shaved, cleaned, and sterilized. Using a Betadine swab (or solution) the injection site, which is 1 in. to 2 in. below and 1 in to 2 in. lateral to the navel, is again sterilized.

The skin is pulled slightly to the center of the abdomen with the thumb of one hand while inserting the needle at an angle of approximately 45° into the injection site with the other hand. The purpose for pulling the skin to one side and placing the needle or catheter at an angle ensures that when the needle or catheter is removed an interrupted channel into the body cavity is established. This reduces bleeding and decreases the chances for infection. A short catheter (2 in.) or needle reduces the chances of perforating the bladder, intestine, or other organs. The location of the needle or catheter can be checked by injecting 1 to 2 ml of saline followed by gentle vacuum with a syringe. If blood, fecal material, urine, or fluid other than saline is aspirated into the syringe, the procedure must be reattempted on the opposite side. Health-threatening consequences could result from organ perforation; thus the veterinarian should be notified immediately and appropriate antibiotic therapy begun.

If an organ is not punctured, thumb pressure on the skin is released, which stabilizes the needle or catheter for injection. As the plunger of the syringe is depressed, attention must be directed at the injection site for possible swelling, which would indicate a subcutaneous or intramuscular dose. If swelling occurs, dosing is stopped and the procedure is repeated. Following completion of the injection, the needle or catheter is removed and pressure is applied to the injection site for approximately 30 sec, followed by observation for any bleeding or swelling at the site.

Infrequent Routes of Dose Administration

Drug delivery systems designed for special therapeutic applications have been evaluated in safety studies but are not considered routine procedures. Such procedures include osmotic

minipumps or other drug-containing implants; topical administration; vaginal, rectal, or colonic administration via suppositories or liquids; and sublingual dosing. Periocular and intraocular dosing (i.e., administration of dosing formulations around the eye or via intravitreal injection) is accomplished in an anesthetized animal, and is an increasingly common route for the development of targeted therapies for degenerative ocular disease. Many of these unique routes of dose administration require surgical procedures or can only be administered to anesthetized animals or animals physically restrained in primate chairs. Such methods are typically utilized only for acute dosing protocols and might not be acceptable for subchronic or chronic toxicity assessments. Inhalation toxicology is a unique area requiring specialized equipment and facilities not usually found in general toxicology laboratories and is thus beyond the scope of this review.

Data and Sample Collection Techniques

All GLP primate toxicology studies require approved protocols including a defined listing of in-life and postlife evaluations to adequately characterize and evaluate the toxicologic and pharmacologic effects of the test article. Various worldwide regulatory agencies provide guidance as to the basic elements of regulatory toxicology study designs, but much is left to the individual researcher in defining (and justifying) the parameters evaluated. Commonly used evaluations include daily clinical observations, body weights, blood and urine collection for clinical chemistry, hematology, coagulation, and urinalysis evaluations. Additional evaluations might include physical examinations, measurement of cardiovascular parameters, ophthalmic examinations (direct or indirect), and serum and plasma test article concentrations for toxicokinetic studies. Occasionally more specialized tests or samples such as menstrual cycle status, pregnancy tests, telemetry-based cardiopulmonary monitoring, nerve conduction velocity, electroencephalograms (EEGs), electroretinograms (ERGs), and tissue biopsy are required. After completion of the dosing (i.e., test article exposure) phase, animals are commonly euthanized, gross necropsies are conducted, organ weights are measured, and a protocol-specified list of tissues are collected, preserved, processed, and evaluated for potential histopathologic changes. Most studies of 4 weeks duration or longer include both terminal phase (i.e., immediately after completion of dosing) and recovery phase groups of animals to assess the potential for the reversal of target organ changes and toxicity. Recovery phases range from 2 weeks to a number of months, depending on a variety of factors including the known toxicology, pharmacology, and tissue distribution, and toxicokinetics of the test article.

Sample collection from great apes is limited to nonterminal and non-life-threatening techniques. Tissues occasionally become available from moribund animals from these species, which are humanely sacrificed, or from animals dying of natural causes.

Both study design and animal inventory planning must consider that primates should only be subjected to one major experimental surgical procedure during their lifetime (Federal Register 1991). A major surgical procedure is defined as one that penetrates a body cavity or is considered invasive in nature (examples include bone surgery, intracranial administration of test article, or medical device implantation). Additional procedures within the same experimental protocol must be approved by the IACUC, include appropriate perioperative measures for analgesia, and be justified by decreasing the overall number of animals used in experimental research.

Clinical Observations

Because primates have complex and highly individualized behavior patterns, obtaining meaningful observation data depends on the experience of the observer, the knowledge of expected observations for the species in a laboratory setting, and an innate familiarity with individual animal responses. The occasional use of group caging makes this task even more complex for more traditional toxicologic evaluations; however, behavior studies prefer the establishment and maintenance of a well-defined animal hierarchy. Ideally, observations should be made at the same time(s)

and by the same observer each day. Many studies include postdose evaluations that coincide with the expected maximum plasma concentrations (C_{max}), which allows the researcher to determine if any effects are present the following day (predose). Changes in behavior or appearance are most important to note. An experienced observer should be quick to note the following (Silverman 1988):

- Lethargy
- Dehydration
- Unusual posture or movement within the cage
- Poor condition of hair coat
- Brightness or dullness of the eyes
- Unusual fluid from body orifices
- Unusual motion within the cage and proprioceptive deficits
- Self-mutilation
- Hyperventilation
- Hyperactivity
- Hyperreactivity (startle responses)
- Apparent pain or discomfort
- Favoring of individual limbs
- Evidence of changes in appetite or water consumption (if possible)
- Watery or discolored stool
- Changes at injection sites (where relevant)
- Any other unusual behavior or appearance

Appetite should be assessed at least once daily, and is commonly conducted prior to offering the morning food ration. Persistent inappetance can result in serious (and possibly life-threatening) hypoglycemia in most Old and New World species, as these animals have limited stores of glycogen and become ketotic with starvation.

Physical Examinations

The physical examination findings can be one of the most important overall criteria to determine acceptability of an animal for study use. These evaluations include, at a minimum, the assessment of the overall physical condition of the animal, body temperature, respiration rate, heart rate, thoracic auscultation, abdominal palpation, integumentary assessment, overall behavior and mentation, and other evaluations that are specified in the testing facility standard operating procedures or study protocol. Specialized examinations, which can include endocrinological profiles or more extensive neurological examinations, might be required, depending on the type of toxicology study (Keeling and Wolf 1975).

Clinical pathology (serum chemistry, hematology, and coagulation parameters) and fecal parasite evaluations are typically included in the general physical examination. Table 9.3 and table 9.4 detail typical normative clinical hematology and serum chemistry data for nonhuman primates commonly used in toxicity evaluation studies.

Body Weights

Toxicologists who are not accustomed to reviewing data from primate studies are often surprised by the degree of inter- and intra-animal variability of body weights. It is not uncommon in a study of 2 weeks duration (or less) to encounter weight changes of 10% or more that are totally unrelated to the experimental protocol. These changes can be minimized by weighing and feeding at the same time each day, acclimating the animals to in-life procedures prior to initiation of dosing, and providing individualized care to animals that might not adapt as quickly to the requirements of the

Table 9.3 Hematology of Nonhuman Primates, Normal Ranges

Test	Units	Squirrel monkey[b] (*Saimiri sciureus*)	Cynomolgus monkey[a] (*Macaca fascicularis*)	Rhesus monkey[a] (*Macaca mulatta*)	Baboon[c,d] (*Papio sp.*)	Chimpanzee[a] (*Pan Troglodytes*)
White blood cells (WBC)	thsd/mm^3	3.4–14.8	4.5–14.0	5.5–15.4	—	7.2–16.8
Red blood cells (RBC)	mill/mm^3	7.1–10.9	4.6–6.5	4.0–6.6	—	4.8–5.8
Hemoglobin (HGB)	gm%	12.9–17.0	10.0–13.1	10.9–15.5	8.7–13.9	12.0–5.8
Hematocrit (HCT)	percent	—	30–41	35.2–47.6	31–43	36.0–49.5
Mean corpuscular volume (MCV)	μ^3	41.4–62.7	57–68	65–79	63–90	64–102
Mean corpuscular hemoglobin (MCH)	µµg	13.9–20.1	18–22	21–27	18–27	20–34
Mean corpuscular hemoglobin concentration (MCHC)	percent	29.2–34.8	30–33	30–34	28–34	27–36
Platelets (PLT)	thsd/mm^3	—	200–550	230–650	225–544	150–450
Reticulocytes	percent	—	0.1–1.1	0–1.9	0.3–2.3	0.5–1.5
Differential						
Segmented neutrophils (SEGS)	percent	13.0–79.0	10–50	32–83	23–78	55–80
Lymphocytes (LYMPH)	percent	19.0–82.0	50–80	34–69	14–76	12–45
Monocytes (MONO)	percent	0.0–6.0	0–2	0–2	0–3	0–5
Eosinophils (EOS)	percent	0.0–22.0	2–8	0–6	0–8	0–2
Basophils (BASO)	percent	0.0–4.0	0–2	0–1	0–1	0–1
Coagulation						
Prothrombin time (PT)	sec	—	8.9–12.9	9.4–13.4	11.5–13.0	10.0–13.4
Activated partial thromboplastin time (APTT)	sec	—	16.9–33.9	19.5–25.3	29.5–38.0	18.9–35.6
Fibrin degradation products (FDP)	µg%	—	< 8	< 8	—	< 8
Fibrinogen (FIBRIN)	mg%	—	150–300	187–411	180–230	139–416

[a] Primate Research Laboratory, NMSU; Colony Historical Data.

[b] Abee (1985).

[c] Hack and Gleiser (1982).

[d] Kelly and Gleiser (1986).

study. Longer term acclimatization to the room, the diet, and the other animals in the room is also advantageous for some types of studies (Renquist 1975).

Anesthetized animals can be weighed on conventional scales; however, the use of a transfer box (tared to provide a net body weight) allows for data collection without the use of a sedating or immobilizing agent. Animals can be readily trained to enter the boxes, particularly if they are housed in cages with squeeze-back mechanisms.

Physiological Measurements

Physiological endpoint measurements have become routine in drug safety evaluations, and are used to determine if acute effects occur that have an adverse effect on the cardiorespiratory and central and perhiperal nervous systems. Historically, the requirement for such measurements resulted from observations of pyrogenic activity of certain proteins and cardiotoxic or neurotoxic effects of monoclonal antibodies. Indications of untoward physiological responses in the past have necessitated a thorough evaluation of these parameters. Comprehensive batteries of physiologic measurements (except neurological examinations) are usually obtained under ketamine immobilization to allow for a more thorough evaluation of all organ and body systems; however, some

Table 9.4 Blood Chemistry of Nonhuman Primates, Normal Ranges

Test	Units	Squirrel monkey[b] (*Saimiri sciureus*)	Cynomolgus monkey[a] (*Macaca fascicularis*)	Rhesus monkey[a] (*Macaca mulatta*)	Baboon[c,d] (*Papio sp.*)	Chimpanzee[a] (*Pan Troglodytes*)
Alanine aminotrans-ferase (ALT)	U/L	4–1612	5–50	5–61	—	0–40
Albumin (ALB)	mg%	—	3.4–5.0	3.6–5.5	3.6–5.5	2.7–5.3
Alkaline phosphatase (ALP)	U/L	6.0–49.0	150–464	45–661	154–1,105	142–624
Aspartate aminotransferase (AST)	U/L	—	5–50	12–63	—	0–40
Bicarbonate (BICARB)	mml/L	—	20–32	96–116	—	25–32
Bilirubin, total (TBILI)	mg%	0.0–1.9	0.1–0.8	0.1–1.2	0.3–0.7	0.1–1.3
Blood urea nitrogen (BUN)	mg%	11.4–42.2	8–28	8–38	9–25	5–22
Calcium (CA)	mg%	4.2–5.8	8.4–11.1	8.1–13.8	8.0–9.6	8.3–12.9
Chloride (CL)	mml/L	103.0–118.0	100–118	16–29	104–118	94–110
Cholesterol (CHOL)	mg%	116.0–272.0	70–178	62–238	68–232	144–304
Creatine phosphoki-nase (CPK)	U/L	—	206–630	22–53	—	9–33
Creatinine (CREAT)	mg%	—	0.5–1.2	0.1–1.5	0.8–1.4	0.1–1.3
Gamma glutamyl-transferase (GGT)	U/L	—	11–50	24–645	—	42–366
Globulin (GLOB)	mg%	—	1.5–4.8	1.5–4.8	2.4–4.4	1.9–4.9
Glucose (GLUC)	mg%	35.0–148.0	48.0–80	27–100	50–129	75–117
Lactic dehydrogenase (LDH)	U/L	230.0–760.0	100–446	43–426	99–488	105–439
Phosphrous (PHOS)	mg%	3.2–9.2	2.1–5.5	2.4–8.7	4.7–7.7	3.0–6.4
Potassium (K)	mml/L	3.5–9.5	3.5–5.0	2.93–5.45	3.2–4.3	3.5–5.3
Protein, total (TPROT)	mg%	—	6.2–7.6	5.9–8.8	5.7–7.8	5.5–7.2
Sodium (Na)	mml/L	144.0–173.0	135–158	135–158	143–158	133–148

[a] Primate Research Laboratory, NMSU; Colony Historical Data.

[b] Abee (1985).

[c] Hack and Gleiser (1982).

[d] Kelly and Gleiser (1986).

measurements can be collected in an alert animal due to the sequence of study-related activities and the need to monitor the animals during the postdose period(s).

Body Temperature (Rectal)

Rectal temperatures are obtained using a digital thermometer coated with lubricant (K-Y Jelly or equivalent). The animal is placed in the prone position on a table or gurney for rectal accessibility. The probe, covered with a lubricated disposable plastic sheath, is gently inserted into the rectum until a body temperature reading is attained. Rectal temperatures should be recorded as quickly as possible after sedation for the most accurate reading. Rectal temperature can be obtained in alert animals using manual restraint, but excitement due to the procedure results in wide variations in temperature compared to measurements obtained from lightly sedated animals.

Blood Pressure

Systolic, diastolic, and mean arterial blood pressure is usually obtained indirectly using a blood pressure machine and cuff designed for small animals or infants. The cuff device is applied to the forearm or tail. The procedure is repeated to confirm the accuracy of the reading, and to provide a mean value of approximately three readings. It should be noted that the most accurate data are obtained through using telemetry and surgically implanted sensors, but such techniques have the disadvantage of requiring surgical implantation of a pressure transducer into the femoral artery. As a result, most toxicology studies (with the exception of studies that include radiotelemetry monitoring in subgroups of animals) use indirect means for obtaining these data. The blood pressure cuff is placed securely around the upper thigh or forearm with the cuff-positioning arrow situated over the medial midfemoral or humeral region. The machine is started and the reading on the gauge is recorded. The blood pressure assessment should be repeated at least two additional times and a mean calculated to ensure an accurate estimate. Blood pressure readings using the cuff method can be affected by movement of the animal, so it is critical that the animal remain as quiet as possible during the recording.

Heart Rate

The standard method for recording the heart rate requires using a stethoscope to hear the heartbeat. Using a stopwatch, the heart rate is counted over a minimum 15-sec period and the appropriate multiplication factor is applied. The heart rate can also be determined by calculations from ECGs or with blood pressure monitors equipped to record this measurement.

Respiration Rate

Respiration rates can be obtained cageside by observing the breathing pattern of the animal. If the monkey was recently removed from the cage for dose administration (or other study-related procedures), sufficient time should be factored into the procedure to allow the respiration rate to return to expected resting values. Calculations from counting over a set period of time are made similar to those used for heart rate data.

Neurological Evaluation

A neurologic examination is a series of subjective observations conducted to evaluate both central and peripheral sensory and motor functions. These evaluations are performed by veterinarians or research personnel experienced in observing monkeys in a laboratory environment. A freely moving animal is initially examined cageside, followed by removal from the cage and assessment of specific neurologic pathways via reflex testing. An example of a grading system is given in table 9.5.

Table 9.5 Example Neurologic Examination Grading System

Grade	Sign
0	Absence of purposeful movement; paraplegia
1	Unable to stand to support; slight movement when supported by the tail; severe paraparesis
2	Unable to stand to support; when assisted moves limbs readily but stumbles and falls frequently; moderate paraparesis and ataxia
3	Can stand to support but frequently stumbles and falls; mild paraparesis and ataxia
4	Can stand to support; minimal paraparesis and ataxia
5	Normal strength and coordination

Additional tests require the animal to be squeezed to the front of the cage or properly restrained outside the cage. Pelvic or thoracic limb flexor reflexes are assessed by exerting pressure with a hemostat to the lateral and medial digits. The integrity of the reflex arc following mild pressure stimulus is graded as responsive (+) or nonresponsive (–).

Patellar reflexes (femoral nerves) are evaluated while the animal is as relaxed as possible. The patellar tendon is lightly tapped with a reflex hammer and subjectively graded as shown in table 9.6.

Table 9.6 Evaluation of Patellar Reflexes

Grade	Sign
0	No reflex
1	Hyporeflexic
2	Normal
3	Hyperreflexic
4	Clonic/tonic

Cranial nerves are evaluated by observing a variety of reflexes and positional responses, which allow the clinician to determine if the test article has a central nervous system effect that can be localized to specific region(s) of the brain. Among these evaluations are pupillary reflexes, assessment of pupillary size (both relative and degree of dilation or restriction), absence or presence of involuntary ocular movements (nystagmus), improper positioning of one or both eyes (strabismus), presence or absence of the menace reflex, and responses to controlled auditory stimuli. Additional tests can be conducted depending on the expected pharmacologic or toxicologic action of the test article.

Ophthalmologic Examinations

Ophthalmologic examinations are becoming increasingly routine in toxicology studies, due to a heightened concern for potential ocular toxicity. The eye is a complex organ that includes functional, neurological, and vascular components that can be highly sensitive to potential toxins. Ophthamologic examinations commonly occur prior to initiation of dosing and prior to scheduled necropsies (or release from study). Documentation of the presence of preexisting abnormalities is critical to determine if changes observed at later time points are related to test article exposure.

The ophthalmic examination involves, at a minimum, the assessment of the health of the adnexa (eyelids, conjunctiva, external surface of the cornea, sclera, and associated surface vasculature). These examinations can be conducted on alert animals using a restraint chair. However, if the examination requires an assessment of the anterior and posterior chambers and associated structures, the animal is chemically restrained with ketamine hydrochloride. Prior to the examination, a few drops of a topical mydriatic agent are placed in the surface of the eye to achieve papillary dilation. When immobilized, the adnexa is thoroughly evaluated. A fundic examination is performed using a direct or an indirect ophthalmoscope, allowing the veterinarian (or other trained professional) to observe the anterior chamber, iris, posterior chamber (vitreous), fundus, optic disc, macula, and the choroidal structures.

Electrocardiograms

ECGs measure the electrical conduction activity of the heart, and are a standardized assessment of cardiac function and health. Examinations should occur prior to initiation of dosing and at one or more time points during the dosing and recovery periods (if included in the study design). Given the potentially transient nature of changes to cardiac electrical activity associated with test article pharmacology and toxicity, ECGs can be collected predose and at a defined time point postdose (typically at the expected time of maximum test article concentration).

ECGs are collected on alert animals using a Plexiglas restraint board. The animal is removed from the cage and manually restrained. In general, the animal is positioned on its back in a horizontal position. The hair is shaved from each site designated for the placement of an ECG lead or electrode. After shaving, the site is wiped with alcohol and the appropriate ECG lead is attached to the skin using an alligator-style clip. The leads are attached to the sites given in table 9.7 to provide a standard six-lead (I, II, III, aVR, aVL, and aVF) ECG recording.

Table 9.7 Standard ECG Lead Placement

Labeled Leads	Lead Placement/Location
Left arm (LA)	Wrist of the left arm
Right arm (RA)	Wrist of the right arm
Left leg (LL)	Calf of left leg
Right leg (RL)	Calf of right leg
V1	4th intercostal space, approximately 4 cm from midsternal line
V2	4th intercostal space, approximately 4 cm from midsternal line (i.e., on left side of symmetrical MV1)
V3	Left midaxillary line in the 5th intercostal space (approximately 1 cm below level of MV2)
V4	7th thoracic vertebra

Both qualitative (assessment of tracings for presence of abnormal complexes) and quantitative (measurement of specific intervals) data can be derived manually or electronically, depending on the collection and assessment methods. The discovery of multiple drug interactions and the potential for cardiac arrest has led to an increased emphasis on quantitative ECG assessments in cardiovascular safety pharmacology. The determination of QT intervals, corrected for heart rate (QTc) has become increasingly common when the test article's pharmacology suggests a potential effect on the heart. There are various formulas used to correct the QT interval, and they are available in the current literature for review and consideration.

Blood Collection and Normative Clinical Pathology Data

Trained and experienced technicians can collect blood samples from alert (unanesthetized) macaques in most situations without danger to either the animal or the technician. If the technicians are not experienced, if the macaques are large males, if the cages are not the appropriate type, or if the blood volume needed is large, animals should be anesthetized prior to venipuncture. Some studies have shown physiological and immunological changes caused by behavioral stress with alert bleeds (Mason 1972). These possibilities must be considered in any study design. In certain instances, macaques and adult chimpanzees and baboons can be trained to submit voluntarily to blood collection procedures by experienced technicians (Hein et al. 1989; Vertein and Reinhardt 1989); however, routine blood collections in the larger species are almost always done under anesthesia (ketamine HCl; Muchmore 1973) because of the danger of injury to technicians by stronger animals and increased activity during blood sampling from alert animals can adversely affect some clinical parameters (e.g., will elevate creatine phosphokinase and corticosteroids; refer to Coe et al. 1985).

Usually blood samples are collected by venipuncture. Occasionally, samples are collected through temporary indwelling catheters when frequent collections are required, as in pharmacokinetic studies. Rarely, samples are collected through long-term indwelling catheters. In general, venipuncture is preferred because of less damage caused to the veins, less need for anesthesia or minor surgery, and a reduced chance of infections. In some instances, samples have been collected several times daily for up to a month by venipuncture without adverse effects if sterile techniques are used (shave and alcohol prep the collection site).

Venipuncture samples requiring small amounts (less than 5 mL) can be collected from the cephalic or saphenous veins. The femoral vein is generally used when large blood volumes are required (greater than 5 mL). For cephalic or saphenous vein collections, animals are restrained as described in the section on IV drug administration. The skin over the vein is shaved and swabbed with alcohol or an iodine solution. Either a syringe and needle or an evacuated container can be used. The needle should be the smallest size compatible with the sample volume; that is, 23 gauge for samples under 1 mL, 22 gauge for l- to 3-mL samples, and so on. Smaller needles cause less damage to the vein and lessen the hazard of hematomas forming. Short-bevel needles are preferred when they are available because they have less chance of slicing the vein wall. After withdrawal of the needle, direct pressure should be maintained on the site for approximately 30 sec. If femoral samples are collected (from anesthetized animals), pressure should be maintained on the site for at least 1 min to minimize the incidence of hematoma formation.

The amount of blood that can safely be removed from a primate during the course of a study depends on the study purpose, the study length, total blood volume (Bender 1955), and the frequency and amount of samples (Keeling and Wolf 1975). When in doubt, hematological parameters should be monitored frequently to avoid adverse effects. If an important endpoint of the study is a hematological evaluation, then the maximum recommended volume should be considered to avoid influencing these endpoints. As a general guideline, up to 10 mL/kg body weight can be removed over a 21-day period, because that is the minimal rate at which blood is replaced. Up to 15 mL/kg over a 21-day period can be removed at one time if additional veterinary monitoring is performed to assure no adverse effect from blood withdrawal. This amount is considered a maximum and can be expected to temporarily change some hematological parameters.

Technicians who work with monkeys on a daily basis find that monkeys quickly adapt to routine blood sample collection. After a time they will offer their arm without being restrained or handled. This learning process can be speeded considerably if rewards are provided to cooperative animals.

Some laboratories return red cells to the animal in an attempt to increase the maximum amount of blood that can be collected. In general, this technique should be avoided. Experience has shown that problems of infection and hematological changes outweigh the benefits gained.

Chronic catheterization can provide a route for serious infections even when the most careful aseptic techniques are used. Moreover, chronically placed catheters frequently develop fibrin flaps over their distal end, effectively blocking blood withdrawal. When catheters are used, their patency can be maintained by keeping them filled with a normal saline solution containing 1 to 4 IU/mL sodium heparin for injection. Regular flushing of the catheter and strict aseptic procedures are required to maintain patency in a chronic catheter for periods longer than a week.

Temporary catheters placed in restrained or anesthetized animals provide a means for collecting very frequent samples over a short period of time. They are introduced by the technique described earlier for IV infusion (also see Bowen and Cummins 1985).

Table 9.3 and table 9.4 summarize normal ranges for hematology and blood chemistry laboratory values in the five most common primate species.

Usually blood samples are collected by peripheral venipuncture. Occasionally, samples are collected through temporary indwelling catheters when frequent collections are required, as in some pharmacokinetic studies that required frequent low-volume, sample collections. Blood samples can be collected through long-term indwelling catheters. In general, venipuncture is preferred because of less damage caused to the veins, less need for anesthesia or minor surgery, and a reduced chance

of infections. In some instances, samples have been collected by peripheral venipuncture several times daily for multiple weeks without adverse effects if sterile techniques are used (shave and alcohol prep the collection site). Laboratory technicians who work with monkeys on a daily basis find that monkeys quickly adapt to routine blood sample collection. Acclimation to the cageside technique, when coupled with positive reinforcement (e.g., treats offered immediately following the sample collection) might actually result in the animal offering a limb without being restrained or handled.

The femoral vein (or artery, depending on the actual vessel of collection) is most commonly used in macaques. Venipuncture can be accomplished using the cephalic or saphenous veins, but these are not as reliable and can produce a relatively poor-quality sample. For all of these routes of collection, animals are restrained as described in the section on IV drug administration. The skin over the vein is shaved and swabbed with alcohol. Either a syringe and needle or an evacuated container can be used. The needle should be the smallest size compatible with the vessel of collection; a 23- or 22-gauge needle is used for the majority of samples. In general, smaller needles cause less damage to the vein and lessen the hazard of hematomas forming, but they also can result in a higher incidence of hemolysis (attributed to increased turbulence and associated vacuum pressure from the syringe). After withdrawal of the needle, direct pressure should be maintained on the site for at least 30 sec to minimize the incidence of hematoma formation or frank bleeding.

The amount of blood that can safely be removed from a primate during the course of a study depends on the study purpose, the study length, total blood volume (Bender 1955), and the frequency and amount of samples (Keeling and Wolf 1975). When in doubt, hematological indicators of red blood cell mass (i.e., red blood cell count, hemoglobin, and hematocrit) should be monitored to avoid adverse effects related to blood loss. Up to 5 mL/kg can be removed at one time if no other samples have been or will be collected for several weeks (Schalm 1975). At our laboratory, the maximum amount of blood volume that can be collected, without veterinary monitoring and oversight, is 10 mL/kg over a 21-day period; this period coincides with the generally accepted life span of the erythrocyte for nonhuman primates. A maximum of 15 mL/kg can be collected over the same period, but the animal must be monitored by the veterinary clinical staff.

Urine Collection

Because monkeys have a tendency to play with their food and water (or watering devices), quantitative timed collections of uncontaminated urine and feces pose a difficult task (Baker and Morris 1979). By placing a collecting pan (with screens optional) under the cage and removing the food and water source, short-term urine samples of reasonable quality can be obtained from a single void specimen. Samples should be collected early in the morning, with pans placed beneath the cage prior to turning the lights on in the room. When the daily light and activity cycle of the room begins, most animals void quickly. When setting up urine collection cages for metabolism studies (or for studies that require urinary electrolyte excretion studies), space is provided under the collection pans to place the collection vessel in ice. This is important when total urine collections for 24 hr are required. Inexpensive styrofoam coolers can be modified and are quite useful for holding the ice and urine collection vessel, and also have the added benefit of protecting the resultant sample from ambient light.

Specialized pharmacokinetic studies might require quantitative urine collection over a limited period of time. This is frequently accomplished by placing the animal in a chair, catheterizing the bladder of females and placing a condom collection device over the penis of males (catheterization of males' bladders is not practical as a routine, alert procedure). If animals are appropriately adapted to the chair, this technique can be used for up to 4 hr. Longer periods of chairing are best interrupted with an exercise period to prohibit any prolonged stress-related changes. Other methods used in a chemically restrained animal include a direct sample collection by suprapubic puncture (Keeling

and Wolf 1975) or an indirect collection by manual bladder expression. Neither method is recommended for repeated or frequent sample collections.

Pharmacokinetic and Toxicokinetic Evaluations

Pharmacokinetic studies involved the collection of serial blood samples to assess the overall absorption, metabolism, and elimination kinetics of a drug. Samples are collected to provide an adequate quantity of the appropriate matrix (i.e., serum or plasma), as well as a sufficient number of time points to allow for calculation of pertinent data. Toxicokinetics involve the mathematical and statistical manipulation of the data to determine the behavior of the drug in the selected animal system.

The following toxicokinetic parameters are typically derived separately for males and females in all groups on the intervals (i.e., day 1 and subsequent time points):

- C_{max} = maximum plasma/serum/matrix concentration
- t_{max} = time of maximum plasma/serum/matrix concentration
- t_{last} = time of last quantifiable plasma/serum/matrix concentration
- AUC_{last} = area under the concentration versus time curve from time zero to the time of the last quantifiable plasma/serum/matrix concentration
- AUC_{0-} = area under the concentration versus time curve from time zero to infinity
- $t_{1/2}$ = apparent terminal elimination half-life

These data, when considered with the remaining study data, provide a more integrated assessment of test article exposure (to include potential accumulation and gender effects) as it relates to clinical observations, clinical pathology, gross necropsy, and histopathologic findings. The reader is encouraged to consult a variety of references that more fully address this subject.

Invasive Cardiovascular Procedures

Direct monitoring of central arterial and venous pressure is the most common cardiovascular measurement requiring an invasive procedure. Normally animals are surgically catheterized in the femoral vein or artery, connected to a direct recording device, and monitored for up to several hours under general anesthesia. IV infusion of ketamine or an inhalant anesthetic such as isoflurane can be used.

Measurement of cardiac output is possible with the thermal dilution technique in large macaques using human pediatric catheters. This technique is not suitable for smaller animals because appropriate catheters are not available at this time. Microspheres can be used to estimate cardiac output in smaller animals (Forsyth et al. 1968). Normal cardiovascular values have been reviewed by Bourne (1975).

Necropsy

Critical to the majority of toxicological procedures is the determination of the cause of death or morbidity of an animal, and to determine if one or more tissues (or organ systems) were adversely affected by test article exposure. The necropsy findings (both gross and histomorphologic) should focus on both the antemortem and postmortem findings of the study.

Owing to the potential zoonotic potential for disease transmission from primates to humans, it is essential that all safety procedures be strictly adhered to during primate necropsies. These procedures include use of gloves (two-layer for prosectors), masks, face shields, disposable surgical gowns, and other specialized protective wear as required for the individual study.

It is essential that a prenecropsy meeting be held with prosectors, pathologist, and study director to familiarize all concerned with the protocol, previous antemortem findings, and so on. This will

enable any recorded abnormalities (noted at the time of clinical observations or physical examinations) to be noted for potential correlation at necropsy.

Although necropsy procedures might vary slightly depending on the facility, species evaluated, and study objectives, the basic steps are relatively standard. The study protocol must be reviewed in detail to determine the specific requirements, organ weights to be measured, tissues collected, and any unique procedures (e.g., frozen tissues, whole body perfusion, or unconventional tissue collection and preservation procedures). The testing facility standard operating procedures detail the most common procedures, and the study protocol is referenced to detail any unique requirements.

Specialty Toxicology

Neurotoxicology

Animal models of neurotoxic insults are valuable to the extent that they provide a sensitive and valid index of the onset or progression of the compound-induced deficits. For deficits that are similar across a wide range of species, such as axonal degeneration in the peripheral nerve (e.g., length-dependent distal axonopathy), adequate models might be limited to rodents. In contrast, there are some neurotoxic deficits that affect portions of the neuroaxis with vulnerability, specialized anatomy, or transmitter distributions that are unique to primates. For these cases, in-life neurotoxicology assessments should include nonhuman primates. For example, in monkeys, apes, and humans the distal projection of bipolar neurons in the dorsal root ganglia (DRG) forms the distal peripheral nerve, and the central projection of many of these cells enters the spinal cord and forms the ascending dorsal columns without synapsing. Thus, axons in the spinal cord of primates can represent the processes of first-order sensory neurons and be damaged by pathology limited to the DRG (e.g., buildup of heavy metals such as platinum). When considering a compound that might induce a central myenopathy (e.g., γ-vinyl Gaba) the sheer volume of the subcortical white matter makes these regions vulnerable in primates. Any assessment of potential central nervous system myelin damage should include assessment of the major ascending tracts, such as the lateral lemniscus, and the corpus callosum in a nonhuman primate model. In addition, for compounds that target specific neurotransmitters, such as those used to treat psychiatric disorders, nonhuman primates must be a part of the preclinical safety evaluation because of the primate-specific pattern and distribution of neurotransmitters, such as NMDA.

The ultimate neurotoxicity of a novel drug is related to the pharmacokinetics of the compound, potentially to the metabolites of its breakdown, and clearly to the degree and speed of its penetration of the blood–brain and blood–nerve barriers. The clinical impact of these elements is best modeled in nonhuman primates.

Developmental and Reproductive Toxicology

Nonhuman primates are useful animal models for male and female fertility, teratology, and peri- and postnatal studies due to their phylogenetic, reproductive, and developmental similarities to humans (Hendrickx and Cukierski 1987; Hendricx and Dukelow 1995). Many pharmaceutical and biotechnology products are under development that can be consumed by women of childbearing age (e.g., birth control products, preterm labor, vaccines used for autoimmune diseases), men during conception, or are immunotherapeutic products that only cross-react with nonhuman primate tissues. Therefore, the need for safety assessments performed in nonhuman primates has increased.

Cynomolgus and rhesus monkeys are the most frequently used nonhuman primates in reproductive toxicity testing. Rhesus monkeys, however, are used less frequently due to seasonal infertility. These studies are labor-intensive because the menstrual cycle of each individual animal must be tracked to determine ovulation for the optimal mating days. In rhesus monkeys, a timed breeding model has been developed using exogenous progesterone to induce an artificial luteal phase for

studies that require dosing of test article during the early gestational days (Phillippi-Falkenstein and Harrison 2003).

Currently, the earliest pregnancy can be consistently detected using ultrasonography and palpation is gestational day 18 to 20. Human test kits that detect the presence of chorionic gonadotropin work well in the great apes. They are of limited value in macaques because of minimal cross-reaction between the reagents and monkey chorionic gonadotropin. Radioimmunoassays for chorionic gonadotropin in macaques are available (Hobson et al. 1975). In general, however, they are only useful for diagnosing pregnancy during gestational days 17 to 33 following conception, because chorionic gonadotropin secretion is limited to that time in macaques (Hein et al. 1989). A urinary chorionic gonadotropin kit is available from NIH at cost (Contraceptive Development Branch, Dr. Gabriel Bialy, Bldg EPN, 600A). Like humans, great apes secrete chorionic gonadotropin throughout pregnancy; however, it is difficult to detect prior to day 13 and after day 90 of pregnancy owing to low levels (Reyes et al. 1975).

The great apes and Old World monkeys (baboons and macaques) exhibit menstrual bleeding at the end of a nonfertile cycle (when the corpus luteum has ceased producing progesterone). New World monkeys do not exhibit menstrual bleeding; thus other endpoints (e.g., serum estrogen, progesterone, FSH, etc.) are necessary to follow the reproductive status of the females. In addition to observations of menstrual bleeding, chimpanzee menstrual cycles can be followed by observations of their sex skin. The perineal area swells dramatically in response to estrogen in the early part of the menstrual cycle and regresses rapidly immediately following ovulation (Graham et al. 1972).

Because menstrual bleeding is minimal in macaques, simple observation of the animal or the cage floor is not always sufficient to detect the presence of bleeding. Daily swabbing of the exterior vagina with cotton-tipped swabs provides an excellent means of following the menstrual cycle. Most female macaques rapidly learn to "present" for the swabbing so that data from an entire room can be collected in a few minutes. The length of the menstrual cycle of female cynomolgus monkeys is 28.6 ± 3.3 days and normally could vary up to 20% of the previous cycle (Oneda et al. 2003).

Ultrasonagraphy can be use to monitor the general condition of the fetus or embryo by monitoring the heart rate and developmental landmarks. Additionally, measurements such as gestational sac, greatest length, humerus and femur length, biparietal diameter, occipitofrontal diameter, head circumference, and abdominal circumference can be collected during gestation and provide an excellent method to determine stage of pregnancy (Conrad et al. 1989; Farine et al. 1988; Tarantal and Hendrickx 1988). Accurate dating of pregnancy (within ± 2 days of parturition) is possible at early stages of pregnancy. This process permits dosing at specific gestational ages without more drastic intervention. Radiography and fluoroscopy are useful for determining potential fetal abnormalities or organ dysfunctions. Both instruments, particularly ultrasound, can be utilized to perform size and functional aspects of cardiac flow, rhythm, and other cardiovascular phenomena.

Female fertility studies in nonhuman primates are performed by monitoring the menstrual cycle and sex hormone (estrogen, progesterone, follicle stimulating hormone, and luteinizing hormone) profile. The pattern of expression of the sex steroid hormones is similar to that in humans (Hotchkiss 1994). Blood from the female monkeys is collected every 2 days during the follicular phase and 3 days in the luteal phase at approximately the same time of day, with the limitation that the volume must be below IACUC criteria. Samples are generally collected for one cycle prior to treatment, one cycle during treatment, and one cycle during the recovery period. Additionally, histopathology is also performed on the reproductive organs.

Parameters evaluated for male fertility studies include sperm count, motility and morphology; testicular volume, and level of testosterone. Typically samples are collected prestudy, during dosing, and during the recovery phase. Typically the duration of treatment in the male fertility study is approximately 90 days, or one sperm cycle. Additionally, histopathology is also performed on the reproductive organs.

Challenges with the use of nonhuman primates in reproductive toxicology studies are: length of gestation (approximately 155–165 days for cynomolgus monkeys; Hendrickx and Dukelow 1995), low conception rate (approximately 25%), spontaneous abortion rate (approximately 17% for rhesus monkeys and 17.8% for cynomolgus monkeys; Hendrickx et al. 1966), small sample size, limited supply, high interanimal variability, and single offspring.

Treatment dose, regimen, and study length are dependent on the potential use of the test article in humans.

Maternal endpoints include clinical observations, food consumption, body weight measurements, hormone profile, toxicokinetic and immunogenicity evaluation of blood and milk, and ultrasonography. For teratology studies (generally gestation day 100) and perinatal studies (generally gestation days 140 and 150), fetuses are obtained via C-section and endpoints evaluated include body weight, teratologic evaluations (external, visceral, and skeletal examination), skeletal examination (e.g., radiography or alizarin red), organ weight, histopatholgy, toxicokinetics, and antibody analysis. Additionally, the placenta is obtained during the C-section and is evaluated.

The central nervous system development in the nonhuman primate is more advanced at birth than it is in humans (Wood et al. 2003). For postnatal studies conducted in nonhuman primates, the infants are obtained from live birth and are generally observed for up to 1 year following birth. The infant endpoints include mother–infant bonding, neonatal muscle tone, neonatal neurobehavioral test battery (based on Brazelton Neonatal Behavior Assessment Scale [Golub 1990; Golub and Gershwin 1984]), organ weight, histopatholgy, toxicokinetics, and antibody analysis.

Safety Pharmacology (Cardiovascular Safety Assessment)

Safety pharmacology studies are designed to evaluate the potential for drugs to produce secondary pharmacological or toxicological effects that are acute and life-threatening. As described in the S7A International Conference on Harmonisation (ICH) guidelines (2001), these types of studies were developed to protect clinical trial participants and patients receiving marketed products from potential adverse effects of pharmaceuticals. In some cases, studies in nonhuman primates provide the best means of assessing the toxicity of prospective pharmaceuticals on physiological functions in relation to exposure in the therapeutic range and at multiples of this range. These types of studies are intended to supplement existing information to satisfy international regulatory guidelines for nonclinical safety evaluation that recommend investigation of potential ancillary pharmacologic actions (S7A, International Conference on Harmonisation 2001; S7B: International Conference on Harmonisation 2002).

The cardiovascular, respiratory, and central nervous systems are considered to be the vital organ systems that are critical for life. According to the ICH guideline, these organ systems comprise the core battery that should be evaluated prior to first administration to human beings by appropriate designed safety pharmacology studies.

The use of telemetry technology in unrestrained nonhuman primates provides a useful model to assess the primary effects of new drugs on ECG and cardiovascular parameters. For the cardiovascular system, the S7A ICH guideline suggests measurement of blood pressure, heart rate, and ECG. Importantly, telemetry provides a greater opportunity for the detection of conduction abnormalities and adverse effects on repolarization by measurement of the QT interval; a heart rate independent prolongation of the interval has been associated with fatal arrhythmias in human beings due to a variety of drug classes. Telemetry technology with totally implantable instrumentation offers advantages over models that require physical or chemical restraint (anesthetized model). The lack of restraint facilitates the collection of more physiologically meaningful data and increases the likelihood of detecting drug-related adverse effects on the cardiovascular system. The integration of the following parameters into the design of the cardiovascular safety pharmacology study

accommodates the assessment of the respiratory and central nervous systems, which satisfies the S7A ICH guideline for adequately evaluating the core battery:

- *Respiratory system:* Respiratory rate (via telemetry or visual assessment) and other measures of respiratory function (e.g., hemoglobin oxygen saturation or tidal volume).
- *Central nervous system:* Motor activity, behavioral changes, coordination, and sensory/motor reflex responses using functional observation battery (FOB) and body temperature via telemetry.

Ocular Toxicology

Nonhuman primates provide an excellent ophthalmology model because of the similarity in ocular anatomy and physiology to the human eye. Similar to humans and unlike other laboratory species, nonhuman primates have a macula and do not have a tapetum lucidum. Ocular test article can be administered to nonhuman primates via several dose routes including topical to the eye, intravitreal injection, and subconjunctival injection.

Standard ocular evaluations in toxicology studies include ophthalmoscopic and biomicroscopic (slit-lamp) examination. More specialized evaluation of retinal vascularity is conducted by fluroscein angiography, intraocular pressure can be measured by pneumotonometry, and retinal conductance is evaluated by electroretinography.

Currently research efforts are targeted to many serious ocular diseases such as glaucoma, diabetic retinopathy, and macular degeneration. A recently developed model in the macaque uses a laser to induce retinal neovascularization (a response by the choroid to the laser lesion) to mimic neovascularization associated with macular degeneration. This model could be used to evaluate potential drugs targeted toward preventing or treating neovascularization (Criswell et al. 2004; Shen et al. 2004).

Immunotoxicology

The drug development pipeline contains an increasing number of compounds with expected pharmacologic immunomodulation, and a few with unintentional immune suppression or activation. Whereas immunotoxicology testing in rodents has been an established process for several decades, comparable methodologies for use in primates have been slow to develop. Some human-targeted small molecule drugs are directed at antigens with cross-reactivity or comparable activity in primates only. Appropriate tools necessary for the identification and characterization of primate immune system effects have thus become paramount to the success of some drug programs.

Traditional primate toxicology evaluations include basic procedures for measuring immunomodulation consisting of physical examination and clinical observations, clinical pathology (hematology), gross and histopathology, immune organ weights, and bone marrow analysis. These methods provide an overview of morphologic changes that might be associated with alterations of the immune system, but provide only general information about functional changes that might be present. Additional immunotoxicology test systems (morphologic or functional) modeled after those used in rodent toxicology are beginning to be more routinely employed in primate immunotoxicology assessments.

Morphologic Evaluation Methods

Cell surface and intracellular antigens expressed by subpopulations of structurally similar immune cells (e.g., lymphocytes) can be evaluated using techniques such as flow cytometry (FCM) and immunohistochemistry (IHC; Lappin and Black, 2003). Both FCM and IHC utilize antigen-binding markers, allowing subsequent identification and quantification of specific cell populations. FCM is routinely employed in primate toxicology to assess changes in peripheral blood immunocyte

subsets that would not be discernable by standard hematology, and can be used for similar quantification of immune tissue cells. FCM allows for rapid acquisition of large amounts of data from thousands of cells, providing statistically relevant information in a relatively short time interval. Additionally, FCM in primates can be repeated in individual animals over the course of the study to help define the onset, persistence, and recovery of effects on immune cell populations. Despite these benefits, FCM of immune tissues requires single cells in suspension for evaluation, so the structural integrity of tissues is generally lost in the preparation of tissue samples. IHC uses similar antigen identification techniques, but requires microscopic evaluation similar to that done with standard histopathology. Because IHC does not require dissociation of the tissue, morphology is preserved, allowing the identification of specific subpopulations and the ability to localize those populations to the tissue. FCM and IHC can be used to enhance the morphologic data derived from standard toxicological study; the additional information derived from these assessments might also provide insight into mechanisms of action of some immunomodulators. The measurement of functional alterations in primates using FCM or IHC has been done on a limited basis, and will likely become more commonplace in the near future.

Functional Evaluation Methods

Assays for functional alterations of the immune system in primates are also available on a limited but expanding basis. The complexity of immune function has prompted the development of more general assay methods looking at whole pathways rather than individual cell functions. Current primate immune functional assays focus on alterations in the adaptive (humoral and/or cellular) and innate components of the immune system, with immunotoxicity programs designed to follow current regulatory recommendations (Committee for Proprietary Medicinal Products 2000; U.S. Food and Drug Administration 2001).

Humoral Immune Response

The humoral arm of the immune system is responsible for specific antigen recognition, antigen processing and presentation, and subsequent antibody production (Goldsby et al. 2000). The T-lymphocyte-dependent antibody response (TDAR) is used to evaluate all components of the humoral system including antigen presenting cells, T-helper lymphocytes, and B-lymphocytes. The TDAR has been shown to be more consistent in primates than the comparable sheep red blood cell assay used traditionally in rodents. The humoral immune system is activated in the TDAR using novel antigens (Keyhole Limpet Hemocyanin [KLH] or Tetanus Toxoid [TT]) administered by intramuscular injection. Competence of the humoral immune system in the presence of an immunomodulator is evaluated by measurement of IgM and IgG antibody responses to the novel antigen.

Cellular Immune Response

The main component of the cellular immune system is T-lymphocyte recognition of small peptide antigens on other cells coupled to membrane molecules encoded by the major histocompatibility complex (MHC; Goldsby et al. 2000). General responses of the cellular immune system to peptide antigen exposure involve cellular activation with the release of soluble mediators (cytokines), inflammation, cytotoxic T-lymphocyte (CTL)-associated cell lysis and/or clonal expansion. Ex vivo evaluation of cell-mediated cytokine release can be done using ELISA-based assays to measure the release of proinflammatory mediators into serum following an appropriate stimulus. *In vivo* testing of the cellular immune response traditionally involves the delayed-type hypersensitivity (DTH) reaction. The DTH test requires the intradermal administration of an appropriate antigen (e.g., Candida, Trichophyton) followed by gross observation of the classic "wheal and flare" reaction or histologic evaluation of the inflammatory reaction at the injection site. T-lymphocyte

clonal expansion represents another method of evaluating function in the cellular arm of the immune system; this assay is typically performed *in vitro* using peripheral blood or tissue cells, and is quantified via changes in relative cell numbers (indicating proliferation) or incorporation of radiolabeled or nonradioactive nucleotides into the nucleus (indicative of DNA synthesis) after exposure of the cells to appropriate antigen stimuli. Similar to morphologically based assays (FCM and IHC), cytokine release, CTL-associated lysis, inflammatory response (DTH) or cell proliferation are measured in the presence of an immunomodulator to determine the impact, if any, on cellular immune function.

Innate Immune Response

Innate immunity represents all immediate and nonspecific reactions to foreign antigens or pathogens, with participation by a wide variety of cell types including neutrophils, macrophages, and natural killer (NK) cells (Goldsby et al. 2000). Neutrophil and macrophage response can be evaluated by function-specific tests in rodents, but most often involves histologic examination in primates. The standard for the assessment of innate immune function has traditionally been the NK activity assay; NK cells attach to and lyse cells expressing foreign or aberrant surface proteins (e.g., virus-infected cells, tumor cells), cells displaying certain antigen–antibody complexes (antibody-dependent cell-mediated cytotoxicity or ADCC), or cells that lack appropriate MHC molecules. For this assay, NK cells are collected from peripheral blood or spleen of animals treated with an immunomodulator *in vivo*, and subsequently tested *in vitro* for lytic activity against MHC-deficient cultured cells (e.g., K562 erythroleukemia cell line). Alterations in NK cell activity are the measured endpoints to determine effects of the immunomodulator on innate immune response. As with all cell-based measurements in primates, the NK cell activity assay is subject to considerable interanimal variability compared to similar testing in inbred rodents. In an effort to optimize this assay in primates, a number of test and quality control refinements have been instituted, including flow cytometry and fluorescence spectrophotometer-based test systems and efforts to more efficiently normalize sample preparation and analysis between animals.

Additional test modalities to evaluate the immune system of primates are more specific and typically involve functional tests of specific components of the immune system (specific cell types or pathways). Many of these assays are used to further refine the pathogenesis of changes identified by the screening tests described earlier; most are not available or widely used outside academic or discovery research laboratories.

PATHOLOGY

A number of texts (Benirschke 1983; Bennett et al. 1998; Dunn 1968; Griesemer 1976; Jones et al. 1993; Scott 1992; Wolfe-Coote 2005.) and journal articles (Kaup 2002; Lowenstine 2003) address the general subject of nonhuman primate pathology. It is, therefore, not the intent of this chapter to provide a comprehensive review of this information. Rather, the intent is to provide a toxicologist who has little or no knowledge of nonhuman primate pathology with enough information to acquaint him or her with the subject for those species most commonly used in toxicology. Also, the intent is to provide adequate references such that an individual can pursue additional reading as needed. Many of the references at the end of this chapter are themselves heavily referenced, and will provide additional information. A useful Web-based resource is the PrimateLit Database (http://primatelit.library.wisc.edu/) maintained by the University of Wisconsin. This searchable database contains much of the world literature on nonhuman primates.

In most instances, nonhuman primates used for toxicological studies are screened for specific disease agents before being assigned to a study. Screening is accomplished by the vendor before shipment, and by the laboratory animal veterinarian after arrival at the using institution. Screening

provides some assurance that the animals will be in good health when the study is started. Should clinical signs of disease be apparent, the laboratory animal veterinarian should be consulted and his or her recommendations followed. Routine screening does not identify or eliminate all pathogenic and latent microorganisms. If a specific study might be compromised by particular biologic agents, the toxicologist should communicate this to the veterinarian so that proper screening can be performed and appropriate animals selected. Depending on the agents to be excluded, specific pathogen-free macaques are available only in limited numbers and are expensive.

This section outlines only the most common diseases in four species: the rhesus (*Macaca mulatta*), the cynomolgus (*Macaca fascicularis*), the squirrel (*Saimiri sciureus*), and the common marmoset (*Callithrix jacchus*). Other species are, of course, used in research but are generally used for studies other than toxicology studies.

Nonneoplastic Spontaneous Diseases

Background Changes

The following are common background histologic findings in rhesus and cynomolgus macaques: mineralization in the brain (corpora amylacea); protein inclusions in transitional epithelium (cytokeratin) of the urinary tract; macrophages in intestinal villi; mineralization of adrenal corticomedullary zone, ovary, and renal papilla; multinucleated cells in renal pelvis; lymphoid infiltrates in salivary glands, brain, prostate, heart, kidney, and other tissues; herniation of intestinal glands into GALT; reticulo-endothelial hyperplasia in spleen; lymphoid hyperplasia of the spleen and lymph nodes; and involution of the thymus. The etiology is generally unknown and the clinical, pathological, and toxicological significance, if any, appear to be minimal.

Bacterial Diseases

Bacterial infections are common in nonhuman primates and can be a significant cause of clinical disease. Most prominent are the enteric organisms followed by the respiratory pathogens. Nonhuman primates can be screened for common bacterial pathogens prior to placing them on study. This process typically requires 6 weeks or longer and will be done by the laboratory animal veterinarian.

Shigellosis, or Bacillary Dysentery

Shigellosis is the most common bacterial disease of captive primates. The rhesus macaque is most susceptible, followed by the cynomolgus. Shigella only affects primates and *Shigella flexneri* and *S. sonnei* are the most common species isolated from nonhuman primates. The sources of infection for nonhuman primates are human carriers, infected food, sick animals, and asymptomatic carriers. Although asymptomatic carriers are common, clinical cases are most often observed when the animals are captured, shipped to a new facility, or otherwise stressed. Shigella is readily spread from active cases to other animals in the same enclosure.

The clinical manifestations are variable. Carriers might be asymptomatic or might have softer and more frequent stools than normal. In more severe cases, the animals sit bent forward with the head between the hunched hind legs, indicating abdominal pain. Progressive dehydration, weakness, and prostration can occur quite rapidly and emaciation might become quite pronounced. In the severe form of dysentery, the general state of health deteriorates rapidly, with anorexia and prostration. In this instance, the stools are usually liquid, containing mucus and frequently gross blood. The feces in shigellosis have a distinctive odor. Prolapse of the rectum is common. The body temperature is subnormal and untreated animals die in 2 to 3 days. Sometimes, the acute form passes into chronic colitis, manifested as a relapsing or chronic debilitating disease with

nonresponsive fluid diarrhea and severe weight loss. Shigella usually cannot be isolated from the feces of animals with chronic colitis. Chronic colitis is usually terminal.

The pathological changes of shigellosis are restricted to the large intestine. Shigella does not become septicemic. These features help differentiate Shigella infections from those caused by Salmonella and Campylobacter, the other common enteric pathogens of nonhuman primates. The serosal surface might be covered with petechial hemorrhages. Shigella characteristically causes purulent hemorrhagic necrotizing inflammation that appears grossly as ulcers, hemorrhage, and fibrinous pseudomembranes on the mucosa. The mucosa is often diffusely edematous. The changes can affect any part of the large intestine and can be focal, multifocal, or diffuse. The shigellae infecting nonhuman primates also can cause dysentery in humans, although instances of proven transmission from monkey to humans are rare. Although the clinical and pathologic features of shigellosis are characteristic, etiologic diagnosis depends on culture.

Campylobacteriosis

Campylobacter species are widespread in laboratory animals and are important not only from the disease seen in animals, but also from the zoonotic possibilities. *Campylobacter jejuni* has been recognized as a cause of diarrhea in humans. As with other enteric pathogens, fecal–oral spread is the prinicipal route of infection. Primates can acquire the infection in the wild, during holding for export, or during transport. The organism has been isolated from several species of primates, including the macaques, baboons, and marmosets. Clinically, signs vary from none to a severe diarrhea. Apparently, the incubation period is from 2 to 5 days. Persistent or intermittent watery diarrhea without blood or mucus is the usual symptom in macaques.

At necropsy, a mild to severe colitis might be seen. The small intestine is sometimes affected as well. Microscopic findings are variable, but usually include infiltration of the mucosa with mononuclear inflammatory cells and hyperplasia of the mucous membrane. The toxicologist must realize that stressful procedures could contribute to clinical campylobacteriosis, and that measures for personal protection should be used. An excellent review of campylobacteriosis has been published by Fox (1982). Etiologic diagnosis depends on culture and response to specific therapy. Campylobacter are frequently isolated from asymptomatic animals.

Salmonellosis

Salmonella species, once common, are now unusual causes of dysentery in nonhuman primates. The disease can occur as a mixed infection with Shigella or Campylobacter. The serotypes involved are variable, but belong chiefly to groups B, C, D, and E. In groups B and D, *S. typhimurium, S. stanley,* and *S. enteritidis* are the most pathogenic. The usual sources of infections are contaminated food and water, usually with rodent or bird feces, healthy carriers, and sick animals. The occurrence of the disease is usually sporadic, but epidemics sometime occur.

Clinically, the disease has an acute beginning characterized by anorexia, prostration, diarrhea with mucus and blood, watery stools, and hypothermia in the more severe cases. At necropsy, the small intestine is frequently involved, in addition to the colon. This is in distinct contrast to shigellosis. Pyogranulomas can occur in the liver and other organs. Etiologic diagnosis depends on culture.

Bacterial Pneumonia

Pneumonia is a common disease of all nonhuman primates, although the incidence in macaques has been greatly reduced with the elimination of the respiratory mite, *Pneumonyssus simicola,* through the widespread use of Ivermectin. Pneumonia is often a complication of some other disease or stressful environmental condition. It can, however, occur as a primary infection, especially in

young monkeys up to 6 months of age. Organisms that can be involved are *Streptococcus pneumoniae, Staphylococcus, Haemophilus influenzae, Bordetella bronchiseptica, E. coli, Pasteurella,* and *Klebsiella pneumoniae.*

Clinically, coughing, sneezing, and rhinitis suggest pneumonia, although caged animals might show few overt clinical signs. An elevated temperature is characteristic. On x-ray the affected areas show increased opacity and the appearance of shadows. At necropsy, the affected portions of the lung lobes, typically the dependent or diaphragmatic lobes, are dark red or consolidated. Typically, the affected lobe will not collapse when the chest is opened. Histologically, an alveolar exudate is usually prevalent and consists predominantly of neutrophilic leukocytes mixed with erythrocytes, desquamated epithelial cells, and fibrin. Although the pathologic features of many bacterial infections are characteristic, definitive etiologic diagnosis depends on culture.

Tuberculosis

From a colony management and zoonotic standpoint, the most important bacterial pathogen in nonhuman primates is undoubtedly *Mycobacterium. M. tuberculosis* and *M. bovis* cause similar disease patterns. TB is primarily a human disease, and nonhuman primates become infected by being exposed to infected humans, often in the country of origin. Old World monkeys (rhesus, cynomolgus) are in general more susceptible to TB than New World monkeys (squirrel, marmoset). TB can rapidly spread throughout a colony of nonhuman primates and humans have become infected from infected monkeys.

As in humans, the primary site of infection, or primary focus, is usually in the respiratory or GI tract, depending on the route of exposure. The infection readily spreads throughout the body by hematogenous and lymphogenous routes. In nonhuman primates, the disease is usually pulmonary and can run a fulminating course. The disease is characterized by numerous firm or hard, light to gray or yellow tuberculous nodules (granulomas) in the lungs, lymph nodes, liver, spleen, and other organs. When sectioned, there is a characteristic caseous necrotic center that helps differentiate tubercles from abscesses, which have a purulent center. In contrast to humans, tubercles in monkeys rarely calcify. Microscopically, the characteristic lesion of TB is the tuberculoid granuloma, which consists of a necrotic center surrounded by macrophages that have undergone epithelioid cell change, multinucleated giant cells, and lymphocytes. Acid-fast stains are used to detect the characteristic acid-fast bacilli, although they might be quite difficult to find. Although the pathologic changes are often characteristic, definitive diagnosis depends on culture or PCR identification of the infectious agent. There are other nontuberculous mycobacterial infections of nonhuman primates caused by *M. avium/intracellularae,* other atypical Mycobacteria, and *M. leprae.* These infections are pathobiologically distinct from TB and should not be confused. A rigid testing program of all nonhuman primates and of all personnel in contact with them is necessary for adequate diagnosis and control of the disease.

A review of the mycobacterial infections of nonhuman primates has been published (Hines et al. 1995).

Viral Diseases

Viral diseases are important to the toxicologist from two points of view. First, the expression of primate viruses might interfere with a toxicity study and can, at the very least, make the data questionable or difficult to interpret. Second, they pose a threat to human health. Obviously, the viral status of the primates must be considered before the toxicologist begins his or her work.

Although there are approximately 75 nonhuman primate viruses that are distinctly simian (Kalter 1983), relatively little is known about them or their impact on their hosts. Indeed, the natural host of several simian viruses is unknown, and their pathogenesis is not understood (Kalter 1983). Two excellent discussions of the simian viruses, their hosts, and the diseases they cause are provided

by Kalter (1986). This section discusses only those that can be hazardous to the conduct or interpretation of a toxicity study or to the individuals conducting that study. In addition, a few viruses that pose a distinct threat to other primates are discussed.

Cercopithecine herpesvirus I (Herpes simiae, B-Virus). Cercopithecine herpesvirus I, otherwise known as *Herpes simiae,* herpes B virus, or simply B-virus, affects only macaques, including the rhesus and cynomolgus. The organism belongs to the herpesvirus group, which is one of the largest of the viral groups. *Herpes simiae* is a natural infection of macaques and is endemic in most captive colonies. It is the macaque homologue of Herpes simplex I infection of humans. As such, it usually causes only minor lesions that resemble fever blisters in macaques and, like many herpesviruses, becomes a lifelong latent infection in ganglia. However, infected monkeys might shed virus in saliva intermittently throughout their life, with or without lesions being present. *Herpes simiae* is spread primarily through bites or scratches. The incidence of infection in primates is age related, being highest in adults and lowest in juveniles, with a rapid increase at about the time of puberty. Like many herpesviruses that infect aberrant hosts, *Herpes simiae* is highly pathogenic for humans, wherein lies its main significance in toxicology.

The clinical manifestation of the disease in macaques is variable. The natural, primary infection closely resembles herpesvirus simplex infections in humans and is usually so minimal as to escape detection. Occasionally, the primary infection results in larger vesicles and ulcers on the oral mucous membranes and esophagus and systemic infection with interstitial pneumonia and necrosis in the liver, spleen, and other organs, typical of many systemic herpesvirus infections in other species. After the primary infection, most animals become infected for life, but seldom show any signs of infection. Macaques can shed virus whether or not they have lesions. Most infected macaques become seropositive and remain so for life. Diagnosis of active shedding can be made by viral culture of oral mucous membranes. Postmortem diagnosis can be made by viral culture of lesions or by culture or PCR evaluation of ganglia. It is important that the toxicologist understand that all personnel in contact with conventional macaques and their blood, fluids, and tissues are potentially at risk for exposure to B-virus. If humans become infected, they develop a severe, often fatal, central nervous system infection. No vaccine is available, although postexposure antiviral therapy has been effective in a few cases.

It would be ideal to use only *Herpes simiae*-free macaques in research. Unfortunately, these animals are only available in limited numbers and they are very expensive. Therefore, until the supply can be greatly increased, prevention of transmission to humans is the only recourse. The toxicologist should become thoroughly familiar with his or her institution's biosafety protocols. Additional information on Herpes B infections can be found in Cohen et al. (2002) ILAR, NRC of the National Academies of Science (2003), and Centers for Disease Control (1987b).

Simian Varicella Virus (Delta herpesvirus, Medical Lake Macaque Vrus, Liverpool Vervet Monkey Virus). This is a group of closely related herpesviruses that naturally infect some species of Old World monkeys, including macaques and African green monkeys, and are homologues of human varicella-zoster virus, the cause of chicken pox. They are transmitted via the respiratory tract and can cause a herpetic rash, depression, and respiratory difficulty. In advanced cases that are studied at necropsy, vesicles on skin, oral mucous membranes, and esophagus are commonly seen. Focal necrosis in the lungs, liver, spleen, lymph nodes, adrenal, bone marrow, and intestinal tract are common. Intranuclear inclusion bodies are present. Like many herpesviruses, simian varicella becomes latent in ganglia. Simian varicalla virus sometimes appears in colonies because latently infected monkeys become stressed or immunosuppressed and have clinical recurrences. When this occurs, they are infectious for other previously uninfected monkeys.

Additional details can be found in Bladely et al. (1973), Roberts et al. (1984), and Mahalingam et al. (1992).

Herpesvirus Tamarinus (Herpes T). *Herpesvirus tamarinus,* first isolated from South American marmosets, infects only New World monkeys and is the squirrel monkey homologue of *Herpes simplex* in humans and *Herpes simiae* in macaques. Squirrel monkeys are the natural reservoir host, show little disease, and become infected for life. Other susceptible monkey species include Saguinus species, Ateles species, and some Cebus species. Transmission of the disease is by contact. Transfer of the virus to susceptible aberrant hosts, such as marmosets or owl monkeys, causes a systemic fatal disease. Fortunately, humans do not appear to be susceptible to infection with *Herpes tamarinus*. Different species of monkeys should never be mixed or allowed to contact one another.

The lesions occurring in owl monkeys and marmosets have been described by Hunt and Melendez (1966) and are typical of systemic herpesvirus infections in many species. The clinical course in marmosets and owl monkeys is rapid, with death occurring in 4 to 5 days. No specific clinical signs are associated with the disease, and there is no treatment.

Hepatitis. Of the common forms of human viral hepatitis, Hepatitis A and B are of most concern in nonhuman primates used in toxicology. Regarding Hepatitis A virus (HAV), one must distinguish between human HAV and the closely related but genetically distinct viruses indigenous in cynomolgus monkeys, African green monkeys, and no doubt other Asian and African species. Owl monkeys and chimpanzees are susceptible to human HAV and have been used as models for its study. Of more concern to toxicologists is the natural HAV that commonly infects macaques, particularly cynomolgus monkeys. The spontaneous disease in rhesus or cynomolgus is not clinically evident; the animals do not stop eating or show any other signs. They do, however, show transient increases in alanine aminotransferase and aspartate aminotransferase levels in the serum. Elevations in these enzymes can cause problems when they occur during a toxicity study (Lankas and Jensen 1987; Slighter et al. 1988). Toxicologists should be careful in ascribing test article toxicity when there is an increased hepatic enzyme activity associated with periportal inflammation due to HAV. The animals seroconvert to a positive antibody status and have transitory immunoglobulin M (IgM) levels during the convalescent period. There is no obvious way of differentiating this disease-induced change from toxic effects.

The pathological lesions in the liver are minimal. There is generalized activation of sinusoidal lining cells, focal hepatocellular necrosis with occasional acidophilic bodies, and cuffs of mononuclear cells in portal areas. The hepatocellular necrosis is most often minimal, although severe cell necrosis can occasionally be found. Proliferation of bile ductules can also be found when portal inflammation is maximal. During recovery, pigment deposits can be seen in Kupffer cells and they persist for some time. Chronic hepatitis never develops. The virus is shed in the feces for an indeterminate period.

The zoonotic potential of the HAVs indigenous to macaques is unclear. Although human HAV has been transferred between humans and infant chimpanzees, there is no clear evidence of transmission of nonhuman primate HAV to humans or of human HAV to macaques. A vaccine for human HAV is available and some have recommended that primate handlers be vaccinated. However, it is unknown whether humans are susceptible to nonhuman primate HAV and whether the vaccine would be protective if they are susceptible.

Genetically distinct indigenous Hepatitis B viruses (HBVs) occur in humans, chimpanzees, gibbons, orangutans, gorillas, and woolly monkeys. Of these species, only chimpanzees are occasionally used in toxicology studies. In addition to their indigenous viruses, chimpanzees are susceptible to human HBV and have been widely used as a model for hepatitis B. Because of this, most chimpanzees currently in captivity have been inoculated with human HBV and might be chronically infected. They therefore pose a potential biohazard for HBV. HBV is spread parenterally, so the precautions in place at most primate facilities should be adequate for protection. A vaccine for HBV is available and is appropriate for those in contact with infected chimpanzees, their blood, or their tissues.

The primate hepatitis viruses have been reviewed by Robertson (2001).

Rhesus Lymphocryptovirus, HVMF1. Nonhuman primate Epstein-Barr-virus-related gammaherpesviruses, including rhesus lymphocrytptovirus in rhesus monkeys and HVMF1 in cynomolgus monkeys, are common natural infections in many species. Most infections are latent and do not produce any readily apparent disease. In immunodeficient animals, lymphocryptoviruses have been associated with lymphoma and with squamous epithelial proliferative lesions on the skin and mucous membranes. The neoplasms are extranodal systemic B-cell lymphomas that resemble immunodeficiency-related (AIDS-associated and transplantation-associated) lymphomas in humans.

Additonal information can be found in Fujimoto and Honjo (1991), Baskin et al. (2001), and Schmidtko et al. (2002).

Callitrichid Hepatitis. This disease of Callitrichidae, the marmosets and tamarins, is caused by a rodent virus, lymphocytic choriomeningitis virus (LCM). LCM virus is endemic in mice worldwide. Callitrichids become infected by the common practice of feeding them neonatal mice or through contact with mouse urine or oral secretions. Infected monkeys might be found dead or might die after showing weakness and anorexia for several days. They can develop siezures and respiratory distress. At necropsy, jaundice, subcutaneous and intramuscular hemorrhage, hepatosplenomegaly, and pleuropericardial effusions are seen grossly. Microscopic changes consist of hepatocellular swelling and necrosis, lymphocytic and neutrophilic infiltrates, acidophilic bodies, and portal phlebitis. Other possible lesions include meningitis, encephalitis, gliosis, necrosis in the spleen and lymph nodes, and interstitial pneumonia. No inclusion bodies are present. The disease is preventable by only feeding mice from sources known to be free of LCM virus (see Montali et al. 1995).

Simian Retroviruses

Simian Retrovirus (SRV, D-virus, Simian Retrovirus Type-D). All type D retroviruses identified to date are of primate origin and are natural infections of animals in the wild. At least five serotypes infect macaques (SRV-1–5). SRV-1 is more common in rhesus, whereas SRV-2 is more common in cynomolgus and pigtailed macaques. Endogenous type D viruses occur in squirrel monkeys and langurs and related proviral sequences have been identified in African and Asian colobines. The endogenous viruses appear to be nonpathogenic. The exogenous viruses infect many species of macaques, naturally occurring infection in the wild being demonstrated in *M. fascicularis* (Indonesia, but not those from the Philippines or Seychelles Islands), *M. nemestrina* (Indonesia), *M. radiata* (India), *M. tonkeana* (Sulawesi), and *M. mulatta* (China). The incidence in captive colonies varies from colony to colony, but can be quite high. Virus can be isolated from peripheral blood mononuclear cells by coculture on Raji cells.

Type D viruses infect B-cells, T-cells (CD4+ and CD8+), macrophages, epithelial cells (salivary gland, intestine, oral Langerhans cells), and choroid plexus. The virus is shed in saliva and transmission requires direct physical contact or contact with fomites. Biting, licking, and grooming are probably the usual modes of transmission, although vertical transmission also occurs. Some monkeys (probably infected near birth) become persistently infected but antibody negative, serving as healthy carriers. Because of this, animals must be screened repeatedly by ELISA and PCR (or culture) to ensure they are virus-free. Experimental formalin-killed whole virus and recombinant vaccines have been used successfully. Strict husbandry procedures to prevent spread by fomites is essential to any eradication program.

Type D viruses induce an immunosuppressive disease in macaques that might be epizootic in previously naive populations or might be enzootic. Exposed animals can develop an antibody response that clears the infection (although virus negative, antibody-positive animals might still harbor virus in bone marrow or gut), or become intermittently virus positive with or without antibodies. They might develop an acute or protracted immunodeficiency disorder with or without fibroproliferative lesions. Retroperitoneal fibromatosis and subcutaneous fibrosarcomas have been

associated with SRV-2. Neutropenia, anemia, and terminal lymphopenia are common. Some animals develop persistent generalized lymphadenopathy. Some eventually develop diarrhea, weight loss, bacterial infections, or opportunistic infections (CMV, cryptosporidia, Candida, Noma). A few B-cell lymphomas have occurred in cynomolgus monkeys, but not in rhesus. The clinical outcome in an infected individual is related to the antibody response. Monkeys that die early in the course of infection have no antibody and high levels of circulating viral antigen. Monkeys that survive with persistent viremia make intermediate levels of antibody and have intermediate levels of viral antigen. Monkeys that clear the infection have high levels of antibody and no antigen. Some animals apparently recover from infection. Neutralizing antibody is thus important in protection against SRV. Lesions that appear to be caused directly by type D viruses include lymphoid hyperplasia, which evolves into atrophy, nonsuppurative enteritis, sialoadenitis, and myositis.

Type D virus has very rarely been detected in humans, but the significance of this is unclear. Type D virus infection of humans is very rare to nonexistent.

SRV has compromised many research studies by causing anemia, altered immune responses, altered expression of cell surface markers, altered cytokine profiles, and nonspecific histological changes. In cynos (but not rhesus), the extensive lymphoid hyperplasia induced by SRV can progress to lymphoma. In toxicologic studies that involve critical measures of immune function or that require that animals be maintained for an extended period of time, it is essential that SRV-free monkeys be used. Unfortunately, it is very difficult to ensure that monkeys are SRV-free. Infected animals can be antibody positive or negative and viremic or not viremic at any particular time point, and their status can change frequently. Therefore, many institutions require at least three negative tests, including tests for antibody and virus (culture or PCR), over a period of several months. Even this regimen has proven to be insufficient in many instances. The best solution is to use monkeys from colonies that are known to be SRV-free. These animals are only available in limited numbers and are expensive, however.

The Type D retroviruses have been reviewed by Lerche and Osborn (2003) and Guzman et al. (1999).

Simian T-Cell Leukemia Virus Type I (STLV-I). Simian T-cell leukemia virus (STLV-I) is closely related (90%–95% homologous) to the human T-cell leukemia virus type 1 (HTLV-I), the etiologic agent of adult T-cell leukemia/lymphoma, tropical spastic paraparesis, and HTLV-associated myelopathy. There is a high incidence of natural infection in many wild and captive Old World monkeys, including baboons, African green monkeys, Patas monkeys, various macaques, and chimpanzees. The incidence of infection correlates with age, reaching a peak in animals over 16 years old, and is higher in females than males. Transmission occurs by sexual contact or parenteral inoculation. Neonatal transmission is probably unusual. Persistent infection without seroconversion has not been observed. STLV-I typically infects CD4+ T-cells in macaques and CD8+ T-cells in African monkeys, but some infected T-cell lines express neither marker. Although most infected animals remain latently infected and asymptomatic for life, STLV-I has been associated with lymphoma and leukemia in baboons and African green monkeys. Most investigators believe STLV-I is not pathogenic in Asian monkeys, including macaques. There is some evidence that STLV-I might alter macaque cell surface markers and cytokine profiles, but any effects STLV-I might have on the macaque immune system have not been well defined. For routine toxicologic studies in macaques, the STLV-I status of animals is probably not very important. However, in studies that require detailed measurements of immune parameters, the use of STLV-I-free macaques should be considered.

Simian Immunodeficiency Virus (SIV). Simian immunodeficiency viruses belong to the lentivirus subgroup of retroviruses and are common natural infections of many species of African nonhuman primates. Wild populations of Asian and South American monkeys are not infected with these viruses. SIV is of particular interest because it is the origin of the human immunodeficiency virus, the cause of human AIDS. SIVs are well adapted to their natural hosts, and do not cause

disease in African monkeys. However, if some isolates of SIV are inoculated into Asian macaques, they develop a disease very similar to human AIDS. This system is widely used as a model for human AIDS. Unless intentionally introduced, SIV is very unlikely to be present in captive populations of cynomolgus or rhesus monkeys, and should therefore be of little concern to the toxicologist.

Additional information may be found in Gardner (2003), Apetrei et al. (2004), and Apetrei and Marx (2004).

Other Viruses. There are numerous other viruses that infect various species of nonhuman primates. A comprehensive listing and discussion of these is beyond the scope of this publication. Modern production facilities, quarantine procedures, and institutional practices should prevent these from impacting toxicologic studies in most cases. One should always remain mindful of the possibility of viral infections, however. The toxicologist should consult a laboratory animal veterinarian or veterinary pathologist with knowledge and experience in primate diseases for more detailed information.

Parasitism

Parasitism is one of the most common infectious problems in nonhuman primate colonies. The incidence of parasitism in animals that are wild caught is much higher than those raised in captivity. However, even animals derived from well-managed, purpose-bred colonies are commonly infected with protozoan and metazoan parasites, although clinical disease due to parasites is uncommon. Many of the background histologic changes commonly encountered in monkeys used in toxicology studies are likely due to parasitism. These include mononuclear and eosinophilic cell infiltrates in some tissues, lymphoid hyperplasia, and granulomas. If one considers metazoan (nematodes, cestodes, and trematodes) and protozoan parasites, along with the species they infect, the number of parasites to consider becomes too large for an adequate treatment in this chapter. Therefore, only the most common infections are discussed. An excellent review of the pathoparasitology of nonhuman primates has been published (Toft 1986). The chapter provides numerous references for those who wish to learn about a specific parasite or group of parasites.

Strongyloidiasis

Parasites of the genus *Strongyloides* are found in both Old World and New World primates. *Strongyloides cebus* has been found in squirrel monkeys and *S. fulleborni* in rhesus and cynomolgus. Only adult female parasites and larvae are found in the intestinal tract. Migrating larvae are, however, found in other organs. The life cycle is complex, consisting of free-living and parasitic forms.

Diarrhea is the most common clinical manifestation of the disease, coupled with dehydration, listlessness, vomiting, and emaciation. Gross lesions are a catarrhal to hemorrhagic enterocolitis. Pulmonary hemorrhage might be seen as a result of migrating larvae. Microscopically, the small intestinal mucosa contains numerous parasites and hemorrhage. In the lungs, hemorrhage is common. This disease is diagnosed by identification of typical larvae in the stool or by demonstration of adult females, eggs, and larvae microscopically.

Oesophagostomiasis

Parasites of the genus *Oesophagostomum* are common nematodes in Old World monkeys. The adult parasites are located in the large intestine and the life cycle is direct. Adult *Oesophagostomum* cause little damage, the lesions being due to the larvae. Continued reinfection results in the sensitization of the host to the parasite. Infected monkeys are usually asymptomatic and the infection goes unrecognized during life. Monkeys with severe infection might show a general unthriftiness and a general debilitation.

Lesions at necropsy consist of smooth brown or black nodules, 2 mm to 4 mm in diameter, which are most frequently seen on the serosal surface of the large intestine and cecum. Older nodules usually are white due to mineralization of the contents. Microscopically, the nodules contain larvae surrounded by brown debris, inflammatory cells, and a fibrous capsule. These are frequently seen incidental findings in monkeys used for toxicology studies.

Pulmonary Nematodiasis

Metastrongylids in the genera *Filaroides* and *Filariopsis* are commonly seen in New World primates, especially in squirrel monkeys. The infection is rare in animals raised in captivity, as the life cycle of the parasite involves the earthworm. The adults are found in the terminal bronchioles, respiratory bronchioles, and pulmonary alveoli, and gross lesions are subtle. When present in fairly large numbers, the pleural surface of the lung has numerous, random, small, elevated, subpleural nodules. Microscopically, there is atelectasis and foci of chronic inflammatory cells.

Filariasis

Filariasis is caused by a wide variety of nematodes that are commonly encountered in New World primates, especially squirrel monkeys. At least 12 different species have been described (Mathiesen et al. 1978) and include four species of *Dipetalonema* and seven species of *Tetrapetalonema*. These parasites live in the abdominal or thoracic cavities, or in the subcutaneous tissues of the host. They also can be found in the mesentery, along the pleural lining of the lung, and inside the pericardium. Typically, parasites in the serous cavities cause a slight peritonitis or pleuritis with fibrinous or fibrous adhesions. In most instances, the infection is subclinical, and diagnosis is based on the demonstration and identification of the adult worms in the body cavities or subcutaneous tissues, or by the characteristic microfilaria in the blood.

Gastrodiscoides hominis

Gastrodiscoides hominis is a fluke that affects *Macaca spp.* and humans. Snails are the intermediate host. Macaques become infected by ingesting metacercariae encysted on vegetation. Gastrodiscoides is usually asymptomatic, although large numbers can cause mucoid diarrhea. Adults are often found in the lumen of the cecum and colon of macaques used in toxicology studies.

Pulmonary Acariasis

Lung mites, especially *Pneumonyssus simicola,* were once an extremely common condition of wild-caught macaques, especially rhesus. With the advent of Ivermectin, lung mites have nearly disappeared from purpose-bred colonies, although older monkeys frequently have residual lesions in their lungs. The infection is usually asymptomatic. Gross lesions consist of variably sized clear, yellow, or brown air-filled cysts randomly located throughout the pulmonary parenchyma. Those located near the surface of the lungs elevate the pleura. Microscopically, the lung mite lesions, or mite houses, are characterized by a dilated airway surrounded by macrophages, giant cells, and eosinophils. Macrophages laden with a golden brown pigment are always present around the lesions.

Balantidium coli

Balantidium coli has been found in Old World monkeys, New World monkeys, and apes. Many animals have *B. coli* in the colon without showing any clinical signs. Balantidium is sometimes associated with diarrhea. Organisms are often found in the lumen of the colon of normal animals and in ulcerative lesions, mucosa, capillaries, lymphatics, and mesenteric lymph nodes of animals

with colitis. It can be a primary pathogen in great apes and pigtailed macaques, but is usually associated with some other pathogen in other species.

Other Diseases

Of course, like all animals, nonhuman primates are subject to a plethora of infectious and noninfectious diseases. These should seldom be of concern to the toxicologist working in a modern, well-managed facility with an animal care program accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and supervised by an experienced primate veterinarian who is a diplomate of the American College of Laboratory Animal Medicine.

Neoplasia

Neoplasia in captive primates is uncommon, compared to other animal species used in toxicology. Neoplasms will very rarely be seen in the young monkeys usually used in toxicology studies. In macaques, uterine leiomyomas (fibroids) and intestinal carcinomas occur with some frequency in older animals. There is a high incidence of B-cell lymphomas in rhesus and cynomolgus macaques that have been experimentally infected with SIV and in those severely immunosuppressed for transplantation studies. There are a few reports of B-cell lymphomas in cynomolgus monkeys infected with simian retrovirus type D. B-cell lymphomas in macaques are associated with concurrent lymphocryptovirus infection. In New World monkeys, colonic adenocarcinomas in the cotton-topped tamirin or marmoset (*Sanguinus oedipus*) are the most frequent and are associated with a chronic colitis syndrome.

The only easily reproducible tumor model in nonhuman primates is the Herpesvirus saimiri/owl monkey lymphoma system. Although nonhuman primates are susceptible to many carcinogens, the induction of neoplasms requires too much time and is too unpredictable to be generally useful as a model. Therefore, nonhuman primates are rarely used in carcinogenicity studies.

Reviews of neoplasms in nonhuman primates may be found in Squire et al. (1979), Lowenstine (1986), Schoeffner and Thorgeirsson (2000), Schmidtko et al. (2002), Paramastri et al. (2002), and Habis et al. (1999).

METABOLISM

Monkeys have been widely used in biomedical research for many years, and their xenobiotic metabolism has also been extensively studied. One could, for example, sacrifice a large number of rats for the sole purpose of isolating cytochrome P-450, but few investigators have been willing to do likewise for the monkey. In addition, monkeys represent a phylogenetic class of animals rather than an individual species. Comparing drug metabolism in rhesus and cynomolgus monkeys is like comparing rats and guinea pigs. There are considerable differences between Old World and New World monkeys (Litterst et al. 1976; Williams 1971, 1974) in terms of specific activity levels, although not necessarily in terms of specific CYP450 isoenzyme activity. One has to be careful of broad generalizations based on study of any one family. This section concentrates on the more common monkeys used in toxicological research. Where such information exists, differences between members of different phylogenetic families are highlighted.

Best characterized in terms of primate CYP450 isoenzyme activity are the cytochromes. Table 9.8 presents the known activities in this species.

Some key parameters of hepatic xenobiotic metabolism in the monkey are highlighted in table 9.9. Concentrations of cytochrome P-450 run between 0.5 and 1.3 nmol/mg protein. There are differences between different species of monkeys. Macaques, such as the rhesus, tend to have the highest concentrations, whereas marmosets tend to have the lowest. Litterst et al. (1976) reported

Table 9.8 Identified Primate Cytochrome P-450 Isoenzyme

Isoenzyme	Isoenzyme	References
1A1	2C9, 2C10, 2C19, 2C20	Guengerich et al. (2003), Weaver et al. (1994), Chhabra et al. (1999), Edwards et al. (1994)
1A2	2D17, 2D19	Van Der Burght et al. (1998); Kastner ands Neubert (1994), Pearce et al. (1992), Ohmori et al. (1994)
2A6	2E1	Ohmori et al. (1998), Ohmori et al. (1993a), Amato et al. (1998), Schulz et al. (1998)
2B6	3A4	Igarashi et al. (1997), Bullock et al. (1995); Ohi et al. (1989)

Table 9.9 Summary of Xenobiotic Metabolizing in Monkeys

Enzyme	Concentration or Activity	Comments and References
Cytochrome P-450	0.5–1.3 nmol/mg-m	Amri et al. (1986), Lindstrom and Whitaker (1987), Lan et al. (1983), Challiner et al. (1981), Litterst et al. (1976), Muller-Eberhard et al. (1983). For Old World monkeys, highest in rhesus, lowest in marmoset.
Cytochrome b_5	0.31–0.32 nmol/mg-m	Roberts et al. (1977)
NADPH:Cytochrome P-450 reductase	80–220 nmol-min/mg-m	Wide range due to methodological and age-related differences. Maloney et al. (1986), Leakey et al. (1983), Litterst et al. (1976)
MMFO Activities		Large differences between squirrel and rhesus monkeys. Litterst et al. (1976), Leakey et al. (1986), Iverson et al. (1982), Challiner et al. (1980), Lan et al. (1983)
Aminopyrine	3.3–9.2 nmol/min/mg-m	
Analine	0.30–0.80 nmol/min/mg-m	
Ethoxycoumarin	0.60–0.90 nmol/min/mg-m	
Ethoxyresorufin	0.03–0.08 nmol/min/mg-m	
Epoxide hydrolase		Pacifici et al. (1981), Pacifici et al. (1983). Species differences, highest in baboon.
Styrene oxide	14–32 nmol/min/mg-m	
UDP-glucuronosyl transferase		Leakey et al. (1983), Litterst et al. (1976)
1-naphthol	16–20 nmol/min/mg-m	
4-nitrophenol	7–25 nmol/min/mg-m	
Glutathione S-transferase		High activity in rhesus monkeys; Summer and Greim (1981)
2,4-dinitro-1-chlorobenzene	2.5–7 μmol/min/mg-c	
1,2-dichloro-4-nitrobenzene ethacrynic acid	7–33 nmol/min/mg-c	
Protein estimates		Lan et al. (1983), Lindstrom and Whitaker (1987)
Microsomal cytosolic	18–36 mg/g liver	

Note: mg-m = mg microsomal protein; mg-c = mg cytosolic protein.

that the amounts of cytochrome P-450 and NADPH:cytochrome C reductase, and microsomal mixed function oxidase (MMFO) activities (with ethylmorphine, aminopyrine, aniline, biphenyl, or benzo[a]pyrene) were higher (roughly 2.5-fold) in rhesus than in squirrel monkeys. The molecular nature of cytochrome P-450 from monkeys has not been as well characterized as that in rats. Ohmori et al. (1984) isolated and purified cytochrome P-450 from (Japanese) crab-eating monkeys (macaques). They observed that the monkey MMFO has activity against several typical MMFO substrates, but antibodies against monkey cytochrome P-450 did not inhibit rat MMFO activity, suggesting that there might be little structural homology between the two species. Using SDS-PAGE techniques, Challiner et al. (1981) compared microsomal preparations of marmosets treated with different inducing agents. Their results suggest that there are at least four different cytochrome

P-450 isozymes in this species. This was confirmed by Kastner and Schultz (1987) using fast ion-exchange protein chromatography. Thus, cytochrome P-450 in monkeys exists, as in all other animal species examined, as a family of different isozymes with differing substrate specificity and sensitivity to different inducing agents.

Ohta et al. (1989) compared the activation of four representative different mutagens in the Ames test using microsomes from rats, dogs, monkeys, and humans. In general, monkey microsomes did not have the highest activity with any of the substrates, and most resembled those of the human with regard to both absolute activity and activity relative to the rat or dog. Interestingly, antibodies against rat cytochrome P-448 had the least inhibitory effect with monkey microsomes. This would suggest that despite the similarity between humans and monkeys with regard to activity, the molecular structure of their cytochrome P-450s might be quite different.

A few studies on direct interspecies comparisons of *in vitro* MMFO activity have been reported. (For a review on species-related differences in MMFO activity, see Kato 1979.) Dohi et al. (1973) reported that the cytochrome P-450 content and the nicotine-metabolizing capability were higher in rhesus monkeys than in dogs. Amri et al. (1986) compared the MMFO of eight different species, including cynomolgus monkeys, to that of humans. The monkeys had essentially the same amount of cytochrome P-450 (approx. 0.75 nmol/mg microsomal protein) as the guinea pig, and more than twice that of the human subjects (0.31 nmol/min). When the maximal velocities were examined with six different model substrates, the monkey consistently had higher activities than the human. In fact, the similarities between the rat and the rhesus monkey were greater than those between the rhesus and the human. In general, the rhesus monkey tends to have higher amounts of cytochrome P-450 than the rat. For example, Lan et al. (1983) noted that rhesus monkeys had cytochrome P-450 concentrations comparable to that of the rat on a protein basis (approx. 1 nmol/mg), but given that the monkey had higher microsomal protein concentrations, monkeys had the higher concentrations on a gram liver basis (18.8 nmol/g).

Comparatively little work has been published on the molecular nature of the other components (e.g., NADPH:cytochrome C reductase, cytochrome b_5) of the MMFO of monkeys. Given both the importance of these enzymes, as established by work done with rodents, and the assumed phylogenetic similarities between humans and monkeys, this lack of research is somewhat surprising. Schmucker and Wang (1986) isolated and purified the reductase from rhesus monkeys and found that it exists as a single protein with a molecular weight of approximately 77 kDa, which is similar to that of other species. There is immunological cross-reactivity between the NADPH:cytochrome C reductases of the pig and rhesus monkey. Using the techniques available at the time, Nobrega et al. (1969) compared the molecular properties of cytochrome b_5 from New World monkeys (*Aluoouatta fusca*), pigs, chickens, and humans. The properties of the enzyme isolated from the mammalian species were quite similar with regard to molecular weight (10,600–11,400), amino acid composition, trypsin digestion pattern, and spectrophotometric spectra. Perhaps the reason that there has been so little work on these enzymes in the primate is that the available data do not suggest that they are sufficiently different to warrant the use of primates further in additional studies.

Definite gender- and age-related (postmaturation or senescent) changes in the MMFO have been identified in rats. Relatively few papers have been published on the subject in monkeys. The data published by Litterst et al. (1976) did not identify any sex-related differences in the MMFO (either in enzyme components or activity), nor for that matter, in glutathione S-transferase, UDP-glucuronosyl transferase, or N-acetyl transferase. Sutter et al. (1985) studied the influence of age on the MMFO in female pigtail macaques (*Macaca nemstrina*) and identified no age-related (2.5–21.0 year) changes in cytochrome P-450 content, NADPH:cytochrome C reductase, or aryl hydrocarbon hydroxylase activity. Maloney and colleagues (1986) examined adult rhesus monkeys ranging in age from 1 to 25 years for age differences in the MMFO. They established that there were no age- or sex-related differences with regard to the amount of cytochrome P-450, MMFO activity with ethylmorphine, or the phospholipid content of endoplasmic reticulum. It would appear, therefore, that age- and sex-related changes in the MMFO are not marked in monkeys. A possible age-related

increase in cytochrome C reductase activity has been identified by Maloney et al. (1986) and confirmed in a follow-up paper (Schmucker and Wang 1986). There is an age-related increase in the specific activity of this enzyme in rhesus monkeys, which is not accompanied by changes in molecular weight or immunoprecipitability. This is in contrast to the situation in the rat (see chapter 3), where several investigators have shown that the specific activity of NADPH:cytochrome C reductase declines with age. The paradox of how there can be increases in the reductase activity that are not reflected in increases in MMFO activity remains to be resolved. Developmental (pre- and postnatal) changes of the MMFO in monkeys have been examined in a few publications, but not thoroughly explored. Dvorchik and colleagues (1976; Dvorchik et al. 1979) established that fetal stump-tail macaques had higher concentrations of cytochrome P-450 and MMFO activity than those of comparably aged rats, but still had less activity than the adult animals. This observation was confirmed by Leakey and colleagues (1986). They compared the MMFO of near-term fetal rhesus monkeys to both adult monkeys, and adult and near-term fetal rats. The concentration of cytochrome P-450 and activity of the MMFO of the fetal rhesus monkey is much higher than that of the fetal rat, both in absolute terms and relative to comparable adult levels. That is, in the monkey, the fetal cytochrome P-450 concentration was 17% of that of the adult, whereas in the rat the comparable figure was 8.5%. Differences of similar magnitude were seen with aminopyrine N-demethylase and ethoxycoumarin O-deethylase activities. Interestingly, neither fetal rats or monkeys had measurable activity with ethoxyresorufin deethylation, a specific substrate for cytochrome P-448-mediated activity. This observation would suggest that this particular isozyme develops well after birth in both species.

Treatment of the monkey mothers with dexamethasone induced an increase in the MMFO (enzyme components and activity toward most substrates) in fetal monkeys, but not in fetal rats. This work clearly indicates that although monkeys are born with an MMFO that is better developed than that of the rat, it is considerably less active than that of the adult monkey. The monkey probably more closely resembles the human in this regard than a rodent. The inducibility by dexamethasone might provide a useful tool for studying drug-related toxicities of the newborn.

The specificity of inhibitors of the MMFO in monkeys has not been as well characterized as in rats on other rodents. In fact, few papers, if any, discuss the use of the classic inhibitors of MMFO activity, such as metyrapone and SKF-525A, in monkeys. Anderson et al. (1982) reported that DPEA, 4,6-diphenyl-1,10-2-[(2,4-dichloro-6-phenyl)phenoxy]ethylamine hydrobromide, at 0.5 mM, effectively inhibited the *in vitro* microsomal metabolism of N-phenyl-2-naphtylamine in rhesus monkeys. Muller-Eberhard and colleagues (1983) reported that allylisopropylacetamide treatment (300 mg/kg/day subcutaneous for 4 days) will result in substantial decreases in cytochrome P-450 and MMFO activity in rhesus monkeys. Unfortunately, the same treatment also inhibits heme oxygenase and increases 8-aminolevulinic acid synthesis, and, therefore, is porphyrogenic. Although interesting, such findings are of limited value to the investigator who wishes to design an experiment involving acute inhibition of the MMFO.

Induction of the MMFO has been examined, but not as exhaustively as in other species. Challiner et al. (1980) reported that phenobarbital (20 mg/kg intraperitoneally for 3 days) caused the expected increases in cytochrome P-450, and the MMFO (*in vitro*) activities toward aminopyrine and ethoxycoumarin, but not ethoxyresorufin, in marmosets. *In vivo*, this induction presented as an increase in the urinary excretion of 6-0-hydroxycortisol. This latter finding suggests a noninvasive method for monitoring for induction in monkeys.

This same group (Challiner et al. 1981) also examined the inducing effect of 3-methylcholanthrene (25 mg/kg intraperitoneally for 3 days) in marmosets. They observed slight but significant increases in cytochrome P-450 and NADPH:cytochrome C reductase, no change in microsomal aminopyrine N-demethylase, a 2.2-fold increase in ethoxycoumarin O-deethylation, and a tenfold increase in ethoxyresorufin O-deethylase. This is fairly typical of how other species respond to 3-methylcholanthrene. Unlike phenobarbital, induction with 3-methylcholanthrene is not associated with an increase in 6-β-hydroxylation of cortisol.

Iverson et al. (1982) reported that cynomolgus monkeys respond to chronic treatment with both Aroclor 1248 and 1254 (2 to 5 mg/kg po, 3 times per week for at least 10 weeks) with an approximately twofold increase in cytochrome P-450 with downward shifts in the absorption maximum. Depending on substrate, increases in activity ranged from 155% (with aminopyrine) to 630% (with ethoxyresorufin) of control. The latter finding is consistent with that seen in other species as ethoxyresorufin is considered to be specifically a substrate for cytochrome P-448-mediated activity.

Thorgeirsson et al. (1978) reported that treatment of male rhesus monkeys with 3-methylcholanthrene (a single 80 mg/kg dose 24 hr before sacrifice) caused a 57% increase in cytochrome P-450, with the expected downward shift in the maximum absorbence (450–448) and large increase (five- to eightfold) in benzo(a)pyrene hydroxylation activity.

Despite the fact that monkeys have been shown to respond to the classic inducing agents (phenobarbital, PCBs, etc.), there are some isolated reports in the literature that might suggest that the monkey is not as inducible as other animal species. Lindstrom and Whitaker (1987) compared the action of an aromatase inhibitor (LY 56110) in the rat, dog, and rhesus monkey. The chemical was a potent inhibitor of MMFO in the rat. Two weeks of treatment results in substantial MMFO induction in the rat, but not in the monkey.

Peroxisomal-inducing agents represent a class of chemicals that induce not only increases in MMFO activity, but also increases in peroxisomal number and associated enzyme activity, primarily in rodents. Hawkins et al. (1987) has reviewed species differences in responses to peroxisomal proliferators (typically antilipidemic agents related to clofibrate or plithalate-plasticizing agents such as di(2-ethylhexyl) plithalate, DHEP) and found that monkeys are typically far less sensitive to such agents than rodents.

For example, Rhodes et al. (1986) compared the effects of DHEP in rats and marmosets. Both species were given DHEP 2,000 mg/kg/day for 14 days. Rats responded with large significant increases in liver weight accompanied by large increases (sixfold) in peroxisomal number and associated enzyme activity (e.g., fourfold increase in pahnitoyl CoA oxidation) that were not seen in marmosets. It would thus appear that in terms of microsomal and peroxisomal proliferation, monkeys are better models for the human responses than are rodents.

Aromatic amines can have three different routes of metabolism in the monkey: aromatic hydroxylation catalyzed by the MMFO, N-hydroxylation catalyzed by the MMFO or the FMFO, and N-acetylation catalyzed by N-acetyltransferase. These pathways are not mutually exclusive. For example, Thorgerisson et al. (1978) reported that hepatic microsomes isolated from rhesus monkeys produce 3-, 5-, and 7-hydroxylated, as well as N-hydroxylated, metabolites from 2-acetylaminofluorene. Radomski and colleagues (1973) compared the metabolism of primary aromatic amines in dogs and monkeys. These chemicals cause bladder cancer in both species. Both species also produce N-hydroxy metabolites (e.g., N-hydroxy-2-naphthylamine from 2-naphthylamine), but excretion of acetylated N-hydroxy metabolites occured only in monkeys. Thus, the N-hydroxy aromatic metabolites are primary bladder carcinogens, and 2-naphthylamine is carcinogenic in both species despite the differences in metabolism. Perhaps such chemicals cause fewer bladder tumors in monkeys than dogs because of the presence of a competing pathway (acetylation). The FMFO has been reported to be active in N-hydroxylation in other species (Ziegler 1988), but the involvement of this enzyme in N-oxidation in the monkey remains to be fully characterized.

Primary amines can be acetylated (and then hydroxylated) by monkeys, but the available evidence suggests wide differences in activity with different substrates. Litterst et al. (1976) reported that *in vitro* cytosolic N-acetyltransferase activity of rhesus monkeys was about 4.9 nmol/min/mg protein with p-aminobenzoic acid, but only 0.01 nmol/min/mg with sulfadiazine. Old World monkeys tend to have higher rates of N-acetyltransferase activity than New World monkeys.

The metabolism of secondary aromatic amines differs from that of primary amines in that the former are not acetylated nor N-hydroxylated by monkeys. As reported by Anderson et al. (1982), who examined the metabolism of N-phenyl-2-naphthylamine (P2NA) by seven different species,

there is no evidence of N-hydroxylation in any species, including the monkey. All seven species formed the same two major metabolites *in vitro*, although the ratio of 6-hydroxy-P2NA to 4'-hydroxy-P2NA did vary. This ratio was 0.4 for rat, monkey and mouse, whereas it was 1.0 for the dog and human. This is another example of how the monkey is not always the best model for humans. Anderson et al. (1982) also reported evidence that the cytochrome P-450-dependent MMFO rather than the flavin-dependent FMFO is responsible for the metabolism of P2NA. This is one of the few papers to address FMFO in monkeys.

Epoxide hydrolase has been studied, but not extensively characterized in monkeys. In general, monkeys have higher epoxide hydrolase activity (against styrene oxide) than rodents and this activity more closely resemble that of humans. Activity ranges from 14.8 ± 2.3 nmol/min/mg protein reported for rhesus (Pacifici et al. 1983) to 31.3 ± 1.7 for the baboon (Pacifici et al. 1981). Epoxides are substrates for both epoxide hydrolase and glutathione S-transferase. Although broad substrate specificity studies have not been done to confirm this point, the work of Pacifici and colleagues suggests that the preferred route of metabolism of epoxides in primates is through epoxide hydrolase, not glutathione conjugation. In comparing primates to rodents in *in vivo* disposition of a specific xenobiotic, one should not be surprised to find a greater percentage of diol metabolites in monkeys (assuming equivalent MMFO aromatic oxidation activity).

The important conjugative enzyme, glutathione S-transferase, has received some, but hardly exhaustive, scrutiny in the monkey. Litterst et al. (1976) compared glutathione activity S-transferase with 1,2-dichloro-4-nitrobenzene in rhesus and squirrel monkeys. They found that activity ranged from approximately 15 to 25 nmol/min/mg cytosolic protein with no species-related differences. Asaoka and colleagues (1977a, 1977b) characterized glutathione S-transferase of the Japanese crab-eating monkey and the rhesus monkey. Using various chromatographic techniques, they tentatively identified five isozymes, with various activities against six typical substrates. Thus, as in other species, glutathione exists as a family of isozymes in other monkeys. The pattern present in rat appears to more closely resemble that of the human as opposed to that of the monkey (Asaoka et al. 1977b). Unlike the situation in rats where there are definite isozymic substrate specificities, one glutathione S-transferase isozyme (iv) in the monkey tended to have the highest activity with all substrates examined (Asoaka et al. 1977b).

This enzyme was further examined by Asaoka et al. (1977a). It has a molecular weight of 48,000, is composed of two identical subunits (which is consistent with the isozymic structure of GSH-T of other animals), and is competitively inhibited by hexachlorobenzene. Summer and Greim (1981) compared the activity of GSH-T from the livers of the rat, rhesus monkey, chimpanzee, and human. The model substrates were 1-chloro2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DNCNB). With CDNB, rhesus had the highest activity (about 6.5 μmol/min/mg cytosolic protein) followed by the chimpanzee (3.2), the rat (1.8), and then human (1.6). With DCNB, the rat had the highest activity (about 51 nmol/min/mg), followed by the rhesus monkey (2.9), chimpanzee (9.2), and then human (4.6). This pattern suggests that (a) compared to the rat, primates have comparable or greater activity of glutathione S-transferase, depending on substrate, and (b) rhesus monkeys tend to have greater activity than humans.

Other conjugative (phase 2) reactions have probably been somewhat better characterized in monkeys than (phase 1) MMFO-catalyzed reactions with regard to similarities to humans. As reviewed by Caldwell (1981), there are some conjugative reactions that have been shown to occur only in humans or monkeys. These include the N1-glucuronidation of methoxysulfonamides, the glucuronidation of cyproheptadine to yield a quarternary glucuronide, and the conjugation of arylacetic acids with glutamine. Williams (1974) extensively reviewed this last reaction. With phenylacetic acid, for example, humans will excrete almost 95% as the glutamate conjugate. Similar results were obtained with monkeys, where, depending on species, up to 90% is excreted as the glutamate conjugate. Interestingly, this is one metabolic reaction where there are not large differences between Old and New World monkeys. Nonprimates such as the rat will produce 80% to 100% phenylacetate glycine conjugate and no detectable glutamate conjugates.

Dulik and Fenselau (1987) have examined some of the aforementioned primate-specific UDP-glucuronosyl transferase reactions *in vitro* using immobilized enzyme preparations. Interestingly, although they confirmed *in vitro* that primates have a much higher activity in the glucuronidation of sulfadimethoxine, the human was still found to have roughly three times the activity of the rhesus monkey. Additionally, the activity toward both p-nitrophenol and cyproheptadine was more similar between the monkey and rabbit than between the monkey and the human.

Others have studied UDP-glucuronosyl transferase activity in the rhesus monkey. Litterst et al. (1976) compared *in vitro* microsomal UDP-glucuronosyl transferase activity in the rhesus monkey to that of the squirrel monkey. With 4-nitrophenol as the substrate, activity in both species were comparable (approximately 6–14 nmol/min/mg), whereas the squirrel monkey had considerably higher activity with 2-aminophenol (approximately 0.5 vs 2.9 nmol/min/mg). When compared to the rat, the rhesus monkey had higher activity with p-nitrophenol and comparable activity with o-aminophenol.

Pacifici et al. (1986) established that the rhesus monkey has a relatively high transferase activity toward morphine. Leakey et al. (1983) established that the monkey has activity against a wide variety of (type 1) substrates, including 4-nitrophenol, and 1-naphthol. Activities in adult females were compared to those of the late-term fetus. With natural (type 2) substrates (e.g., steroid hormones or bilirubin), enzyme activities of the fetus were less than 5% than those of the adult, whereas with synthetic substrates (e.g., 4-nitrophenol) activities ran 40% to 120% of those of the adult.

These results clearly suggest that UDP-glucuronosyl transferase exists as a family of isozymes with differential development in the rhesus monkey. Also, unlike the rat, intrauterine exposure of rhesus monkeys to dexamethasone (10 mg/kg subcutaneous given to the pregnant animals 1, 2, and 3 days prior to near-term cesarean section) greatly enhanced the fetal hepatic UDP-glucuronosyl transferase activity toward the type 2 substrates. For example, activity against bilirubin was increased from 3 to 74 nmol/mg/min. Hence, despite the evidence that UDP-glucuronosyl transferase exists as a family of isozymes in both rodents and primates, the genetic and developmental controls are different. As mentioned elsewhere (chapter 9), there is stereospecificity in the activity of primate UDP-glucuronosyl transferase. For example, when the activities were examined using either (R) or (S) loraepam as substrates, the rabbit had no stereospecificity, the monkey exclusively glucuronidated the (R) isomer, and human preparation preferred the (R) to the (S) isomer by approximately 4:1 (Dulik and Fenselau 1987).

Extrahepatic metabolism has not been extensively or systematically examined in monkeys. There are some isolated reports in the literature. For example, Pacifici et al. (1986) demonstrated that the UDP-glucuronosyl transferase present in the gut plays a major role in the metabolism of morphine in the rhesus monkey. They also discussed extrahepatic styrene oxide metabolism in primates (Pacifici et al. 1981; Pacifici et al. 1983). Thorgeirsson et al. (1978) reported the presence of aromatic hydrocarbon hydroxylase in monkey lungs. However, the current state of the literature does not permit any integrative generalization. Therefore, the subject is not discussed further.

Two processes involved in disposition are mentioned here, even though they are not metabolic processes: plasma protein binding and biliary excretion (see also chapter 1). Although they are not metabolic processes, they can play a major role in disposition of a chemical. A tightly bound drug, for example, will not be as rapidly metabolized. There can be wide species differences in plasma protein binding, as reviewed by Cayen (1987). The rhesus monkey is probably the best model for humans. In general, plasma protein binding is greatest in the human and lowest in the mouse. For example, clofibrate is 97% and 95% protein bound in human and rhesus monkey plasma, respectively, whereas it is only 35% bound in the mouse.

The gut flora often play a role in xenobiotic metabolism and toxicity, as reviewed by Rowland (1988). The gut flora of primates differs considerably from that of rodents, and thus might lead to apparent qualitative differences in metabolism. For example, only humans and Old World monkeys have been shown to aromatize quinic acid to benzoic acid. If monkeys are treated with neomycin to suppress gut flora, quinic acid is excreted unchanged (Williams 1974). It would appear that Old

World monkeys would be the better model for humans in studying the involvement of gut flora in xenobiotic metabolism. Gut flora are also important because the interplay between gut metabolism and biliary excretion that govern enterohepatic circulation. As reviewed by Levine (1978), most xenobiotics appear in the bile as metabolites, particularly glucuronides. As reviewed by Cayen (1987), monkeys and humans are relatively inefficient in the process of biliary excretion because the molecular weight cut-off for transportation is much lower in rodents than in primates (Levine 1978). As reviewed by Calabrese (1988), primates have much lower gut flora O-glucuronidase activity, so the conjugated chemicals that are excreted in bile are less likely to be hydrolyzed and reabsorbed in primates.

Two generalities can be drawn from this work: (a) enterohepatic circulation of a xenobiotic is more likely to occur in rodents than in primates, and (b) in primates, a greater percentage of metabolites will be excreted in the urine than in the feces; however, once a glucuronide metabolite is excreted into the primate gut, it is less likely to be reabsorbed than in the rat.

Isolated hepatocytes have been used extensively to study xenobiotic metabolism in smaller species. Extensive discussion of this *in vitro* methodology is outside the scope of this book, but it deserves a mention here because of the opportunity it affords to decrease the use of monkeys. Gee et al. (1984) have described a method of isolating hepatocytes from squirrel monkeys. Cell viability was comparable to that of other species. The *in vitro* metabolism of tolbutamide, as a model chemical, was studied and both the rates of metabolism and spectrum of metabolites determined. They concluded that isolated hepatocytes from the rat, rabbit, dog, and monkey formed the major metabolites reported to occur *in vivo* at relative rates similar to intact animals. Interestingly, they also concluded that the rabbit rather than the monkey was the better model for human metabolism of tolbutamide. The important point is that they were able to come to that conclusion using relatively few animals.

In a classic review, Caldwell (1981) concluded that the monkey was the best model for predicting metabolism in humans. His conclusion was based on the fact that there are fewer biochemical differences between the rhesus monkey and humans than for other species. This was confirmed by review of the literature for 32 different chemicals. Differences in routes of metabolism, however, might or might not be of practical importance. For example, Cayen et al. (1985) examined the pharmacokinetics of tolrestat in rats, dogs, and monkeys, and concluded that both the dog and the monkey were equally good models, despite large differences in metabolite profiles, for the pharmacokinetic behavior (AUCs and terminal half-lives) of this drug in humans. The dog excreted most of the drug (82%) unchanged in the urine, whereas the monkey excreted mostly conjugated tolrestat (49%) or conjugates of other metabolites (32%). In determining which species might be the best model for humans, one should examine both the routes of metabolism and *in vivo* pharmacokinetic behavior of the parent chemical. The monkey might not always be the best or only model for humans.

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CHAPTER 10

The Minipig

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CONTENTS

TOXICOLOGY

The use of pigs (*Sus scrufa*) in biomedical research is well established. In toxicology, although the use of pigs in the United States was historically limited to dermal studies, since the mid-1990s

they have become very popular for pharmaceutical studies in place of dogs and primates. They have been extensively used for surgical (Swindle et al. 1988) and physiological (primarily cardiovascular, renal, and digestive) research (Clausing et al. 1986; Khan 1984) for years.

Until relatively recently, their use in toxicity testing was uncommon except in the testing of veterinary or herd-management drugs intended for use in swine or in dermal toxicity and absorption studies. This reluctance to use the pig as a model has been based primarily on body mass, which leads to the need for significantly more test compound. Because of their well-accepted physiological (and newly identified metabolite) similarities to humans, minipigs are becoming increasingly attractive toxicological models (table 10.1). In fact, they are already more frequently used in nutritional toxicology studies (Clausing et al. 1986). Among the more common experimental animals, pigs are the only one whose use is on the increase (Khan 1984). Their expense (both in procurement and maintenance) and their relatively large size have mitigated against their use in more general toxicity testing, but the development of minipigs has resulted in a strain of more manageable size. In addition, the increase in expense in the use of dogs, as well as the perceived lay opposition to their uses, makes minipigs even more attractive as a nonrodent species for general toxicity studies. The dog is a far more common companion animal and many of the recent developments in animal care and use laws have made specific provisions about the care of dogs. Minipigs have been shown to be more sensitive to a wide variety of drugs and chemicals (e.g., carbaryl, methylmercury) than dogs (Khan 1984). The U.S. Food and Drug Administration (FDA) has kept its own breeding colony of minipigs since the early 1960s. In short, there are scientific, economic, and sociological reasons that make minipigs good toxicological models. The reader is referred to an excellent short review by Phillips and Tumbleson (1986) that puts the issue of minipigs in biomedical research into the context of modeling in general. Table 10.2 presents the advantages of the minipig.

Table 10.1 The Minipig in Toxicity Testing

Due to the many advantages, mini- and micropigs are real alternatives to the use of nonrodents (dogs, ferrets, and primates)
Minnesota minipig introduced in 1949
Body weights at age 2 years
Yucatan minipig 70–90 kg
Yucatan micropig 40–45 kg
Göttinger micropig 35–40 kg
Use in general toxicity testing and reproduction, teratological and behavioral toxicity (aspects of public acceptance as species for testing)

Table 10.2 Main Advantages of the Minipig

Similar to humans in
Cardiovascular anatomy and physiology
Ventricular performance
Electrophysiology
Coronary artery distribution
Human skin
Thickness and permeability
Pigmentation
Allergic reaction
Reaction to burning and distress
Gastrointestinal system and digestion
Renal system
Immune system (FDA: “better than rodents”)
P-450 total enzyme activity (especially CYP2E1, CYP3A4)

Several breeds of miniature swine have been developed. In the United States, these include the Yucatan micro- and minipigs, the Handford, the Sinclair, the Pitman-Moore, and the Hormel. The

Yucatan and the Sinclair tend to be the most commonly used. Panepinto and Phillips (1986) have discussed the characteristics, advantages, and disadvantages of the Yucatan minipig in some detail. In Europe, the Göttinger minipig is extensively used. At sexual maturity (4–6 months) the typical minipig weighs 20 to 40 kg, as compared to 102 kg for the more common pig and 8 to 15 kg for the dog. Micropigs weigh about 14 to 20 kg at sexual maturity. The minipig and the dog have comparable life spans; for example, Peggins et al. (1984) reported that the average life span for miniature swine is 15 to 17 years. The average beagle dog might have a life span of 8 to 12 years. Most of this discussion focuses on the purpose-bred minipigs, primarily the Yucatan and the Sinclair.

Husbandry

Housing

A general review of handling and husbandry have been described by Panepinto (1986) and Swindle et al. (1988). Young weanling pigs can be kept for short periods of time (up to 1 month) in standard dog cages with the floor modified with narrow mesh to account for the smaller foot of the pig. After that, however, their rapid growth generally makes such caging inappropriate. Larger stainless steel cages would be extremely expensive. Standard dog runs could have enough floor space to be converted for pigs, but smooth flooring does not provide appropriate footing for pigs and needs to be covered with wood chip bedding (Swindle et al. 1988). Although pigs are very social, they do not have to be group housed, as discussed by Barnett and Hensworth (1986); individually housed swine show little evidence of a chronic stress response. Insufficient space, on the other hand, can cause chronic stress in pigs. Hunsaker et al. (1984) have described an inexpensive caging system for miniature swine that is appropriate for toxicology studies. The flooring and walls are constructed of 0.50-cm welded wire coated with polyvinyl chloride polymer. As described, the unit has sufficient room for two pigs, separated by a partition. These units are relatively inexpensive and provide more than sufficient floor space (about 17 ft^2 per pig) to meet the recommendations for pigs.

Water and Feed

Like all animals, pigs should be permitted free access to potable water, preferably from a municipal water supply intended for human consumption. Drinking water intended for pigs does not have to be filtered or deionized. Various diets have been described. Because of their size (i.e., high maintenance charges and test article demand), pigs have seldom been used for chronic studies in which the possibility of waterborne environmental contaminants could influence a study.

For miniature swine, the consistent use of a certified chow from a major manufacturer is recommended (Swindle et al. 1988). Free access to feed is not recommended, as pigs will eat to excess. Available feed should be restricted to approximately 4% of body weight per day to prevent the animals from becoming obese.

Restraint and Dosing

In general, minipigs are docile, and easily socialized and trained. Barnett and Hensworth (1986) recommended a socialization regimen of 2 min of gentle interaction (e.g., stroking, etc.). Pigs, like most experimental animals, are rarely simply kept and fed, but have to be occasionally restrained so samples can be taken and other measurements made. Restraint methods designed for commercial swine should not be used for laboratory swine. Panepinto et al. (1983) have described a sling method that provides restraint with minimal stress. The most frequently mentioned dosing routes in the literature are dietary admixture, dermal (topical), gavage, and intravenous (IV) injections. Generally, minipigs are restrained in a sling while being dosed by an active route such as gavage. If the experiment requires the implantation of, for example, an indwelling catheter, minipigs can be anesthetized with ketamine (20 mg/kg intramuscular) as described by Swindle et al. (1988).

Clinical Laboratory

Clinical chemical and hematological parameters for minipigs have been studied. Ranges for some of the more commonly examined parameters from Yucatan minipigs are summarized in table 10.3 and table 10.4 (from Radin et al. 1986). Parsons and Wells (1986) have published similar data on the Yucatan minipig. Brechbuler et al. (1984) and Oldigs (1986) have published on the Göttinger minipig. Middleton and coworkers have published extensive lists (organized by age and sex) on the hematological parameters (Burks et al. 1977) and serum electrolytes (Hutcheson et al. 1979) for the Sinclair minipig. In general, the clinical laboratory pictures of the various strains are quite similar. No real differences between sexes have been identified, but age can be very much a factor. For example, serum creatinine can be 33% higher in 3-month-old as compared to 18-month-old Sinclair minipigs (based on data reported by Burks et al. 1977). As with other species, health status, feed composition, feeding regimen, fasting state, season, time of day, and so on can affect clinical laboratory results in the minipig. Toxicological experiments should not be run without concurrent controls.

Table 10.3 Minipig Clinical Chemistry Parameters in Different Strains

Parameter	Yucatan	Göttinger
Glucose (mmol/L)	3.75 ± 0.64	5.98 ± 1.01
Urea (mmol/L)	7.84 ± 2.64	3.19 ± 1.15
Creatinine (mmol/L)	155 ± 16	52.2 ± 11.1
Total protein (g/L)	74 ± 9	54.0 ± 4.6
Albumin (g/L)	50 ± 6	26.2 ± 6.0
Bilirubin total (µmol/L)	3.42 ± 1.37	—
Triglycerides (mg/L)	267 ± 134	565 ± 250
Total cholesterol (mmol/L)	1.85 ± 0.38	1.62 ± 0.38
γ-Glutamyl transpeptidase (U/L)	61.6 ± 11.2	—
Alanine aminotransferase (U/L)	72.5 ± 13.6	—
Aspartate aminotransferase	40.3 ± 5.9	—
Na^+ (mmol/L)	140.5 ± 4.2	142.3 ± 3.00
K^+ (mmol/L)	4.1 ± 0.3	3.94 ± 0.32
Cl^- (mmol/L)	103.1 ± 4.3	101.3 ± 3.6
Ca^{++} (mmol/L)	2.62 ± 0.18	2.58 ± 0.16
$PO_4^=$ (mmol/L)	2.41 ± 0.26	1.61 ± 0.30

Note: Data are *M* ± *SD*. From Parsons and Wells (1986), Brechbuler et al. (1984), Oldigs (1986).

Table 10.4 Minipig Hematological Parameters in Different Strains

Parameter	Yucatan	Göttinger
Red blood cell (10^6/mm^3)	7.61 ± 0.15	7.0 ± 0.80
Hemoglobin (g/dL)	14.87 ± 0.18	14.9 ± 1.2
Hematocrit (%)	44 ± 0.5	44.6 ± 4.1
Mean corpuscular volume (fL)	58.5 ± 0.8	64.4 ± 3.7
Mean corpuscular volume (pg)	19.8 ± 0.3	21.4 ± 1.3
Mean corpuscular hemoglobin concentration (g/dL)	33.9 ± 0.3	33.2 ± 0.8
White blood cell (10^3/mm^3)	12.73 ± 0.41	12.6 ± 3.0
Lymphocytes (10^3/mm^3)	7.25 ± 0.24	5.75 ± 1.52
Neutrophil (per mm^3)	4.47 ± 0.24	5.27 ± 1.29
Eosinophils (per mm^3)	534 ± 57	517 ± 31
Monocyte (per mm^3)	422 ± 35	945 ± 71
Basophils (per mm^3)	89 ± 15	63 ± 1.3
Platelets (10^3/mm^3)	—	441 ± 119

Note: From Burks et al. 1977 (12-month-old, sexes pooled), and Radin et al. (1986).

General Toxicity Testing

Are minipigs an appropriate species for the general toxicity testing of new drugs and chemicals? This question is perhaps best addressed by comparing the toxicity of known chemicals in minipigs with that observed in other animals. Unfortunately, relatively few examples of the use of minipigs in a safety assessment package have been published. In one of the few such examples, Van Ryzin and Trapold (1980) published on the toxicity of proquazone (a nonsteroidal anti-inflammatory drug [NSAID]) in rats, dogs, and minipigs. Rats in general are exquisitely sensitive to NSAIDs, and proquazone was no exception; dosages of 25 mg/kg/day (13 weeks) and above caused evidence of gastrointestinal (GI) toxicity. In dogs, dosages as high as 75 mg/kg were without effect, and higher dosages caused emesis, anorexia, and anemia, but no GI lesions. In a longer term study, however, evidence of gastric damage was produced in the dog. In minipigs, dosages ranged from 6 to 94 mg/kg/day (26 weeks). Dosage-related mortality, diarrhea, and gastric ulceration were observed at all levels. In this particular example, if the minipig had been used in place of the dog, somewhat different conclusions regarding the safety of proquazone would have been reached. Generalizing from this single case, minipigs might be more similar to rats than to human beings in their response to NSAIDs.

The toxicity of relatively large numbers of chemicals has been investigated in regular swine (summarized in table 10.5). These studies did not use minipigs, but still could be used to infer the toxic syndrome in minipigs. After all, if pigs cannot with reasonable certainty predict the toxicity in a different breed of pig, how reliable can they be in predicting the toxicity of chemicals in human beings? In general, these publications suggest that the toxic syndromes produced in pigs reliably predict the toxicity of drugs and chemicals in human beings. There are some notable quantitative and qualitative differences. As mentioned, pigs appear to be more sensitive to the GI effects of NSAIDs (a quantitative difference with humans). Pigs primarily develop methemoglobinemia (a qualitative difference with humans) in response to acetaminophen rather than liver damage (Artwhol et al. 1988).

Reproductive Toxicity and Teratogenicity

Although the rat and the rabbit will probably remain the mainstay of reproductive developmental toxicity testing, the minipig has several attractive features that might make it an appropriate model when one of these other species is not. The estrus cycle is approximately 20 days, which certainly makes the minipig a more convenient nonrodent model than dogs for reproductive toxicity studies. The gestation period is about 114 days and the critical period for organogenesis is days 11 through 35 (Hayama and Kokue 1985). Average litter size is about six, with weaning in about 5 weeks. The piglets are born quite well developed, and make very good models for behavioral teratogenicity testing. These aspects would make the pig more attractive than the monkey for teratogenicity testing. Other aspects of the porcine reproductive system make pigs good models for other types of research as well. Sows have the epitheliochorial type of placenta, which blocks the transplacental passage of proteins, and therefore the newborn piglet is free of antibodies. The minipig is, therefore, also an excellent model for gnotobiotic research, as discussed by Mandel and Travnicek (1987). For example, Kim et al. (1980) used the gnotobiotic minipig model in their studies of natural killer cells and antibody-dependent cellular toxicity.

Numerous examples of teratogenic studies with minipigs can be found in the literature. Ivankovic (1979) studied the teratogenic effects of N-alkylnitrosureas using the Göttinger minipig. He demonstrated, for example, that a single dose of (70 mg/kg intraperitoneally) ethylnitrosourea given 13 days postcoitus to the sow causes severe bone malformations in the piglets. This chemical class is also teratogenic to rats and hamsters.

Hayama and Kokue (1985) have published on the natural incidences of various malformations and functional deficits in the Göttinger minipig; many other practical aspects of teratogenicity

Table 10.5 Summary of Literature on Toxicity in the Pig

Chemical (References)	Syndrome	Comments
3-nitro-4-hydroxyphenylarsenic (Rice et al. 1985)	Subchronic dosing leads to exercise-inducible muscle tremors and colonic convulsions.	Controlled lab study, dietary admixture, Landrace pigs, 20 kg
Lead Lassen and Buck 1979)	Only mild clinical signs despite blood levels of 240 μg/ml and decreases in aminolevulinic acid dehydratase (ALAD), hemoglobin, and Hematocrit (HCT).	Controlled lab study, oral administration in solution, crossbred 6-week-old pigs, 17–24 kg
Polybrominated biphenyls (Howard et al. 1980)	200 ppm in diet for 12 weeks led to decreases in lymphocyte mitogen response in sow and piglets (4 weeks postpartum). No changes in bactericidal activity. Large increases in pre-β lipoprotein.	Controlled lab study, dietary admixure, two-generational study of immunotoxicological effects
Zearalenone (James and Smith 1982)	10 μg/g feed for 4 weeks caused increases in uterine weight with no effect on growth or feed efficiency.	Controlled lab study, dietary admixture Yorkshire gults, included comparisons with rats
Chloropyrifos (Scheidt et al. 1987)	Exposure of newborn piglets prior to healing of umbilical and tail wounds led to severe signs of organophosphate insecticide toxicity: lethargy, ataxia, salivation, and diarrhea.	Controlled lab study to follow up a case report, gravid, crossbred sows, aerosol exposure
Acetaminophen (Artwhol et al. 1988)	Acutely, major clinical sign of toxicity was methemoglobin formation. Relatively mild effects on liver; primarily dose-related increases in glycogen (500–2,000 mg/kg IV over 90 min).	Controlled lab study, IV infusion, crossbred male and female swine
Gossypol (Hascheck et al. 1989)	Severe cardiotoxicity: diffuse myofiber atrophy with perinuclear vacuolation; liver damage (marked centrilobular congestion and necrosis).	Clinical report, feedlot swine problem traced to cottonseed supplementation of feed
Fenbendazole (Hayes et al. 1983)	When given at 200 mg/kg for 14 days, caused transient leukopenia and increased serum sorbitol dehydrogenase, but not histopathological lesions.	Controlled lab study, oral gavage, female Yorkshire pigs, 18–24 kg
Aflatoxin B_1 (Osuna and Edds 1982)	0.2 mg/kg/day for 10 days causes increased serum alkaline phosphatase, sorbitol dehydrogenase, prothrombin time, and partial thromboplastin times. Decreases in total protein and β-, γ-globulins.	Controlled lab study, emphasis primarily on clinical pathology parameters
Tumeric oleoresin (Bille et al. 1985)	60, 296, 1,551 mg/kg/day. Dose-related increases in thyroid and liver weights; pericholangitis, hypoplasia of the thyroid, changes in epithelial cells in kidney and bladder	Controlled lab study, dietary admixture, crossbred swine
Diacetoxyscirpenol (Weaver et al. 1981)	0, 2, 4, 8, and 16 ppm. Decreases in feed consumption and weight gain at all dosages; multifocal proliferative, gingival, and lingual lesions; Glandular and mucosal small intestine hyperplasia.	Controlled lab study, dietary admixture, crossbred weanling pigs
Toxaphene (DiPietro and Haliburton 1979)	Ataxia, lethargy, depression, diarrhea, seizures, increased rectal temperatures.	Clinical case report, findings attributed to improper use of a topical preparation
T-2 Toxin (Lorenzana et al. 1985)	Cyanosis, anorexia, lethargy, pneumonia, necrotic lymph tissue, necrotizing gastroenteritis, and other lesions; depressed lymphocytes and macrophage function, but no effect on red blood cells.	Controlled lab study

testing in minipigs are discussed in their review article, which is highly recommended reading. They studied pyrimethamine (a folic acid antagonist, given in the feed at an average of 3.6 mg/kg/day on days 11–35 of gestation) and established that it causes a high incidence of major malformations such as cleft palate, clubfoot, and micrognathia.

Dexter et al. (1983) have examined Sinclair minipigs, which will voluntarily consume (i.e., do not avoid) alcohol, as a potential model for human fetal alcohol syndrome (FAS). They reported that alcohol (20% in drinking water) causes a progressive decrease in litter size and piglet weight, but does not result in the more common FAS-related malformations such as microcephaly and narrowed palpebral fissures.

Dermal Toxicity

Although rabbits are commonly used for the assessment of primary dermal irritation, pigs have generally been considered to be better models for the more sophisticated study of dermal permeability and toxicity. As reviewed by Sambuco (1985), human and porcine skin are similar with regard to sparseness of the pelage; thickness and general morphology; epidermal cell turnover time; and size, orientation, and distribution of vessels in the skin. The particularly thin haircoat and lack of pigments of the Yucatan minipig make it particularly ideal for dermal studies. The size of the animal also provides the additional practical advantage of abundant surface area for multiple site testing.

Sambuco (1985) has described the sunburn response of the Yucatan minipig to ultraviolet (UV) light, suggesting that this species would also make a good model in phototoxicity as well as photocontact dermatitis studies. Thirty 12-cm sites were demarcated, permitting the study of 15 different dermal dosages of UV radiation.

Mannisto et al. (1984) have published a series of articles on the dermal toxicity of the anthralins in the minipig. In one experiment, 24 sites per minipig were used to assess the acute dermal irritation of various concentrations to four different chemicals per site. The range of concentrations tested permitted them to calculate the median erythema concentration and median irritation concentrations with relatively few animals. They were able to show clear differences between anthralin congeners (antipsoriatic drugs) with regard to irritation. When compared to other species (mouse and guinea pig) the response of the minipig was the most similar to humans in that in both species these chemicals are delayed irritants, and several days postexposure can pass before the maximal irritant response is presented.

In a second experiment (Hanhijarvi et al. 1985), the chronic, cumulative dermal effects of anthralin chemicals were studied in minipigs. Using only 12 animals, they were able, by having 32 sites per animal, to study the effects of two different chemicals (dithranol and butantrone, both anthralins) in three different formulations at three different concentrations each. The protocol also included observations for systemic toxicity, clinical laboratory measurements, plasma drug analyses, and gross and histopathological examinations.

In a third report (very similar to the second), Hanhijarvi et al. (1985) clearly demonstrated that the type of vehicle can greatly influence irritation in that dithranol was clearly more irritating when applied in paraffin than when applied in a gel. They were also able to demonstrate that although dithranol was less irritating than butantrone acutely, the cumulative irritations (mean scores at the end of 6 months of applications six times per week) were quite similar (Mannisto et al. 1986). There was no evidence of systemic toxicity or of test article in plasma with either species.

Cardiovascular Toxicity

In general, the published literature consistently maintains that the cardiovascular systems of swine and humans are very similar. For example, as reviewed by Lee (1986), swine, including minipigs, have a noticeable background incidence of atherosclerotic lesions, and swine fed high-lipid diets will develop even more extensive atherosclerotic lesions. High-lipid diets will produce lesions similar to advanced atheromatous lesions seen in humans. Although few drugs or chemicals have been shown to cause atherosclerosis, this information has three general applications to toxicology and pharmacology. First, the feeding regimen of minipigs should be carefully controlled in

general toxicity studies to minimize the incidence of arterial disease, especially in long-term studies. Second, the pathologist should be aware of the natural background of this disease when preparing a diagnosis. Third, the minipig could provide a convenient model for the study of atherosclerotic disease and the screening of potential therapies.

The minipig has been used to study cardiotoxicity. Van Vleet et al. (1984) reported that minipigs were the only species studied other than dogs to develop cardiac damage in response to large doses of minoxidil. In both the pig and the dog, minoxidil cardiotoxicity is characterized by vascular damage (with hemorrhage in the arterial epicardium) and myocardial necrosis (mostly of the left ventricular papillary muscles). Interestingly, in the dog the atrial lesion is largely restricted to the right atrium, whereas in the pig it is restricted to the left atrium. These lesions can be produced in roughly 50% of the minipigs given 10 mg/kg of minoxidil for 2 days and sacrificed 48 hr after the last dose (Herman et al. 1988; Herman et al. 1986). Herman and colleagues have published extensive descriptions of minoxidil-induced lesions in minipigs in comparison to those produced in dogs (Herman et al. 1988; Herman et al. 1989). The right versus left arterial difference is believed to be due to differences in the anatomical pattern of coronary circulation between two species (Herman et al. 1988).

Minipigs are also sensitive to the cardiotoxic effect of doxorubicin. When given six IV injections of either 1.6 or 2.4 mg/kg of doxorubicin at 3-week intervals, minipigs develop cardiac lesions similar to those seen in dogs, rabbits, and other experimental animals (Herman et al. 1986). The lesion is characterized by cytoplasmic vacuolation and varying degrees of myofibrillar degeneration and loss. Thus, the minipig is sensitive to the cardiotoxic effect of two well-known and extensively studied chemicals. Therefore, it is a suitable nonrodent species for the general assessment of cardiotoxicity.

Advantages and Disadvantages

There are two disadvantages to the use of minipigs. The first is their size. Although minipigs are smaller than regular swine, at maturity they are generally larger than beagle dogs. The second is their expense: They are not only larger than dogs, but currently carry higher purchasing costs. Among the advantages are the facts that they are long-lived, cooperative animals with well-defined physiological and metabolic characteristics. As they are not either popular companion animals (like dogs) or physically similar to humans (like monkeys), minipigs are not specifically discussed in animal "welfare" laws like the other two species. Depending on their final form, new animal welfare regulations could make the space and maintenance costs for dogs and monkeys very prohibitive. This could make minipigs increasingly more attractive as a nonrodent species for general toxicity testing.

PATHOLOGY

Pathological evaluation of toxicity studies is not only concerned with the recognition of lesions caused by treatment directly, but also with the identification of spontaneous lesions that might have increased in severity or frequency in treated animals. It is therefore vital to be aware of the background and incidentally occurring lesions that can be observed during macroscopic and microscopic examinations of organs from laboratory animals in routine toxicity studies.

The pig offers many important advantages in pharmacological and toxicological research due to its general physiological resemblance to man, but the cost of housing and handling associated with using normally sized pigs is an obstacle to its extensive use as an experimental animal. Consequently a number of strains of miniature pigs have been developed through selective breeding.

The use of minipigs has thus aroused increased interest during recent years, as it provides a viable alternative to dogs or nonhuman primates. Particular similarities to humans include its

cardiovascular anatomy and physiology, skin, GI system and digestion, renal system, and immune system. The costs of animals and housing are comparable to those for beagle dogs (and thus much cheaper than for primates), although some procedures such as dosing and blood sampling might require more manpower, adding slightly to the cost of a toxicity study. A more practical consideration is the fact that minipigs are not specifically discussed in animal welfare laws, as are dogs and monkeys.

The procedures needed to conduct minipig toxicity studies do not differ much from those used for dogs, although naturally some aspects of housing and feeding must be modified. Minipigs can be housed individually or in small male or female groups, but beause they are by nature social animals some contact with other pigs is always necessary. In group housing the social hierarchy should also be considered (Bollen et al. 1998). Because ad libitum feeding leads to obesity, restricted feeding schedules must be implemented (Ritskes-Hoitinga and Bollen 1998).

Minipigs are used in all standard preclinical studies: repeat-dose, single-dose, teratology, fertility assessments, and absorption, distribution, metabolism, and excretion (ADME) studies. This also includes all methods of drug administration: oral intubation, dietary, inhalation, dermal including administration to experimental wounds, injection (subcutaneous, intramuscular, intradermal, intravenous, intraperitonal, epidural), and continuous IV infusion with ambulatory infusion pumps carried in a jacket.

The most commonly used breeds are the Yucatan, Hanford, Sinclair, Hormel, and Göttingen minipig (Swindle et al. 1994). The information provided here focuses on Göttingen minipigs, as this is the main breed employed at Scantox A/S, Denmark, where the data were obtained. The Göttingen minipig is also widely used in Europe and bred in Denmark.

A general overview is provided, covering systemic findings, an outline of the main steps of the necropsy procedure (including a list of standard organs sampled), and a summary of macroscopic and microscopic findings based on Scantox A/S data. The following sections then discuss typical microscopic findings system by system. For each, our own findings are given, as well as a comparison with humans or other laboratory animals where appropriate.

The data from Scantox A/S are based on findings from more than 150 untreated Göttingen minipigs used in oral and dermal studies in the period 1997 to 2001. Ages were from 3 months to 1 year. In the next section, data from 150 of these are provided in tabular form.

General Overview

Systemic Findings

The Göttingen minipig has been shown to be free of a range of viral, parasitic, fungal, and bacterial diseases (including *Streptococcus suis*; Hansen et al. 1997; Madsen et al. 2001; *www.minipigs.dk*).

Hemorrhagic syndrome is the common term for an important systemic condition spontaneously occurring in Göttingen minipigs. Its etiology is unknown. In humans the corresponding syndrome is known as von Willebrand's disease, and is here an autosomal trait that leads to massive mucosal hemorrhage often resulting in death (Strauss and Bloom 1965). The syndrome is also seen in the normal pig, which was among the first animal models for this type of genetic hemorrhagic disease (Hogan et al. 1941).

In minipigs, the affected animal usually exhibits poor condition prior to necropsy. Main macroscopic findings consist of generalized multiple petechial hemorrhages in the subcutis of the entire body and in the mesentery. These hemorrhages are easily detected in the mucosal or visceral surfaces of the internal organs, such as heart, lung, kidney, urinary bladder, and lymph nodes. Further observations might include enlarged pale kidneys, thickened urinary bladder with hemorrhagic mucosa and content, enlarged and reddened lymph nodes, and an edematous thymus. Microscopic findings consist of slight to marked multifocal hemorrhages in various tissues (aorta and aortic

arch, heart, kidney, liver, gall bladder, pancreas, lung, lymph node, skeletal muscle, skin/subcutis, stomach, and small/large intestines, urinary bladder). These main changes can be accompanied by diffuse hemorrhagic edematous cystitis, interstitial nephritis, hemorrhagic parenchymal necrosis of liver, reactive lymphoid hyperplasia of lymph nodes, or increased bone-marrow cell density. In surviving animals hemosiderin deposits can be seen in these organs as remains after hemorrhages.

Necropsy Procedure

Macroscopic examination is performed after opening the abdominal, thoracic, and cranial cavities, respectively. The appearance of the organs is observed in situ.

The standard tissue list (covering the requirement of international guidelines for toxicology studies) includes adrenal glands, aorta (thoracic), bones (medial condyles of right femur including knee joint), brain, epididymides, esophagus, eyes with lenses and optic nerves, gallbladder, heart with aortic arch, kidneys, liver (all main lobes), lungs (cranial and caudal lobes), lymph nodes (right mandibular and mesenteric), mammary gland, muscle (right quadriceps femoris), ovaries, pancreas, parathyroids, pituitary, prostate, salivary glands (right parotid and mandibular), sciatic nerve, seminal vesicles, skin, small (duodenum, jejunum, and ileum) and large (cecum, colon, and rectum) intestines, spinal cord (thoracic and lumbar), spleen, sternum (for bone marrow), stomach, testes, thymus, thyroid, tongue, trachea, urinary bladder, uterus (horn and cervix), vagina, and vertebrum (thoracic and lumbar). Additional samples are taken from any tissue that appears abnormal macroscopically. Details of any abnormalities are recorded with respect to size, color, texture, and other descriptive characteristics.

Sampled tissues are fixed in neutral phosphate-buffered 4% formaldehyde, except for eyes (Davidson's fixative) and testes (Bouins fixative). The lungs are infused with fixative via trachea. Tissues are processed using standard techniques and embedded in paraffin wax. Sections are cut at a nominal 5 μm and stained with hematoxylin and eosin (H&E). After formaldehyde fixation osseous tissues are decalcified with acetic acid.

Summary of Macroscopic and Microscopic Data

A summary of the most commonly observed background or incidental macroscopic and microscopic changes at Scantox A/S in the period between 1997 and 2001 is given in table 10.6 and table 10.7, respectively. Macroscopic data are taken from 124 animals, and the microscopic data are based on 150. All were untreated Göttingen minipigs from 2-, 4-, 13-, 26-, and 52-week oral and dermal studies. As can be seen, spontaneous pathological changes are not very frequent and do not seem to depend on sex or age within the age range studied. They are generally focal and mild in nature.

Cardiovascular System

Anatomically the heart of pig is similar to man's, the main exception being the presence of a left azygous vein draining the intercostal system into the coronary sinus (Swindle et al. 1986). The coronary artery's blood supply to the heart is almost identical to that of humans in anatomy and function, and the aorta also has vaso vasorum. As in man, pig has no collateral vessels in the myocardium (Bloor et al. 1992), leading to increased susceptibility to cardiac infarcts.

All these anatomical parallels have led to the significant interest in using pig hearts for human transplants. The size of an adult human's heart corresponds to that of a pig of between 40 kg and 50 kg.

As regards handling procedures, the blood vessels and the two atria tend to be more friable in the pig than in other species, especially in neonates, and vasospasms are therefore more likely during manipulations. Blood samples for toxicokinetics, hematology and clinical chemistry are routinely collected from the bijugular trunk near the entry to the thoracic aperture or from the right

Table 10.6 Most Common Background and Spontaneously Occurring Macroscopic Changes Observed in 124 Control Males and Females of Göttingen Minipig in 2-, 4-, 13-, 26-, and 52-Week Oral and Dermal Studies Between 1997 and 2001

Organ/Finding	Male	Female
Epididymides		
Cyst	1	—
Gall bladder		
Thickened	2	—
Kidney		
Cyst	—	1
Liver		
Marked lobular pattern	—	1
Lung		
Red foci	1	3
Solidifications	1	1
Lymph node (mandibular)		
Enlarged	—	1
Reddened	2	2
Pituitary (pars distalis)		
Red foci	1	—
Small/large intestine		
Red foci	2	1
Spleen		
Gray-white foci	1	—
Submandibular gland		
Edema	—	1
Thymus		
Reddened	4	5
Thyroid		
Red foci	1	3
Urinary bladder		
Reddened mucosa	4	—

Table 10.7 Most Common Background/Incidentally Occurring Microscopic Changes Observed in 150 Control Males and Females of Göttingen Minipig in 2-, 4-, 13-, 26-, and 52-Week Oral and Dermal Studies Between 1997 and 2001

	2-Week		4-Week		13-Week		26-Week		52-Week	
	M	F	M	F	M	F	M	F	M	F
	7	7	25	25	35	35	4	4	4	4
Cardiovascular system										
Heart										
Mononuclear cells (interstitial, focal) (minimal)	—	—	1	1	—	—	—	—	—	—
Digestive system										
Tongue										
Myositis (focal; minimal to slight)	2	—	—	5	2	1	1	—	—	2
Ulceration (focal; slight)	—	—	—	—	1	—	—	—	—	—
Salivary glands										
Mononuclear cells (interstitial, focal; minimal to slight)	—	—	1	1	3	2	—	—	2	—
Mineralization (glandular, focal; minimal)	—	—	—	—	—	1	—	—	—	—
Esophagus										
Mononuclear cells (minimal)	1	1	1	—	—	—	—	—	—	1
Stomach (glandular)										
Inflammation (mucosal, focal; minimal)	1	—	3	2	—	1	—	—	—	—
Erosion (focal; minimal to slight)	1	—	—	—	—	1	—	1	—	—

Table 10.7 Most Common Background/Incidentally Occurring Microscopic Changes Observed in 150 Control Males and Females of Göttingen Minipig in 2-, 4-, 13-, 26-, and 52-Week Oral and Dermal Studies Between 1997 and 2001 (continued)

	2-Week		4-Week		13-Week		26-Week		52-Week	
	M	F	M	F	M	F	M	F	M	F
	7	7	25	25	35	35	4	4	4	4
Small intestine										
Inflammation (focal/diffuse; minimal to marked)	5	2	—	—	—	—	—	—	—	—
Peritonitis (focal; minimal to slight)	—	—	—	—	—	2	—	—	—	—
Large intestine										
Inflammation (focal/diffuse; minimal to moderate)	4	1	—	—	1	—	—	—	—	—
Arteritis (chronic, focal; minimal)	—	—	—	—	—	—	—	—	—	1
Liver										
Mononuclear cells (parenchymal/portal; minimal	1	2	1	3	4	6	—	—	1	1
Necrosis (parenchymal, focal; minimal to slight)	—	—	—	1	—	—	—	—	—	1
Single cell necrosis (minimal)	—	—	—	1	—	—	—	—	—	—
Fibrosis (interlobular; minimal)	—	—	—	—	1	—	—	—	—	—
Gallbladder										
Cholecystitis (diffuse; marked)	—	—	—	—	1	—	—	—	—	—
Pancreas										
Mononuclear cells (interstitial, focal; minimal)	—	—	—	1	1	—	—	—	—	—
Hemorrhage (interstitial; minimal)	—	—	—	1	—	1	—	—	—	—
Arteritis/periarteritis (necrotizing; minimal)	—	—	—	—	—	1	—	—	—	—
Endocrine system										
Adrenal glands										
Mononuclear cells (focal; minimal to slight)	2	—	3	5	4	5	—	—	2	2
Vacuolation (cytoplasmic, cortical, focal/diffuse; slight)	—	—	—	—	—	1	—	—	—	—
Accessory cortical tissue	—	—	—	—	—	1	—	—	—	—
Thyroid										
Hemorrhage/inflammation (capsular)	1	2	8	8	10	12	—	1	—	—
Inflammation (interstitial, focal; minimal to slight)	—	—	—	—	—	3	—	—	—	—
Hematopoietic and lymphatic systems										
Mandibular lymph node										
Sinusoidal hemorrhage (focal/diffuse; minimal to moderate)	—	2	3	4	6	3	—	—	—	1
Abscess (chronic)	—	—	1	—	2	—	—	1	—	—
Mesenteric lymph node										
Sinusoidal hemorrhage (focal; minimal)	1	—	—	—	2	1	—	—	—	—
Spleen										
Macrophages (increased; slight)	—	—	1	—	1	—	—	—	—	—
Arteritis (necrotizing, focal; minimal)	—	—	—	—	—	1	—	—	—	—
Thymus										
Hemorrhage/inflammation (capsular)	4	5	5	5	8	9	1	1	1	—
Hyperplasia (lymphoid; moderate)	—	—	—	1	—	—	—	—	—	—
Atrophy (cortical; slight)	—	—	—	—	1	—	—	—	—	—
Integumentary system										
Skin										
Mononuclear/inflammatory cells (focal; minimal to moderate)	1	—	1	2	5	8	2	—	—	—
Crust (focal; minimal to slight)	—	—	—	—	4	6	2	1	—	—
Edema (subepidermal, focal; minimal to slight)	—	—	1	—	1	6	—	—	—	1
Hyper/parakeratosis (focal/diffuse; minimal)	—	—	—	—	3	4	—	—	—	—

Table 10.7 Most Common Background/Incidentally Occurring Microscopic Changes Observed in 150 Control Males and Females of Göttingen Minipig in 2-, 4-, 13-, 26-, and 52-Week Oral and Dermal Studies Between 1997 and 2001 (continued)

	2-Week		4-Week		13-Week		26-Week		52-Week	
	M	F	M	F	M	F	M	F	M	F
	7	7	25	25	35	35	4	4	4	4
Musculoskeletal system										
Skeletal muscle										
Myonecrosis/myositis (focal; minimal to moderate)	1	1	—	2	3	3	—	1	—	—
Femur										
Serous atrophy of fat cells (minimal to moderate)	1	—	—	—	8	1	2	—	3	—
Tibia										
Serous atrophy of fat cells (minimal to moderate)	1	—	—	—	7	1	—	—	2	—
Vertebrum (lumbar)										
Serous atrophy of fat cells (minimal to moderate)	—	—	—	—	1	—	2	—	—	—
Nervous system										
Brain (cerebrum)										
Mononuclear cells (meningeal, focal; minimal)	—	—	1	—	—	1	—	—	—	—
Brain (cerebellum)										
Mineralization (focal; minimal)	—	—	—	—	2	—	—	—	—	—
Male reproductive system										
Testes										
Hypoplasia (tubular, focal; minimal to slight)	3	—	6	—	13	—	2	—	1	—
Granuloma (spermatic; minimal)	—	—	—	—	1	—	—	—	—	—
Epididymides										
Oligospermia (slight to moderate)	—	—	1	—	1	—	1	—	1	—
Prostate gland										
Mononuclear cells (interstitial, focal; minimal)	—	—	1	—	1	—	—	—	—	—
Mineralization (glandular, focal; minimal)	—	—	—	—	1	—	—	—	—	—
Prostatitis (subacute, diffuse; moderate)	—	—	—	—	1	—	—	—	—	—
Seminal vesicle										
Inflammation (subacute, focal; minimal)	1	—	—	—	—	—	—	—	—	—
Peri/arteritis (chronic, focal; minimal)	—	—	—	—	—	—	—	—	1	—
Female reproductive system										
Ovary										
Mineralization (interstitial, focal; minimal to slight)	—	2	—	—	—	4	—	—	—	—
Cervix/vagina										
Hypoplasia	—	—	—	—	—	1	—	—	—	—
Respiratory system										
Trachea										
Hemorrhage/inflammation (adventitial)	1	1	1	1	4	1	—	—	—	—
Lung										
Macrophages (alveolar, focal; minimal to slight)	2	1	6	4	6	7	—	—	—	—
Mononuclear cells (perivascular, focal; minimal)	2	—	1	1	9	6	—	1	—	—
Mineralization (alveolar, focal; minimal)	—	—	4	2	1	3	—	—	—	1
Interstitial pneumonia (focal; minimal)	2	2	—	—	—	2	—	—	—	—
Foreign body granuloma (focal; minimal)	—	—	2	2	1	—	—	—	—	1
Hemorrhage (alveolar, focal; minimal to moderate)	—	—	3	1	—	—	—	—	—	—
Alveolitis (focal; minimal)	—	—	—	—	—	2	—	—	—	—
Edema (alveolar, focal; slight)	—	—	1	—	—	—	—	—	—	—
Bronchopneumonia (focal; moderate)	—	—	—	—	—	—	—	—	1	—

Table 10.7 Most Common Background/Incidentally Occurring Microscopic Changes Observed in 150 Control Males and Females of Göttingen Minipig in 2-, 4-, 13-, 26-, and 52-Week Oral and Dermal Studies Between 1997 and 2001 (continued)

	2-Week		4-Week		13-Week		26-Week		52-Week	
	M	F	M	F	M	F	M	F	M	F
	7	7	25	25	35	35	4	4	4	4
Pleura										
Pleuritis (focal; minimal to slight)	—	—	—	1	1	—	—	—	—	—
Urinary system										
Kidneys										
Mononuclear cells (interstitial, focal; minimal to moderate)	1	1	6	4	9	13	1	2	1	2
Mineralization (tubular/papillary, focal; minimal)	—	—	2	—	7	5	—	—	3	2
Tubular basophilia (focal; minimal to slight)	2	2	2	1	2	6	—	—	—	—
Eosinophils (pelvic, focal; slight)	—	—	—	—	—	1	—	—	—	—
Casts (tubular, focal; minimal)	—	—	—	—	1	—	—	—	—	—
Cyst (medullary, focal)	—	—	—	—	—	1	—	—	—	—
Glomerulonephritis (focal; minimal)	—	—	—	—	1	—	—	—	—	—

or left jugular vein. The relative deepness of these blood vessels (Swindle et al. 1988) complicates blood sampling (cf. thyroid gland).

Pigs play a significant role as an animal model of cardiac disease. The coronary artery can quite simply be clamped, inducing myocardial infarct. Alternatively, administration of a lipid-rich diet will lead to atherosclerosis. In the minipig, this is mainly located in the abdominal aorta and in the coronary arteries (Jacobsson 1989). The Göttingen minipig breed has been demonstrated to be more susceptible to the effects of a lipid-rich diet than the domestic Swedish Landrace (Jacobsson 1986).

Only minipigs and dogs show cardiac lesions after treatment with vasodilating antihypertensive drugs such as minoxidil (failed attempts include normal pigs, mice, rats, rabbits, and monkeys). Both species develop left ventricular papillary muscle necrosis, myocardial hemorrhage and inflammation, and vascular damage in the atrial epicardium (Herman et al. 1989).

The dog is also an important model for heart research, but using pigs does provide some important advantages. Minipigs show greater tolerance toward treatment with nonsteroid anti-inflammatory drugs (NSAIDs), antihypertensive agents, and sympathicomimetic drugs. For sympathicomimetic drugs, in contrast to dogs, no cardiotoxicity is seen in pigs except for an increase in heart rate. Minipigs do not develop arteriopathy with endothelin-receptor antagonists. They are furthermore not prone to vomiting.

Blood Vessels

Arteritis and periarteritis are regularly seen in Göttingen minipig. One generally sees inflammatory infiltration in all layers of the vessel wall, associated with necrosis and fibrin accumulation (fibrinoid necrosis; figure 10.1). Chronic forms can also be observed as thickening of the artery wall caused by fibrotic tissue. Arteritis and periarteritis mainly affect individual arteries of small or medium size, and although generally observed at minimal levels can occasionally reach moderate. A variety of organs can be affected, in our experience most commonly epididymides, heart, intestines, kidney, lung, spleen, stomach, and urinary bladder. Similar lesions occur spontaneously in beagle dogs and rats. Kemi et al. (1990) suggested that they can be a result of an immune-mediated mechanism in beagle dogs, and Yu et al. (1982) suggested that food restriction might reduce the incidence of periarteritis in rat.

Also phlebitis, acute or chronic, is occasionally seen in the minipig. Most commonly it occurs in the mesenteric vessels.

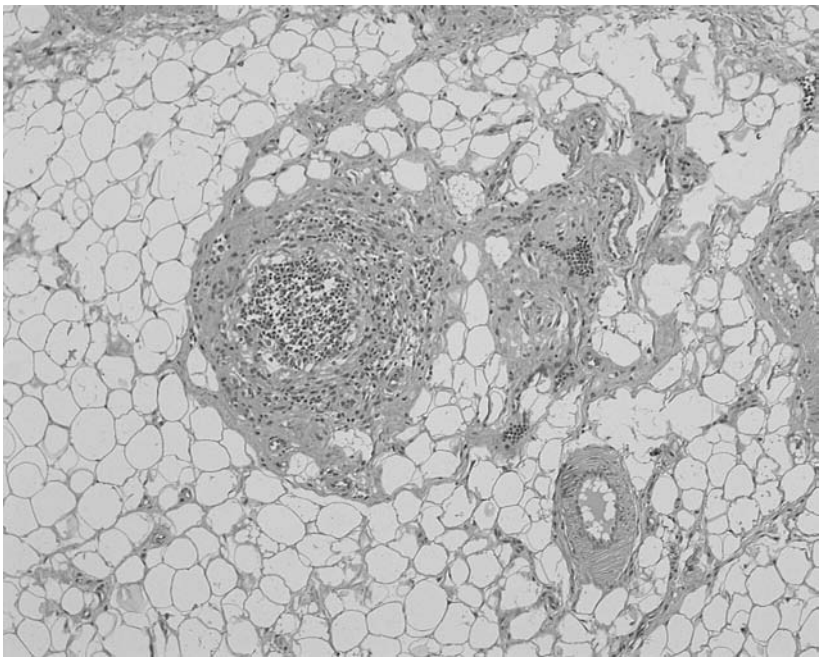

Figure 10.1 Chronic arteritis in a medium-sized artery in the submucosa of the rectum. Inflammatory infiltrate in the lumen and all layers of the vessel wall (H&E, ×10).

Heart

Spontaneously occurring cardiac lesions are very rare in the Göttingen minipig. Occasionally focal mononuclear infiltrates (mainly lymphocytes) can be seen in the interstitial tissue. Necrosis or inflammation of myocardium is seen very occasionally. When found, such lesions appear mild and focal, and are characterized by myofiber necrosis associated with inflammatory cell infiltrates.

Digestive System

Pigs and humans are both omnivores, and although the pig's digestive system has some anatomical differences from that of humans, the physiology of digestion is nevertheless rather similar. Comparable metabolic functions, intestinal transport times, and nutrient absorption characteristics have made pigs very useful in basic nutritional research (Swindle and Smith 1998).

Particularly the stomach and the small intestine have very few differences from humans. Pigs have similar gastric cell types, villi, secretions, pH changes, and transit time. The main differences are found for the large intestine: The cecum and colon are somewhat larger in the pig, and the colon is arranged in a series of coils.

Canulation techniques can be applied both in the GI tract and in the blood circulation allowing the collection of samples of digesta, blood, or tissue.

Tongue

Minimal focal acute or chronic myositis of striated muscle is commonly seen in Göttingen minipigs dosed by oral gavage. The cause of this lesion is most likely damage caused by the mouth stick inserted into the mouth for introduction of the gastric tube.

Minimal erosive or ulcerative changes at fungiform papilla of nonkeratinized stratified squamous epithelium are very rare.

Salivary Glands

The salivary glands of pig are large and consist of paired sets of parotid (serous), sublingual (mucous), and mandibular (serous + mucous) glands (Schantz et al. 1996).

Interstitial mononuclear or periductular chronic inflammatory infiltrates and mineral deposits within the acini can often be observed at minimal levels in the Göttingen minipig.

Periglandular edema of mandibular glands (jellylike material surrounding the gland) is quite a common necropsy finding. The edema is sometimes discernible microscopically in the interlobular tissue.

Minimal focal acinar atrophy of salivary tissue occurs very rarely.

Stomach

Inflammation of the glandular stomach is quite commonly seen in the Göttingen minipig. It is usually minimal, consisting of acute inflammatory infiltrates in the upper lamina propria of the mucosal layer. Erosion is also relatively common at minimal to slight levels, characterized by loss of mucosal epithelium (leaving the basement membrane more or less intact) and associated with acute inflammation in the underlying propria (figure 10.2). At these levels, the changes can be considered incidental, but treatment might cause higher levels or incidence (Barker et al. 1993). The acute and chronic ulcers that might occasionally be seen consist of tissue damage extending into the deep lamina propria. These lesions in the glandular stomach are usually located in the cardiac and pyloric region, and at the duodenal–pyloric junction.

Erosive and ulcerative changes of the nonglandular stomach are occasionally observed, generally at minimal to slight levels.

Small and Large Intestines

The changes occur mainly in the intestinal mucosa. Agonal congestion is very commonly observed, and minimal to moderate focal hemorrhage of the mucosa and submucosa is relatively

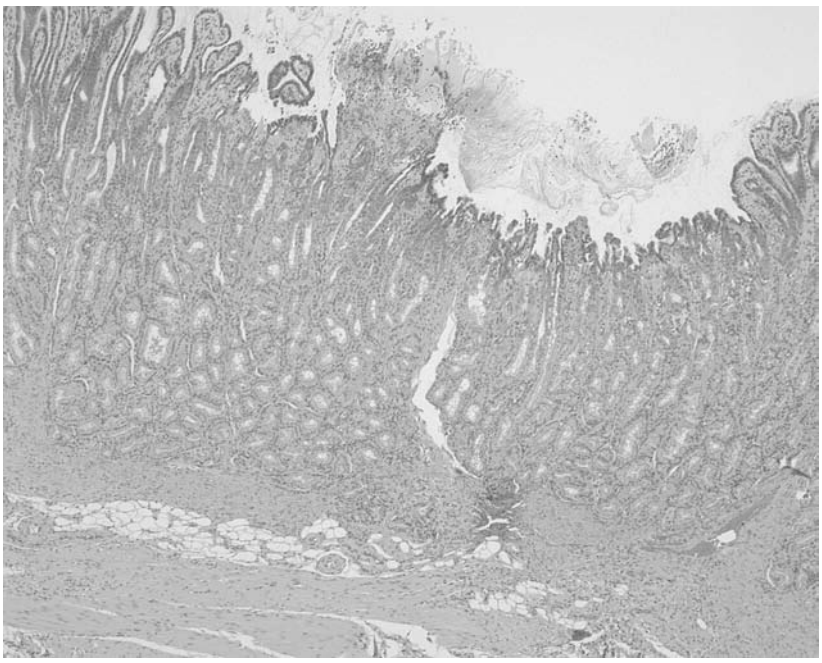

Figure 10.2 Mild focal acute erosion at the pyloric–duodenal junction of glandular stomach. Loss of mucosal epithelium leaving the basement membrane more or less intact (H&E, ×5).

common, as are focal or diffuse acute and chronic inflammation. Inflammation can reach moderate levels, and consists of increased amounts of inflammatory cells in the lamina propria and cellular debris in glands. It is occasionally associated with erosive or ulcerative changes of the mucosal epithelium.

Proliferative or inflammatory lesions involving the mesentery and the serosal surface of the abdominal organs such as ileum, cecum, and colon is occasionally seen. They can reach moderate levels.

Liver

The liver of minipigs consists of six lobes and is lobulated by rather thick fibrous septae (Schantz et al. 1996). Mildly increased interlobular or subcapsular fibrous tissue can occasionally be observed, giving some enlargement of stroma and disorganization of the surrounding hepatic parenchyma.

Liver commonly shows focal or multifocal mononuclear cell infiltration, including granulocytes, in the parenchyma and portal areas. It can be associated with single-cell necrosis.

Randomly distributed small foci of parenchymal necrosis can occasionally be seen. The necrotic areas are usually characterized by eosinophilic hepatocytes that have lost cellular detail. They can be associated with inflammatory infiltrates and, more rarely, hemorrhage. In the final stage fibrotic repair is seen, in some cases accompanied by granulomas.

Cytoplasmic vacuolation of hepatocytes due to lipid accumulation can sometimes be observed. These mild focal or multifocal clear, sharp, empty vacuoles usually have no specific zonal pattern.

Clear cell foci characterized by enlarged hepatocytes with clear cytoplasm in the perinuclear region is seen very occasionally in the Göttingen minipig. The clear cytoplasm is a result of glycogen accumulation.

Gallbladder

Chronic necrotizing cholecystitis, hypoplasia, and aplasia of the gall bladder are characteristic findings in the Göttingen minipig (Svendsen, Skydsgaard et al. 1998).

Of the three, chronic cholecystitis is most commonly observed. Macroscopically the gallbladder is diminished, with thickened walls and thick or absent bile. Microscopically it is usually characterized by a necrotizing, hemorrhagic mucosal layer with granulomatous inflammation extending into the muscular layer (figure 10.3).

A hypoplastic gallbladder can be difficult to discern in macroscopic examination. Microscopically it appears as loose connective tissue rich in blood vessels, leading to the disappearing of mucosal crypts and flattening of the mucosal epithelium.

Pancreas

The pancreas is rather large in both the minipig and the pig, extending from the spleen to the proximal duodenum. The islet cells are relatively indistinct histologically (Schantz et al. 1996).

Not many lesions occur spontaneously in the pancreas. Small foci of mononuclear cells can sometimes be seen in the interstitial tissue.

The Endocrine System

Adrenal Glands

The adrenal glands are located near the cranial poles of both kidneys and the right gland is intimately associated with the wall of the postcava in pigs (Swindle and Smith 1998).

Figure 10.3 Chronic necrotizing cholecystitis in gallbladder. Note completely necrotized mucosa (upper half of the figure) with underlying fibrosis (H&E, ×2.5).

The most common background finding consists of minimal focal or multifocal mononuclear cell infiltration, mainly lymphocytic, and usually located in the cortex. In the zona reticularis of cortex, diffuse cytoplasmic vacuolation can furthermore be seen.

Accessory cortical tissue is observed occasionally. It consists of a portion of the cortex either completely detached from the rest of the gland or attached to it in an enclosing fibrous capsule.

Pituitary Gland

Small cysts can be found in the pars distalis, but are quite rare. They are lined by ciliated columnar epithelium and contain eosinophilic material.

Pars distalis can also occasionally contain mineralized cells, generally randomly distributed and not associated with inflammation.

Thyroid Gland

Accidental mechanical damage of the thyroid gland, or of neighboring organs such as thymus, esophagus, and trachea, is a common occurrence due to the customary procedure of sampling blood from the bijugular trunc by insertion of a needle (cf. the section on the cardiovascular system). The thyroid is located on the ventral surface of the trachea at the thoracic inlet. Such damage consists of capsular and intralobular inflammation characterized by fibrosis, hemorrhage, and macrophages containing pigment (siderin) (figure 10.4). It can reach marked levels leading to necrosis. Depending on the severity, clinical-chemical analysis of thyroid hormone levels can show large variations (Rinke 1997).

Ultimobranchial cysts can sometimes be seen. They are lined by squamous epithelium and distended by keratin and cellular debris. In rats such cysts are considered a congenital anomaly (Boorman et al. 1990).

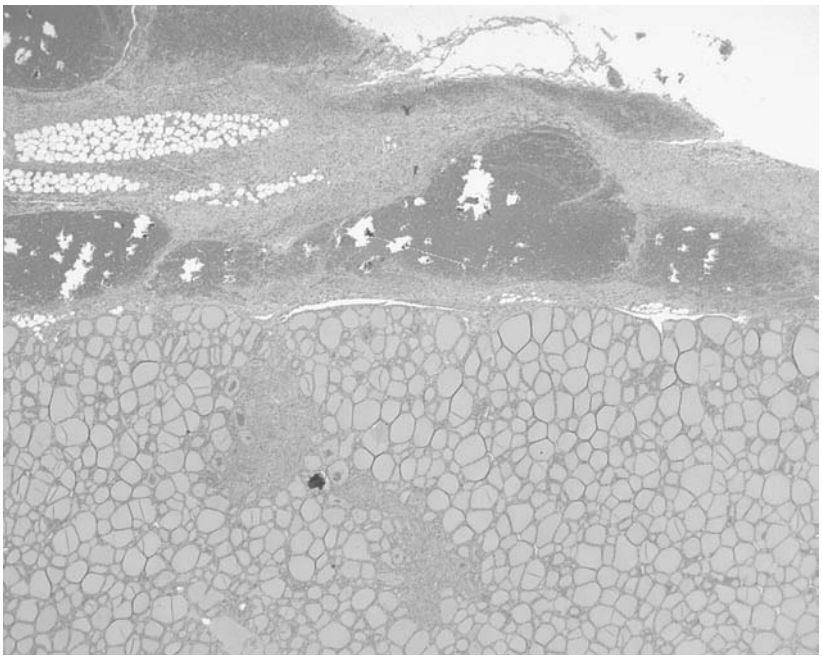

Figure 10.4 Diffuse capsular and intralobular inflammatory, hemorrhagic, and fibrotic changes in the thyroid (H&E, ×2.5).

Parathyroid Glands

The pig is the only species without parathyroid glands attached to, or inside, the thyroid gland, and the glands can therefore be difficult to locate in necropsies. One can be found on either side of the body, close to the bifurcation of the arteria carotis, embedded in the cranial or middle thymus, fat, or connective tissue.

The Göttingen minipig can show cysts, albeit very rarely. The cysts are usually single, and lined by flattened epithelium.

Hematopoietic and Lymphatic Systems

Microscopically the most frequently observed lesion in the Göttingen minipig is, as in other species, focal mononuclear cell infiltration consisting mainly of lymphocytes with smaller amounts of macrophages and plasma cells (Madsen et al. 1998; Svendsen, Skydsgaard, et al. 1998). Generally the infiltration is found interstitially. It could be an indication of a normal immunological potential (Madsen et al. 1998).

Location of iron deposits has been investigated in the Troll minipig (Rinke 1997) and in the Göttingen minipig (Madsen et al. 1998). They have been found in mononuclear phagocytes (e.g., Kupffer cells in liver, reticulocytes in bone marrow, endothelial cells in adrenocortical sinuses), in mesangial cells in the glomeruli and glomerular tufts in kidney, and in the sinuses of local lymph nodes. To prevent development of severe anemia during the first 3 weeks of life, newborn piglets are routinely administered colloid iron preparations parenterally, most often in the neck region. The deposits described are remains after this anemia prophylactic injection of a colloid iron preparation shortly after birth (Svendsen, Bollen, et al. 1998). With age the deposits might eventually disappear.

Lymph Nodes

The lymph nodes of pigs have a unique histological structure, with centrally located cortical tissue and germinal centers (figure 10.5; Nicander et al. 1993).

Routine examinations in toxicological studies generally sample the mandibular and mesenteric lymph nodes. Further investigations can be carried out on the GI and respiratory tracts, as these are of major importance in the lymphatic system (gut-associated lymphoid tissue [GALT] and bronchial-associated lymphoid tissue [BALT], respectively).

Sinusoidal hemorrhage is common in the mandibular lymph node, and is likely to be caused by bleeding from needle puncture during blood sampling, but could alternatively be an agonal phenomenon.

Single small abscesses are relatively common in the mandibular lymph node, usually as a result of local irritation or inflammation. In general they are chronic, characterized by a caseous and calcified center with a surrounding thick layer of fibrotic tissue.

Reactive histiocytosis is relatively common in the Göttingen minipig. It is usually minimal and characterized by dilated sinuses containing variable numbers of histiocytic cells. Lymphocytes and plasma cells might also be present.

Hyperplasia of lymph nodes is occasionally observed. It generally refers to increased numbers of lymphoid cells in the germinal centers (follicular hyperplasia) with the overall lymph node structure still preserved. It is also common to see changes in spleen and in the gut-associated lymphoid tissue.

Granulocytic cell series, particularly eosinophils (confirmed by smear preparations), can sometimes be seen, particularly in the mesenteric lymph nodes at minimal level. Their numbers can increase when there is a hemopoietic demand.

Spleen

Minor amounts of cells of the granulocytic series and macrophages are common and difficult to quantify, however they are described when prominent.

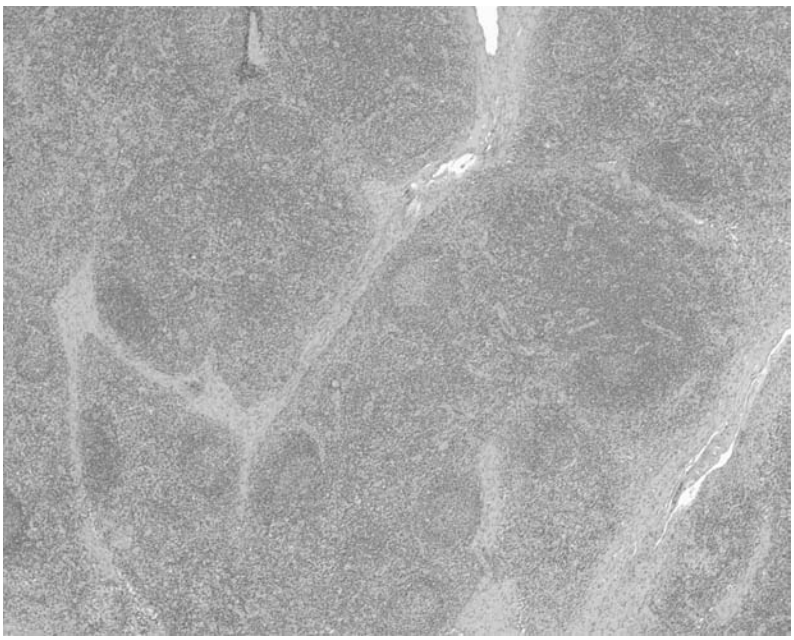

Figure 10.5 Normal structure of a mandibular lymph node in Göttingen minipig (H&E, ×5).

Atrophy of the spleen is very rare as a nonspecific reaction, but it can occur in response to stress or weight loss. It is characterized by lymphocyte depletion of the periarteriolar lymphoid sheaths and marginal zones. Congestion in the spleen occurs as an agonal phenomenon depending on the mode of death.

Thymus

In the minipig the thymus is located in the cranial thorax and neck lying along the trachea. For that reason capsular hemorrhage and inflammation is commonly seen due to blood sampling (cf. the section on the thyroid gland).

Cortical atrophy or hypoplasia can occasionally be observed. It involves thinning of the cortex and lymphocyte depletion. Atrophy can be associated with inflammation or stress.

Lymphoid hyperplasia of the medulla can occur very occasionally in the Göttingen minipig. The medulla becomes crowded with lymphocytes and in advanced cases the number of germinal centers decreases.

Integumentary System

Pig skin has been shown to be anatomically, physiologically, biochemically, and immunologically similar to human skin (Lavker et al. 1991; Zhang and Monteiro-Riviere 1997), and compares favorably to other laboratory animal species as a model in dermal toxicity studies. Today approximately one in three pig toxicity studies in this laboratory involves dermal test article administration.

Macroscopically, pig skin, like that of humans, is firmly attached to underlying structures. There is only limited hair cover, making pig a very important model for the epithelial healing of split-thickness wounds by the proliferation of hair-follicle epithelial cells (Chvapil and Chvapil 1992). The surface pH is 6 to 7, compared to approximately 5 in humans (Meyer 1996).

Microscopically, most features correspond closely to those in human skin. The epidermis, for example, has the same number of cell layers in the viable zones and in the stratum corneum, and as the only animal, pig shows the same rete ridge structure as man. Also, epidermal cell membrane glycoproteins (integrins) form a similar pattern to those of human skin (Zhang and Monteiro-Riviere 1997). Minor differences include the thickness of the epidermis, which is 70 μm to 140 μm in pigs compared to 70 μm to 120 μm in humans (Meyer et al. 1978), and a dermis that is slightly more vascularized in humans. Montagna and Yun (1964) have reported dissimilarities between the skin types including a high content of alkaline phosphatase in the epidermis and sebaceous glands of pigs, and the absence of eccrine sweat glands on the body surface of the pig.

Rodents provide an important alternative to the use of minipigs, particularly the albino rat and mouse. After hair removal, any local effect of a test compound on the skin can be evaluated. The skin of rodents is very thin compared to the skin of humans. Nonrodent alternatives are, however, more scarce. Rabbits are not entirely suitable due to the many particularities in their physiology. Beagle dog skin is both hairy and pigmented, making clinical evaluation of local reaction difficult. Some primates constitute suitable models, but their use involves both practical and ethical problems.

The Sinclair minipig strain has proven to be a useful model of the pathogenesis of melanoma growth and regression, due to their disposition for spontaneously occurring cutaneous malignant melanomas, and the pathologic similarities to human melanomas (Oxenhandler et al. 1982). Also the Yucatan strain has proven to be a useful model in photodermatology studies (Sambuco 1985). UV radiation was shown to induce sunburn cells in the epidermis, with increasing numbers in response to increased exposure. The response was shown to depend on the suberythemal exposure. These findings agree with the behavior in human and mouse.

Skin and Subcutaneous Tissue

Commonly observed nonspecific histological background findings in the Göttingen minipig include mild focal inflammatory infiltrates, intra- and intercellular edema, crust on the epidermal surface, and acanthosis of epidermis. In dermal studies the incidence of these changes can be increased due to method of treatment; for example, by the mechanical action associated with compound application.

Occasionally inflammation of the sebaceous glands and hair follicles is seen. Such changes might be associated with nutritional or hormonal factors, as has been suggested for rats (Platt 1965).

Microabscesses can be seen in the dermis after trauma in response to foreign material or inflammation.

Musculoskeletal System

The pig is rarely employed in studies of the musculoskeletal system, due to the massive nature of the system in this species and the characteristics of quadriped locomotion (Swindle and Smith 1998).

It is important to avoid misinterpretation of treatment-related effects with myotoxic potential and parasitic invasion. This is exemplified by severe changes observed in Troll minipigs, in the form of hyaline degeneration and a lymphohistiocytic reaction in the striated skeletal muscles (Rinke 1997). It occurred in the hind limbs, tongue, and retrotonsillary tissue. Single intracellular sarcosporidia cysts were associated with the affected areas.

Bone

Serous atrophy of bone-marrow fat cells is common in the Göttingen minipig (Svendsen, Skydsgaard et al. 1998). The degeneration of these cells gives a homogeneous eosinophilic appearance and is generally accompanied by a reduction of the hematopoietic tissue (figure 10.6). This lesion is classified as minimal or slight when it is still in the proximal part of the epiphysis of the bone. More rarely, moderate or marked grades are given when the lesion moves to metaphysis and diaphysis. Serous atrophy appears to occur at any age and in either sex, but might be more frequent in males. The precise cause of serous atrophy is unknown, but it occurs in minipigs under normal physiological conditions. Barker (1993) described serous atrophy in calves as a consequence of emaciation and protein–energy malnutrition.

Skeletal Muscle

Minimal to moderate focal or multifocal chronic myositis or myonecrosis is quite common (figure 10.7). Its focal nature and location suggest that it could be considered nonspecific, possibly a result of trauma (Madsen et al. 1998).

The pig is a good model for studying the local effect of intramuscular injections (Svendsen 1988).

Nervous System

The porcine brain is relatively large with structures typical of those of other species. The similarities with human brain development, topical, histologic, and vascular anatomy make them useful as general mammalian models (Swindle and Smith 1998). However, the function of the central nervous system has been studied relatively little. Surgical access to the brain and spinal cord is complicated by the massive nature of the cranial and spinal bone structures.

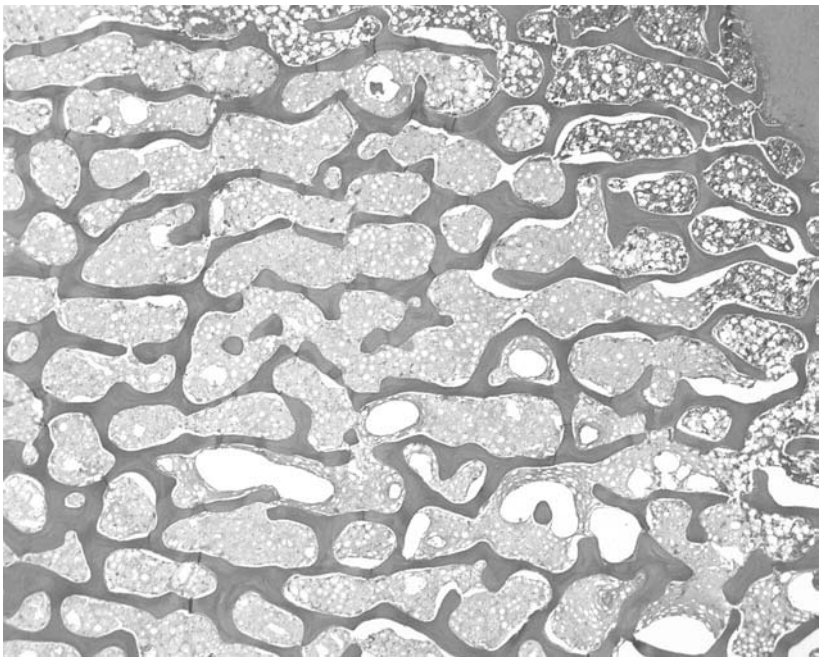

Figure 10.6 Serous atrophy of bone marrow fat cells in femur (H&E, ×2.5).

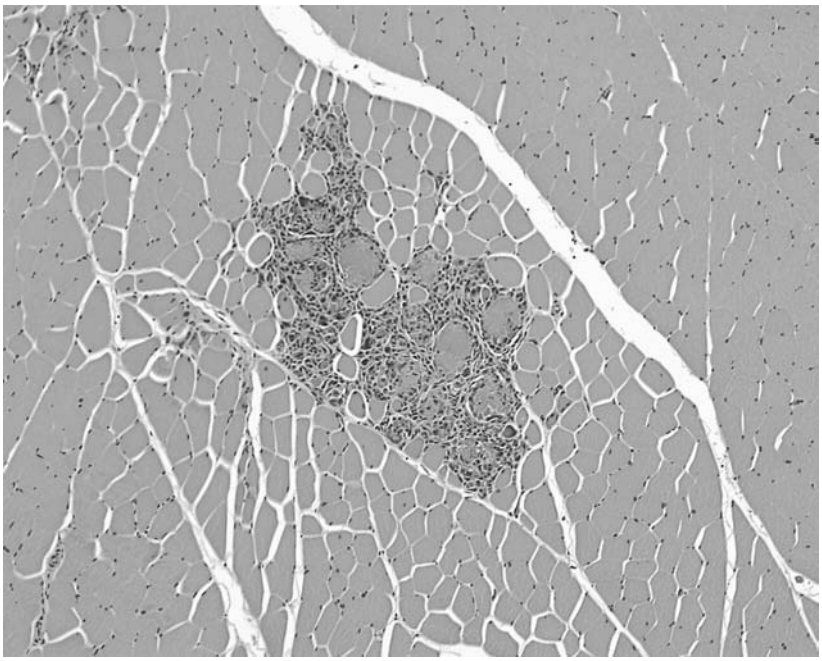

Figure 10.7 Focal myonecrosis of skeletal muscle.

The minipig can be useful in research on Parkinson's disease and provides an economical alternative to primate models (Mikkelsen et al. 1999). In the Göttingen minipig, for example, Parkinson symptoms such as muscle rigidity, hypokinesia, and impaired coordination can be induced. Treatment

consists of subcutaneous *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration at a dose of 1 mg/kg/day, for 6 days; impaired coordination develops within 5 days.

Brain

Large amounts of mineralized areas, particularly in the cerebral and cerebellar leptomeninges, are commonly observed in the Göttingen minipig. Svendsen, Skydsgaard et al. (1998) mentioned that these mineralized foci are genuine rather than artefacts representing skull bone sawdust.

Minimal focal or multifocal mineralized foci located in the pia mater of the meningeal membranes can also be seen rather commonly. They are possibly capillaries and their associated pericytes.

Male Reproductive System

The general structure of the male reproductive system is the same as for humans, but the predominant accessory sex glands differ (Swindle and Smith 1998). They consist of seminal vesicles, the prostate gland, and bulbourethral glands.

Male Göttingen minipigs reach sexual maturity at 3 to 4 months (7–9 kg).

Testes

Unilateral or bilateral testicular tubular hypoplasia or atrophy is a common background finding in the Göttingen minipig (figure 10.8), mainly at minimal levels, but can be moderate. Grading is based on the number of hypoplastic tubules. No abnormalities are visible macroscopically.

The hypoplastic or atrophic tubules are seen microscopically, usually intermingled with normal seminiferous tubules. The abnormal tubules are generally lined by (a) Sertoli cells, (b) Sertoli cells with a basal layer of stem cells, or (c) spermatogonia with no mitotic activity. Their diameters can be diminished, and vacuolation of their germinal epithelium or multinucleate giant cells in their lumen can also be observed.

In moderate cases, the number of interstitial cells (Leydig cells) might seem increased. However quantitative histological studies in bulls have revealed that the actual volume occupied by the interstitial cells might be less than it appears. Also, in severe cases, concurrent degeneration of Sertoli and interstitial cells was observed. It was proposed that degeneration of Sertoli cells could influence the maintenance and regulation of the interstitial cells (Ladds 1993).

The cause of testicular tubular hypoplasia and atrophy in the Göttingen minipig is not known. It has been observed in all types of toxicological studies and does not vary with age for (at least) the first year. In bulls, extensive studies indicate a frequency of testicular hypoplasia on the order of 0.5% to 1%. It can sometimes be much higher, as in the Swedish Highland breed, where an occurrence of 30% has been recorded. This has been shown to be due to genetic factors (Ladds 1993).

Epididymides

Oligospermia or aspermia is a common finding subsequent to testicular hypoplasia or atrophy in the Göttingen minipig. Spermatic granulomas occur very rarely in the minipig.

Prostate Gland

Small foci of mononuclear cells, predominantly lymphocytes, are frequently found interstitially in the prostate of the Göttingen minipig. Alveolar mineralization is also commonly observed.

Focal minimal to moderate amounts of acute or subacute inflammation in the prostate glands can be seen occasionally in older Göttingen minipigs. No cause of this was evident, and in spite

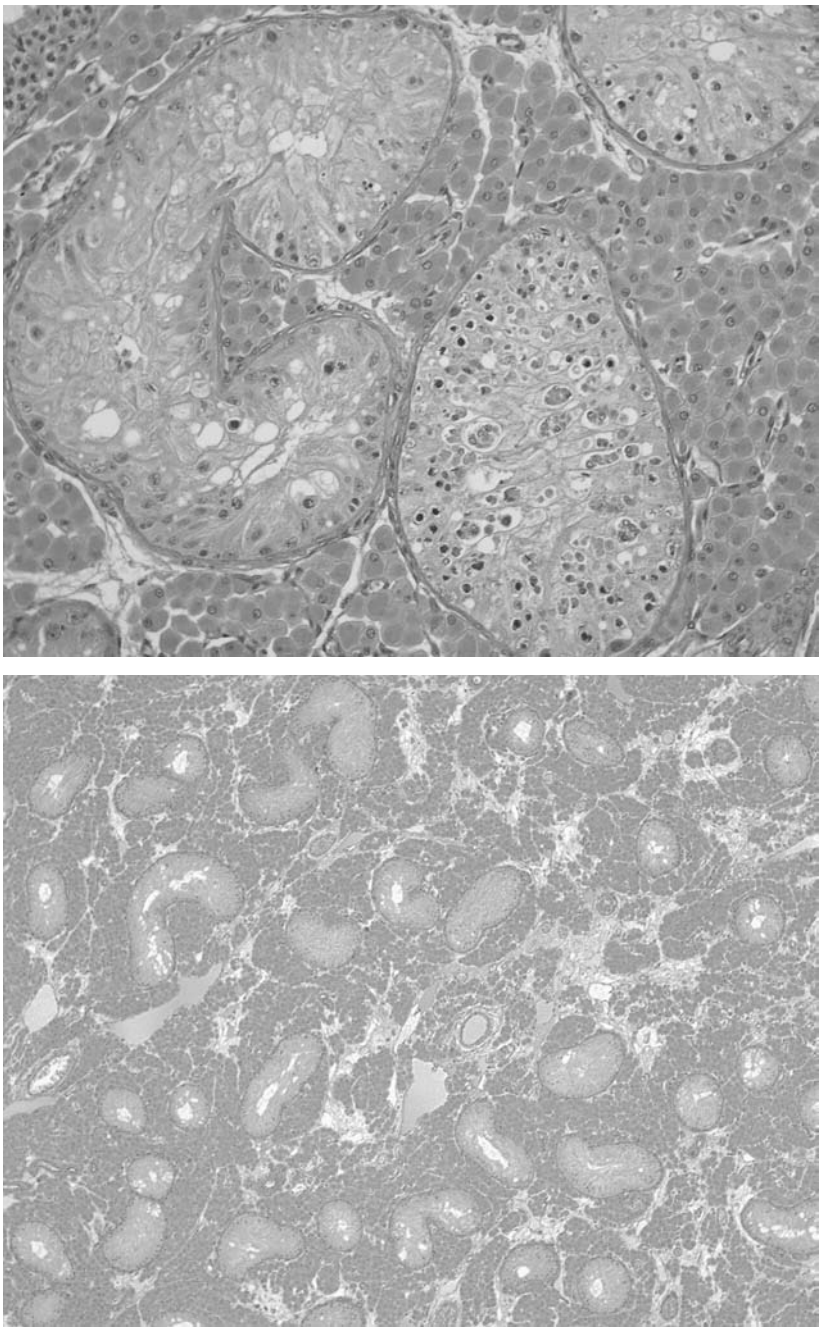

Figure 10.8 (a) Tubular hypoplasia or atrophy in testes (H&E, ×20); (b) Increased number of interstitial cells (Leydig cells) (H&E, ×5).

of what could be expected, no involvement of the urinary bladder, the ureter, and the renal pelvis was observed.

Female Reproductive System

The female reproductive system has a bicornuate uterus with torturous fallopian tubes. The fallopian tubes of an adult female are of the same diameter as those of humans, but much longer.

Pigs have an estrous cycle of 20 to 21 days (Swindle and Smith 1998). Female Göttingen minipigs reach sexual maturity at 4 to 5 months (9–11 kg).

No important background or incidental changes occur in the female reproductive system. In the uterus, focal minimal myometrial hemorrhages and follicular ovarian cysts can occasionally be seen.

Respiratory System

Functional studies of the airway, including neurochemical anatomy and smooth muscle function, make the minipig useful in models of acute respiratory distress syndrome and asthma. The neonatal development of the lungs and airways is useful for extrapolation to humans (Brown and Terris 1996).

Trachea

Adventitial hemorrhage and inflammation is commonly seen due to blood sampling as explained for the thyroid.

Lung

The lungs are composed of apical, middle, and diaphragmatic lobes with an additional accessory lobe for the right lung. The interlobular fissures are incomplete (Swindle and Smith 1998).

Microscopically there is quite a lot to note in the Göttingen minipig. However they are focal and mild lesions.

The most commonly seen lesions in the lungs are multifocal mononuclear infiltrates (predominantly lymphocytes and macrophages) present in the interstitial tissue or perivascularly. Alveolar macrophages are also commonly found (figure 10.9). These macrophage populations are regulatory cells controlling inflammatory, immune, and repair processes through release of a wide array of cytokines and other regulatory molecules.

Mineralized material in alveoli is another common finding, possibly as a result of some debris material.

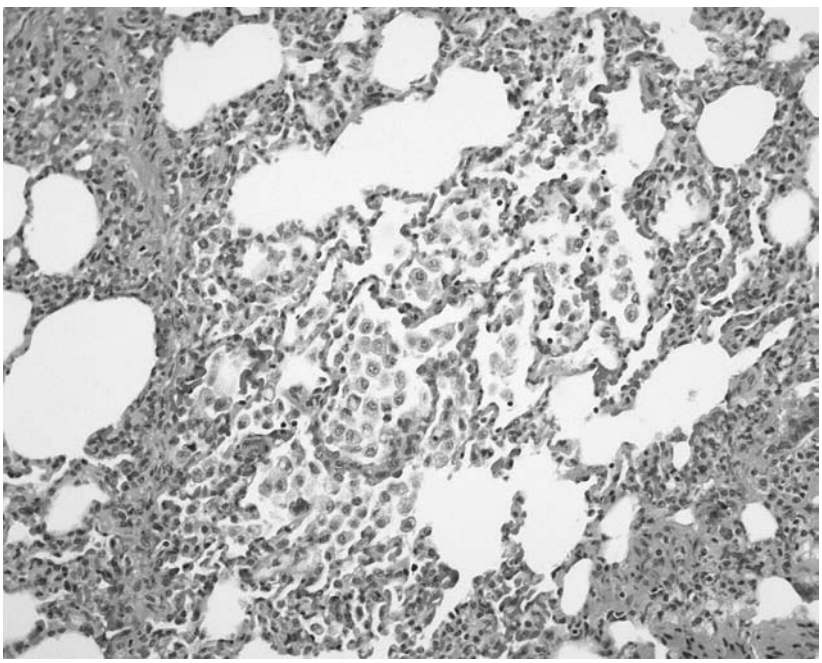

Figure 10.9 Alveolar macrophages in lung (H&E, ×20).

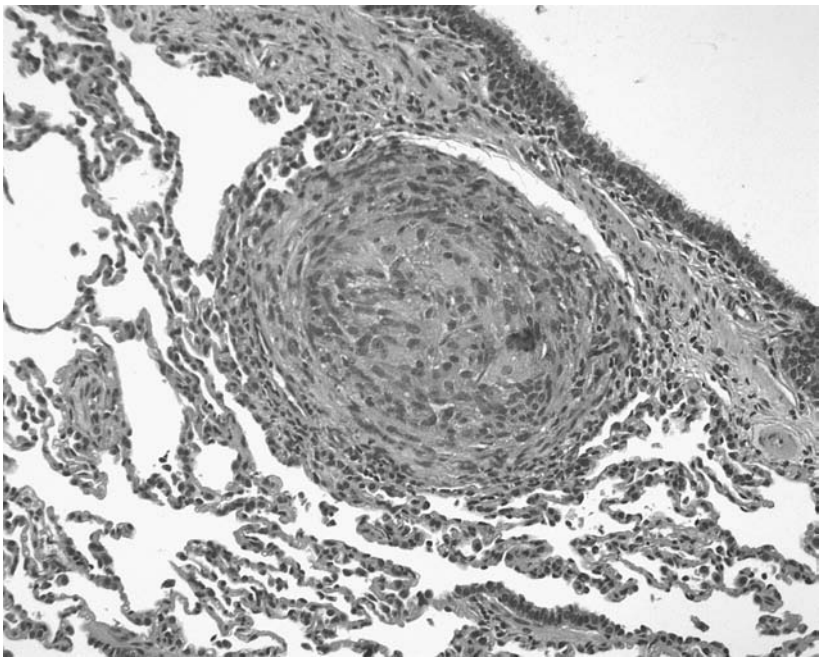

Figure 10.10 An interstitial granuloma in lung (H&E, ×20).

Interstitial pneumonia (alveolitis; figure 10.10) is relatively common. It results from diffuse or patchy damage to the alveolar septa. In the Göttingen minipig, it is generally focal or multifocal, mild, and chronic. The general characterization of the lesions are alveolar accumulations of mononuclear cells, mostly macrophages, and interstitial thickening by lymphoid infiltrates and fibrous tissue. However, an acute form of interstitial pneumonia has been observed. Most interstitial pneumonias in animals are infectious in origin and are caused by viral, bacterial, fungal, or parasitic diseases. Because Göttingen minipigs are free of a range of viral, parasitic, fungal, and bacterial diseases (Hansen et al. 1997; Madsen et al. 2001), these conditions could be a result of stress factors.

Rinke (1997) has noted that nearly half of their examined animals revealed pneumonic alterations, which were severe in some cases in the Troll minipig. Histologically they showed the whole spectrum of general lung pathology; even purulent or abscessing components were found. These lesions are due to a mycoplasmic infection, usually secondarily infected by such bacterial agents common in swine livestock as *Haemophilus pleuropneumoniae, Bordetella bronchioseptica, Pasteuralla multocida,* and others (Dungworth 1993). To avoid such occurrences, optimal hygienic conditions are indispensable.

A variety of foreign-body granulomas can be found fairly commonly in the lungs of the minipig. They are usually hair and particles of diet.

As in other laboratory animals, congestion and hemorrhage in the lungs is a commonly observed agonal phenomenon in the minipig, related to mode of death.

Arterial thrombus, hematoma, and abscess can be found occasionally.

Pleura

Inflammation of pleural surfaces (pleuritis) is observed occasionally. The inflammation is usually minimal, focal, and chronic. It is characterized by fibrosis and mesothelial cell proliferation. Pleuritis can be associated with the inflammation of the lungs or it could be the result of inflammation extending from the thyroid due to blood sampling.

Urinary System

The pig kidney has anatomical and physiological characteristics resembling those of humans, moreso than even primates. For that reason, it is important in the study of pharmacological agents. As for humans, the pig kidney is multirenculate and multipapillate (Swindle and Smith 1998).

Kidney

Of the different organs, the kidney most commonly shows focally accumulated mononuclear cell infiltration, mostly minimal, consisting of lymphoid cells in the interstitial tissue.

Tubular basophilia is regularly observed, particularly in the proximal tubules, and mostly in the cortical part of the kidney (Svendsen, Skydsgaard et al. 1998). It is characterized by basophilic, flattened tubular epithelium sometimes associated with mononuclear cell infiltrates in the surrounding areas. Tubular basophilia is probably the most frequently encountered characteristic of induced nephropathies. It can follow degenerative conditions or represent excessive cell turnover (Gopinath et al. 1987). Because of the focal and mild nature of the change, it is considered to represent de- or regenerating tubules in the Göttingen minipig.

Focal mild interstitial inflammation and fibrosis (interstitial nephritis) can be seen on occasion, usually located in the renal cortex. These focal and mild lesions can be attributed to tubular defects of resorption or secretion. To see these inflammatory changes at moderate or marked levels is extremely rare.

Small foci of mineralization observed as amorphous basophilic deposits in H&E sections are commonly seen in the Göttingen minipig, mostly in the papilla.

Solitary cysts and dilated tubules with lumenal proteinaceous casts are occasionally found, usually in the cortex and medulla. They are associated with flattening of the tubular epithelium (figure 10.11).

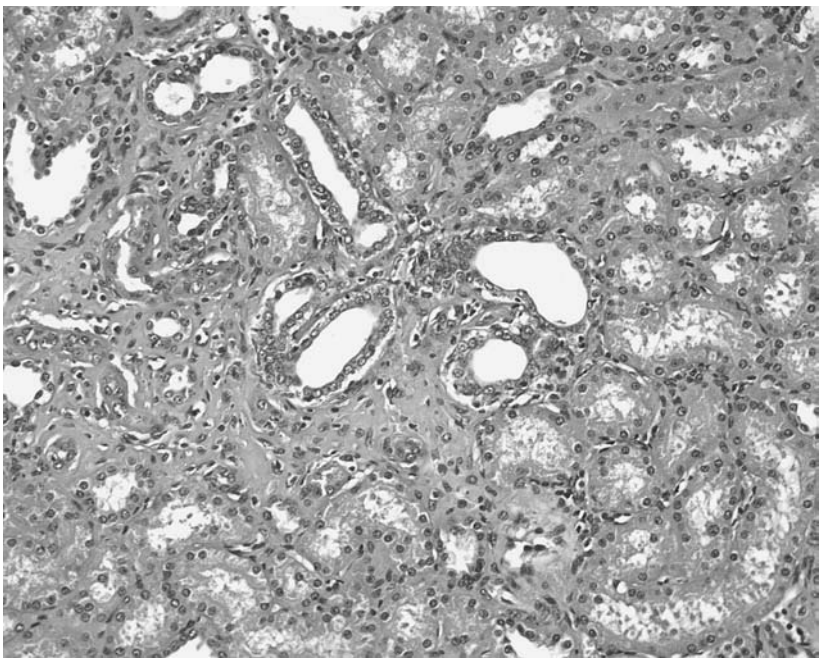

Figure 10.11 Focal area of tubular basophilia in the renal cortex. Dilated tubules with flattened basophilic epithelium and interstitial fibrosis (H&E, ×40).

Urinary Bladder

Small foci of inflammatory infiltrates can occasionally be observed in the mucosa and submucosa. Inflammation of the urinary bladder (cystitis) is observed on rare occasions, secondary to kidney problems.

METABOLISM

Minipigs are increasingly being used in pharmaceutical and toxicological studies, thus their metabolism have recently attracted considerable attention. The reactions catalyzed by biotransforming enzymes are generally divided into two groups: phase 1 and phase 2 reactions. Phase 1 reactions involve the mono-oxygenases such as cytochrome P-450 complex, and flavin-containing mono-oxygenases (FMOs). The cytochrome P-450 complex consists of several subfamilies with different substrate specificities, different inhibitors, and inducers. Several of these subfamilies, as summarized in Table 10.9, (CYP1A, CYP2A, CYP2B, CYP2C, CYP2E, and CYP3A) have been characterized for the minipig during the past few years using hepatocyte microsomes (Anzenbacher et al. 1998; Bogaards et al. 2000; Skaanild and Friis 1999). Two strains of pigs are commonly used, the Göttingen minipig and the Yucatan mini (micro) pigs. In table 10.8, total cytochrome P-450 and the activity of reactions catalyzed by different cytochrome P-450 isoenzymes are presented for both Göttingen minipigs (4 males, 4 females, 4 months) and Yucatan minipigs (3 males, 3 females, 9–10 months). The average total cytochrome for all Göttingen minipigs was more than two times higher than the human values, 0.81nmol/mg protein (Skaanild and Friis 1999) versus 0.33nmol/mg protein (Shimada et al. 1994).

CYP1A

The O-dealkylase activity of 7- ethoxyresorufin, a known substrate for the CYP1A subfamily, was sex related for both minipig strains. The female contained two to four times more than males, whereas the opposite was found for humans (Bogaards et al. 2000), where females contained three

Table 10.8 Total Cytochrome P-450 Content and Cytochrome P-450 Mediated Mean Enzyme Activities in Minipigs and Humans

Enzyme Activity	Sex	Gottingen Minipig[a]	Yucatan Micropig[c]	Human[b]
Total cytochrome P-450		0.81		0.31
7-ethoxyresorufin O-dealkylase activity	F	0.08	0.116	0.03[c]
	M	0.02	0.06	0.09[c]
Coumarin 7-hydroxylase activity	F	0.35	0.97	0.433
	M	0.005	0.18	
Mephenytoin 4-hydroxylase activity		ND	< 0.0004	0.032
Debrisoquine 4-hydroxylase activity		ND		
Bufuralol 1-hydroxylase activity		0.72	0.64	0.07
Dextromethorphan O-demethylase activity		4.4		0.125
Chlorzoxazone 6-hydroxylase activity	F	6	0.71	0.216
	M	1.25	0.29	0.216
Testosterone 6-hydroxylase activity		0.87	4.45	1.69
Nifedipine oxidase activity		1.8		0.645[d]

Note: All activities: nmol/mg prot/min; F = female, M = male, ND = not detectable.

[a] Skaanild and Friis (1999), Skaanild and Friis (submitted).

[b] Chauret et al. (1997).

[c] Bogaards et al. (2000).

[d] Shimada et al. (1994).

Table 10.9 Minipig Cytochrome P-450 Isoenzymes, Substrates, and Inhibitors

Subfamily	Substrates[a, b, c]	Inhibitors[d]
CYP1A	Ethoxyresorufine	Alfa-napthoflavone
CYP2A	Coumarin	Troleandomycin Methoxypsoralen
CYP2B	7-ethoxy-4-trifluoromethylcoumarin	
CYP2C	Diclofenac	
CYP2D (CYP2B)	Dextromethorphan Bufuralol	Orphenadrine[a]
CYP2E (CYP3A)	Chlorzoxazone	Troleandomycin Diethyldithiocarbamate
CYP3A	Testosterone Nifediphine	Ketoconalzole

[a] Skaanild and Friis (1999), Skaanild and Friis (submitted).
[b] Anzenbacher et al. (1998).
[c] Bogaards et al. (2000).
[d] Madden et al. (1998).

times less than males. The activities ranged from 0.02 to 0.12nmol/mg/min. Anzenbacher et al. (1998) found that the activity in male minipigs was about seven times less than the human activities. Western blotting using antihuman antibodies estimated the apoprotein level for the Göttingen minipig, and a positive correlation between enzyme activity and protein level was found. The mRNA concentration was also measured semiquantitatively using RT-PCR (Skaanild and Friis 1999). The results from the RT-PCR did not correlated with either the activity or the protein level. This might indicate that the regulation of the expression was translational or pretranslational. Alfa-napthoflavone, a known inhibitor of CYP1A, strongly inhibited the 7-ethoxypreresorufin O-dealkylase (Bogaards et al. 2000; Madden et al. 1998). The inhibition of CYP1A was stronger in humans (80%–100%) than in minipigs (60%–80%) when incubated with 1μM α-napthoflavone.

CYP2A

The CYP2A activity was determined using coumarin as test substrate. A sex-dependent activity was also seen for the coumarin 7-hydroxylase activities in both minipig strains. Göttingen minipigs, especially, showed differences, with the male having 70 times lower activity than females (0.005 nmol/mg protein, 0.35nmol/mg protein; Skaanild and Friis 1999). The Yucatan minipigs showed a five times difference (0.18 nmol/mg protein, 0.97 nmol/mg protein; Bogaards et al. 2000). The human activity was the same for both sexes (0.433 nmol/mg protein) about the same as the Göttingen female. Again the apoprotein level and the mRNA were determined for the Göttingen minipig (Skaanild and Friis 1999). Positive correlations were found among enzyme activity, apoprotein level, and mRNA, indicating a transcriptional regulation of the gene. The activity of CYP2A could be inhibited by both troleandomycin and methoxypsoralen, two known inhibitors of CYP2A activity (Madden et al. 1998). Inhibition with antihuman CYP2A6 was strong (60%–80%), indicating that this isoenzyme is the main one responsible for the coumarin 7-hydroxylase activity (Bogaards et al. 2000).

CYP2B

Several CYP2B substrates have been used to measure the activity of this isoenzyme. The 7-pentoxyresorufin O-demethylase and the debrisoquine 4-hydroxylase activity were not present (Anzenbacher et al. 1998; Skaanild and Friis 1999). However, the 7-ethoxy-4-triflouromethylcoumarin O-dealkylase activity, another CYP2B-specific activity, was reported in the Yucatan minipig. It was found that females and males contained about the same activity (0.353 nmol/mg protein,

0.286 nmol/mg protein), and they were comparable with human values. The enzyme activity for both humans and pigs could be inhibited moderately (30%–60%) with antirat CYP2B1.

CYP2C

The CYP2C subfamily consists of several enzymes. In humans, four different enzymes have been defined (Guengerich et al. 1997): CYP2C8, CYP2C9, CYP2C11, and CYP2C19. The diclofenac 4-hydroxylase activity specific for human CYP2C9 was measured in Yucatan minipigs (Bogaards et al. 2000), whereas the mephenytoin 4-hydroxylase activity, a CYP2C19-specific reaction, was estimated in both strains. The diclofenac 4-hydroxylase activities were estimated to be 0.06 nmol/min/mg protein for males and 0.02 nmol/mg/min for females. These activities were much smaller than those in humans (1.5–1.9 nmol/mg/min), and the activity of the other CYP2C isoenzyme was not detectable. Immunoblotting using antirat CYP2C11 did react with the porcine CYP2C protein and the highest protein level was found for the males. The human diclofenac 4-hydroxylase activity can be strongly inhibited (80%–100%) by 10 μm sulphaphenazole, whereas the minipig activity could only be inhibited 15% to 30% using the same concentration (Bogaards et al. 2000).

CYP2D

Three different test substrates for CYP2D6 have been used to characterize the minipig CYP2D. The Göttingen minipig possessed no debrisoquine 4-hydroxylase activities, but could biotransform dextromethorphan to dextorphan. Bufuralol 1-hydroxylase activity was found in both pig strains, but it is questionable whether these activities are catalyzed by the CYP2D isoenzymes family, as the reaction cannot be inhibited by either antihuman CYP2D6 or guanidine and quinine, two known chemical inhibitors of CYP2D6 (Bogaards et al. 2000). The dextromethorphan O-demethylase activity could, on the other hand, be inhibited by orphenadrine, a CYP2B inhibitor. The dextromethorphan and bufuralol enzyme activities correlated with the CYP2B protein level in the Göttingen minipig, indicating that CYP2B isoenzymes might be responsible for these enzyme activities (Skaanild and Friis, submitted). The CYP2D apoprotein could be measured only using polyclonal antihuman CYP2D6, but not with monoclonal antibody. The CYP2D6 apoprotein concentration did not correlate with enzyme activities.

CYP2E

The chlorzoxazone 6-hydroxylase activity was found to be sex related in both minipig strains, with the females possessing the highest activity. In humans, however, no differences between the sexes were seen (Chauret et al. 1997). The Göttingen minipig activity was higher (F = 6 ng/min/mg protein; M = 1.23 ng/min/mg protein) than the activity in Yucatan minipigs (F = 0.771 ng/min/mg protein; M = 0.29 ng/min/mg protein) and humans (0.22 ng/min/mg protein; Chauret et al. 1997). The apoprotein level and mRNA concentration of CYP2E did not correlate with the enzyme activity, indicating that the regulation of the gene expression could take place at all steps in the biosynthesis of the protein or that this substrate is not specific for the minipig CYP2E (Skaanild and Friis 1999). The human chlorzoxazone 6-hydroxylase activity could be moderately inhibited by antirat CYP2E1, whereas the minipig activity was not inhibited. Ketoconazole and troleandomycin (TAO), two CYP3A inhibitors, could, on the other hand, inhibit the activity up to 70% to 80% (Madden et al. 1998). This also indicates that chlorzoxazone is not a specific CYP2E substrate in minipigs, but also a CYP3A substrate.

CYP3A

Testosterone was used as test substrate in both minipig strains, whereas nifedipine was only used when testing the enzyme activity in Göttingen minipigs. The Yucatan pig had a higher enzyme

activity than the Göttingen minipig, 4.45 nmol/min/mg protein compared to 0.87 nmol/min/mg protein. The testosterone 6-hydroxylase activity in humans was 1.69 nmol/min/mg protein. The nifedipine oxidase activity correlated well with the testosterone 6-hydroxylase in the minipig, and these activities correlated with the apoprotein level measured using antihuman CYP3A in the immunoblotting assay (Skaanild and Friis 1999). Ketoconazole, a strong inhibitor of human testosterone hydroxylase, was also a strong inhibitor of minipig testosterone hydroxylation (Bogaards et al. 2000; Madden et al. 1998).

A third strain of pigs, the Hanford minipig, has also been used, but their cytochrome P-450 has not been characterized that extensively. Peggins et al. (1984) examined the MMFO in a broad range (10 months–12 years) of Hanford minipigs. They identified definite age-related differences. The amount of cytochrome P-450, the MFO activity with aniline, and p-chloro-N-methylaniline were all significantly higher in middle-age (5–8 years) versus young (less than 4 years) minipigs. Freudendale et al. (1976) examined Hanford minipigs in the 2- to 8-month range, and obtained somewhat different cytochrome P-450 (approximately 0.95 nmol/mg protein) values than did Peggins et al. (1984; approximately 0.5 nmol/mg protein). This can be compared to the cytochrome P-450 values obtained for 4-month-old Göttingen minipigs (0.81 nmol/mg protein) examined by Skaanild and Friis (1999). The reported ranges for aniline hydroxylase (about 50 nmol/min/mg protein) were similar in the papers analyzing Hanford minipigs. These results cannot be compared with the two other minipig strains as no values have been reported. Hence, the available data on the MFO of young Hanford minipigs is fairly consistent.

The induction of the different minipigs' P-450 isoenzymes has been studied either *in vivo* or using liver microsomes or hepatocyte cultures. Early work reporting cytochrome P-450 induction characteristics was done by Mueller et al. (1980). They compared the effects of *in vivo* Aroclor induction on the subsequent responses in the Ames *Salmonella* mutagenicity test of seven different species with five different known mutagens. Animals were treated with a single dose of Aroclor 1254 (500 mg/kg intraperitoneally in sesame oil) and sacrificed 5 days later. The minipig ethylmorphine assay responded in the same fashion as rats and mice, with large increases (3.9-fold) in demethylase activity. Liver fraction from untreated minipigs had low activity in the Ames assay with benzo(a)pyrene, cyclophosphamid, and diethylnitrosamine. In contrast, liver preparations from induced animals greatly increased activity (five- to tenfold) in the Ames assay with these mutagens. This is a pattern very similar to that seen in rats. Thus, the MFO of the minipig is inducible and the resulting changes in the metabolism might not be dissimilar from those produced by rats. More recent work on induction of cytochrome P-450 isoenzymes has been carried out. Kaltenbach et al. (1996) examined the pharmacokinetics of the fungicide itraconazole in Yucatan minipigs (3 females, 1 year). They discovered that the coadministration of rifampin resulted in an 18-fold decrease in the maximum concentration of itraconazole in serum. This was probably caused by an induction of CYP3A activity, as rifampin is a potent inducer of these isoenzymes. In another report, Yucatan minipigs (6 castrates, 6 males, 20 kg) were fed ethanol, 5g/kg body weight per day, to assess possible links among ethanol-induced oxidant stress, expression of hepatic cytochrome P-450, and sex steroid status. The isoenzyme level of CYP2A, CYP2E, and CYP3A was determined using immunohistochemical methods. In control pigs the CYP2A and CYP3A were more dominant in castrated animals than in males. Ethanol feeding increased the hepatic content of all three CYP isoenzymes. The most significant increase occurred in CYP2E and CYP3A in the males, and in CYP2E and CYP2A in the castrated animals. Significant correlations between the levels of the different CYP isoenzymes, protein adducts, and plasma levels of sex steroids were found. The protein adducts were formed between proteins and the metabolites of acetaldehyde, malondialdehyde and 4-hydroxynonenal. Another report (Stiborova et al. 2001) studied the adduct formation between DNA and metabolized aristolochic acid (AA) for humans, minipigs, and rats using liver microsomes. The DNA adduct profiles were found to be the same for all three species. Further studies using inhibition with alfa-naphthoflavone and furafylline revealed that most of the microsomal activation of AA could be attributed to CYP1A1 and CYP1A2. Induction of CYP1A and

CYP3A in cultured hepatocytes by model inducers have been compared in minipigs (Yucatan: 1 male, 1 female, 15 weeks), humans, Sprague-Dawley rat, and beagle dog (Lu and Li 2001). Omeprazole was a potent dose-dependent inducer of CYP1A activity in minipig, human, and beagle dog hepatocytes, but not in rat hepatocytes. Dexamethasone and rifampin induced human CYP3A4, with rifampin being the more potent. Conversely, in rat hepatocyte, dexamethasone was a potent CYP3A inducer, whereas rifampin was not an inducer. Rifampin, but not dexamethasone, induced CYP3A in minipig and beagle dog hepatocytes. These species differences indicate that minipig hepatocytes mimic the human induction pattern better than rat.

The FMO has traditionally been studied in hog liver obtained from slaughterhouses (Tynes and Hodgeson 1984). Interestingly, when FMO activity is compared between species, substrate specificities are found to be generally very similar (Tynes and Hodges 1984). Rettie et al. (1990) have isolated and studied the FMO from Yucatan minipigs. As with the enzyme studied from other species, the hepatic enzyme exists as a single isozymic species, is active with both demethylaniline (N-oxide formation) and alkyl p-tolyl sulfides (sulfoxidation), and is enantioselective in metabolite formation. It would thus appear that the minipig does not differ significantly from conventional pigs in the presence or activity of FMO.

The phase 2 biotransformation reactions (e.g., conjugations, acetylation, and sulfation) have not yet been characterized for Göttingen and Yucatan minipigs, but activities in conventional pigs can be used to help infer the expected values until more complete and specific information appears in the literature on minipigs. However one of the conjugation reactions, the glucuronosyl transferase using 4-nitrophenol as substrate, was measured in Hanford minipigs and found to be higher in middle-aged (5–8 years) pigs compared to young pigs (less than 4 years; Peggins et al. 1984). The pig microsomal activity was similar to the activity in rat microsomes, whereas the activity in normal pigs was about double (4.51, 5.5, and 9.38, respectively). The glutathione S-transferase activity in the minipig has been compared to the activity in rats. Kirby et al. (1980) reported that ethylene dibromide was rapidly metabolized and cleared by pigs in a fashion similar to that for rats. As ethylene dibromide is a substrate for the rat liver glutathione S-transferase system, that of minipigs must have some characteristic in common with that of the rat.

Pharmacokinetics

One of the earliest papers that compares *in vivo* pharmacokinetic behavior of a specific chemical in the minipig to another animal studied the toxicology and pharmacokinetics of cyclotrimethylenetrinnitramine in rat and minipig (Schneider et al. 1977). Rats convulsed within the first several hours after receiving this chemical, whereas minipigs convulsed at 12 hr to 24 hr. This is consistent with the observation that at 24 hr postdosing (100 mg/kg po), the plasma levels were 3.0 μg/ml in rats and 4.7μg/ml in minipigs. The latent period for convulsion development was more similar between minipigs and humans than between rats and humans, indicating that the minipig is a more suitable model for studying human metabolism of nitramines. More detailed reports measuring the plasma concentration of drugs, protein binding, and clearance have since been published. Bailie et al. (1987) studied the pharmacokinetics of acetaminophen, vancomycin, and antipyrine in Hanford miniature pigs (5 males, 14–21 kg). On separate days they were IV doses acetaminophen 40 mg/kg, vancomycin 40 mg/kg, and antipyrine 15 mg/kg. Blood samples were collected and the plasma concentrations versus time profile revealed that the disposition of acetaminophen was monoexponential, whereas the disposition of vancomycin and antipyrine was biexponential. The volume of distribution (Vd) for the three drugs in pigs was the same. Vd for acetaminophen in pigs was smaller than that for humans, whereas Vd for the other two drugs was smaller in humans (table 10.10). The half-lives of the drug were longer in humans than in pigs, especially for antipyrine, and the clearance in pigs was faster than clearance in humans. Again the difference was pronounced for antipyrine with a 20-fold difference. Cockshott et al. (1992) studied the pharmacokinetics of the anesthetic propofol in rats, dogs, rabbits, and minipigs (4 male Alderly Park). Following single

bolus dose to all animals, blood samples were collected 12 hr to 24 hr postdose. The serum concentrations data fitted either a two- or tri-exponential function depending on the time of sampling. Propofol was distributed into a large initial volume (1–2 L/kg) and extensively redistributed (Vd = 2–10 times body weight) in all species. Clearance by all species was rapid, ranging from about 30 to 80 ml/kg per min in rats, dogs, and pigs to about 340 ml/kg/min in rabbits. The pharmacokinetics and metabolism of diclofenac, an NSAID, has been studied using 4 male Yucatan miniature pigs (Oberle et al. 1994). From earlier studies in other animal species it was known that the metabolism of this drug was species dependent, and large differences between dog and human were observed. Compared to human values the absolute bioavailability of an oral-administered buffered solution was high (97%–107%) in pig compared with 50% in humans. The total plasma clearance, after IV dosing, in the minipig was five times slower than in humans (57 ± 17 compared to 252 ± 54 ml/hr/kg) and the plasma level of the different hydroxy metabolites of diclofenac was considerably lower in minipigs. These results suggest slower metabolism or enterohepatic recirculation of the parent drug in minipigs, indicating that the pig was not an appropriate model for studying the metabolism and pharmacokinetics of diclofenac in humans. In contrast to this, a pharmacokinetic study of the β-lactams cefepime, cefpirome, and meropenen showed that the pig was a better model than rats and monkeys. It was concluded that the minipig was an adequate animal model for studying the pharmacokinetics of these β-lactams (Elkhaili et al. 1997), as $T_{1/2}$, C_{max}, and CL_p were the same for humans and pigs. They also found that it was a reliable model to investigate the influence of the method of administration (direct IV vs. continuous infusion) on tissue penetration of β-lactams as well as on the dynamics of killing of bacteria. Different administration routes were also studied by Tse and Laplanche (1998). They investigated the role of administration route in absorption, metabolism, and disposition of an acetylcholinesterease inhibitor (SDZ ENA 713). The minipigs (12 males, Göttingen, 4 months, 7.2–8.3 kg) were given a single oral, a single IV, and two different dermal doses (3 pigs per group). The oral doses were rapidly and efficiently absorbed (93%), and the bioavailability of the parent drug was low (0.5%), apparently due to extensive first-pass metabolism. The half-life was 56 hr after oral dosing and slightly shorter after IV dosing (48 hr). After dermal administration to virgin skin, absorption was low (8%), but daily application of placebo patches for 10 days and then dosing at the same site increased the absorption to 17% to 19%. This is probably due to abrasion and hydration of the skin after repeated applications and removal of the adhesive patches. The bioavailability of dermal doses was 20 to 40 times higher than that of oral doses, showing that different administration routes give rise to different pharmacokinetic parameters.

Table 10.10 Total Body Clearance (L/h/kg) of Some Drug in Minipigs, Dogs, and Humans

Drug	Minipig	Dog	Human	References
Acetaminophen	0.54	0.24	0.3	Bailie et al. (1987)
Vancomycin	0.35	0.18	0.066	Bailie et al. (1987)
Antipyrine	0.64	0.42	0.034	Bailie et al. (1987)
Cefepime	0.12	0.13	0.11	Elkhaili et al. (1997) Gardner and Papich (2001)
Cefpirome	0.11	0.19	0.11	Elkhaili et al. (1997)
Meropenem	0.3	0.24	0.24	Elkhaili et al. (1997) Harrison et al. (1989)
Meloxicam	0.043	0.01	0.01	Busch et al. (1998)
Moxifloxacin	0.645 (1.39)	0.222 (1.33)	0.132 (1.39)	Siefert et al. (1999)

New antibacterial drugs with a broad spectrum are constantly being developed, some of the latest being the 8-methoxyquinolones. The pharmacokinetics of one of these, moxifloxacin, has been compared for humans and other species such as mice, rats, rhesus monkeys, dogs, and minipigs (3 females, Göttingen, 1–2 years, 10–17 kg; Siefert et al. 1999). The pharmacokinetics was inves-

tigated after IV and oral administration of moxifloxacin-HCl to animals and human volunteers. The results indicated a clear dependence on the species. Moxifloxacin is absorbed quickly (rats, dogs, humans > monkey): The major portion of the dose reached the systemic circulation within the first 2 hr. In the minipig, however, absorption was slower. The plasma protein binding was minimal at 55% to 71% (human > monkey, rat, minipig > mouse > dog); the derived AUC_{norm} was higher in humans than in all animal species (human > dog > minipig, monkey > rat > mouse). Total plasma clearance for all species was higher than that for humans, whereas the renal clearance for the animals was comparable to the renal clearance for humans. The Vss for the animal ranged from 4.9 to 2.7 l/kg, with monkeys having the highest and dogs the lowest, closest to those of humans. Allometric scaling based on body weight and different pharmacokinetics parameters such as Vss, total body clearance, and mean residence time showed a good correlation.

The pharmacokinetics of meloxicam, belonging to another group of drugs (NSAIDs), was investigated in several species including minipigs (4 males, 13–18 kg, 1 male and 1 female Göttingen minipig, 13 kg), mice, rats, and baboons (Busch et al. 1998). The plasma concentration versus time profiles for meloxicam in rats and dogs were comparable to that of humans, whereas there were marked differences between humans and mice, minipigs, and baboons. The excretion balance, on the other hand, in minipigs resembled that in humans, with almost equal concentrations being eliminated in the urine and the feces. As in humans, meloxicam circulated mainly in the form of the parent compound in the plasma of mice, rats, dogs, minipigs, and baboons. The main metabolite in rats, minipig, and humans were a 5`-hydroxymethyl derivate and a 5`-carboxy metabolite. The pharmacokinetic profile of meloxicam in rats, however, most closely resembled that of humans with $T_{1/2}$ the same as for humans, whereas the minipig $T_{1/2}$ was about 10 times longer (13.4 hr compared to 121 hr).

The pharmacokinetics of two model drugs—atenolol, a beta-blocking agent with small extent of first-pass biotransformation of the parent drug, and 5-aminosalicylic, an antiphlogistic agent with a rapid biotransformation in humans—were compared in humans, dogs, and minipigs (5 Göttingen, average weight = 24.5 kg) (Kvetina et al. 1999). The pharmacokinetics parameters, such as $T_{1/2}$ (table 10.11), AUC, and c_{max}, for atenolol were comparable for both humans, dogs, and minipigs. The same parameters for acetylsalicylic acid, however, were not quite the same for the three species. $T_{1/2}$ was about two times longer in humans than in the dogs and minipigs, whereas the AUC was 10 times higher in dogs than in humans and pigs. In humans and minipigs, acetylsalicylic acid was metabolized to N-acetyl-5-aminoacetylsalicylic acid and the pharmacokinetics parameters for this metabolite were comparable in humans and minipigs. This metabolite could not be detected in dogs. This study indicates that for drugs with relatively small first-pass effect of biotransformation, there is a greater choice of suitable animal species for studies of comparative pharmacokinetics (as, e.g., in the case of atenolol, where the pharmacokinetics parameters were similar for humans, minipigs, and dogs). On the other hand, when first-pass effect is more pronounced, the situation is

Table 10.11 Plasma Elimination Half-Lives (min) of Some Drugs in Minipigs, Dogs, and Humans

Drug	Minipig	Dog	Human	References
Acetaminophen	62	107	120	Bailie et al. (1987)
Vancomycin	88	102	330	Bailie et al. (1987)
Antipyrine	63	78	726	Bailie et al. (1987)
Cefepime	876	65	1,080	Elkhaili et al. (1997) Gardner et al. (2001)
Cefpirome	774		1,070	Elkhaili et al. (1997)
Meropenem	53	45	50	Elkhaili et al. (1997) Harrison et al. (1989)
Meloxicam	6,270	144	84	Busch et al. (1998)
Moxifloxacin (po)	660	540	720	Siefert et al. (1999)
Moxifloxacin (iv)	342	514	780	Siefert et al. (1999)

more complicated, as the choice of the suitable species depends much more on the mechanism of biotransformation of the given drug and on the enzymes of biotransformation. It therefore depends on the activity and availability of the biotransforming enzymes in the animal species.

The pharmacokinetics and metabolism of a vitamin D has been studied both *in vivo* and *in vitro* using rats and minipigs (3 males and 3 females, 10 kg) and liver microsomes isolated from the two animal species and humans (Kissmeyer and Mortensen 2000). The pharmacokinetic parameters were the same after one or several doses in the two animal species. A good correlation between the metabolic profile obtained *in vitro* and *in vivo* was found, which is important, as most knowledge of the hepatic metabolism in human must be based on the *in vitro* finding if the drug has not been clinically tested.

Until recently the pharmacokinetics and metabolic studies in minipig have involved oral, dermal, and IV routes of administration. Koch et al. (2001) have investigated the pharmacokinetics of verapamil, a calcium channel blocker, using IV and inhalation as routes of administration. Minipigs (2 males and 2 females, Göttingen) were given an IV injection (0.5mg/kg) and blood samples were collected. Twelve days later, when no verapamil could be detected in the blood, the same animals were exposed for 1 hr to an aerosol concentration of ~50 mg/m^3 verapamil. The total estimated dose after 1 hr was 0.79 mg/kg. The verapamil plasma profile revealed that the pharmacokinetic parameters such as clearance time, initial distribution volume, and partitition coefficient, for respiratory delivery of the test substance are almost the same as for IV administration. The plasma level increased immediately without delay after the start of the exposure, suggesting a fast transfer of the active substance from epithelium into blood.

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CHAPTER 11

Alternative Species

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CONTENTS

This book has concentrated on the eight most commonly used laboratory animals. As illustrated in table 11.1, many other types of species are used in biomedical research and toxicological assessment that have not been discussed (see chapter 13 for a more complete discussion on animal model selection). For example, the chicken is a common model for organophosphate insecticide-induced neurotoxicity (Murphy 1986), but we have made no mention of the chicken in this book (nor do we again). Discussing all uses for all species used would have resulted in an encyclopedia, not a convenient source book. These less commonly used species, however, should not be totally ignored. The practice of good science demands that a species be appropriate to the question being pursued. The principle of responsible use of animals demands that we consider the replacement of higher animals with lower species. Here I discuss some less commonly used species that could be used as replacements for other species in some types of toxicity testing: earthworms and fish.

Table 11.1 Example of Uncommonly Used Animal Species

Species	Model or Applications
Armadillo	Leprosy
Aplysia	Behavioral studies
Bear	Sleep/hibernation
Bat	Hearing, sonar, microcirculation
Chicken	Insecticide toxicity, renal excretion
Cat	CNS physiology
Crab	Toxicity screen
Fish	Drug metabolism, carcinogenicity, environmental impact
Hydra	Teratogenetic testing
Insects (cricket, fly)	Genetic damage
Japanese quail	Environmental studies
Lobster	Peripheral nerves
Nude mouse	Antitumor drug testing
Owl	Sleep physiology
Pigeon	Behavioral problems
Pig	Surgical models, cardiovascular studies, dermal absorption studies
Salamander	Severed limb regeneration
Sheep	Acute respiratory distress syndrome (ARDS) and other pulmonary phenomenon

EARTHWORMS

Earthworms are invertebrate, cold-blooded animals that collectively belong to the phylum *Annelida,* class *Oligochaeta,* and order *Megadrili.* Earthworms have been one of the more common species used to test chemicals for potential hazardous impact on the environment. The U.S. Food and Drug Administration (FDA), for example, includes protocols for the study of earthworms in its *Environmental Assessment Handbook.* As reviewed elsewhere (Chengelis 1990), earthworms could also be used for lethality assessment or rankings in place of rodents. The more common species of earthworm used, and their sizes, used in such testing are listed in table 11.2. There is some debate as to which species is best for testing purposes. Some investigators prefer *Lumbricus terrestris* because of its larger size and relative sensitivity (Dean-Ross 1983). Others prefer *Eisena foetida* (and the majority of publications mention this species) because of its small size, prevalence in the environment, and relatively low cost (Neuhauser et al. 1986). Although there are differences in sensitivity (Neuhauser, Durkin, et al. 1985), this appears to be more a matter of individual preference. The use of *L. rubellus* has advantages in an urban area because it is easier to obtain, it is still the same size as *E. foetida,* and it is free of the objectionable odor that often accompanies *E. foetida.* See table 11.3 for a comparison of acute toxicity for various chemicals in *L. rubellus* versus *E. foetida.* In many instances, *L. rubellus is* more sensitive, and therefore might be a more appropriate model for lethality screening.

The basic biology of earthworms has been reviewed by Laird and Kroger (1981) and Roberts and Dorough (1985). Worms differ sufficiently from the mammals with which most toxicologists are familiar that a brief review is necessary here.

Earthworms are highly specialized for life in the soil. The outermost barrier of the body is a thin chitinous cuticle, under which is an epidermal layer that contains mucous secretory glands and nerve receptor cells. Some of these are the light receptors that make earthworms photophobic. Locomotion is affected by contractions of the two layers of muscle cells that are underneath the epidermis, a circular layer and a longitudinal layer. The circular muscle layers are responsible for the segmented appearance of earthworms. The central nervous system consists of two ganglia per segment interconnected by a double nerve cord. The circulatory system is a closed loop system

Table 11.2 Earthworm (Phylum *Annelida,* Class *Chaetopoda*) Species Commonly Used in Environmental Impact Testing

Species	Common Name	Length (cm)
Allolobphora caliginosa	Field worm	5–20
Eisena foetida	Manure worm	5–12.5
Lumbricus rubellus	Red worm	5–12.5
Lumbricus terrestris	Night crawler	10–30

Table 11.3 Earthworm Comparative Toxicity

	LC_{50} (μg/cm²)	
Chemical	*Eisena foetida*	*Lumbricus terrestris*
Carbofuran	0.30	0.31
Aldicarb	3.20	0.02
Carbaryl	9.00	0.28
Malathion	13.5	0.27
Parathion	14.8	1.21
Acephate	851	692

Source: From Roberts and Dorough (1984).

with five hearts and two primary vessels. Respiration is by passive diffusion across the body walls. Earthworms have a coelom, the fluid-filled cavity situated between the body wall and the digestive tract. It permits the worm to crawl in one direction while food is passing through the digestive tract in the other. Interestingly, although hermaphroditic, earthworms do not self-fertilize. They mate, with two exchanges of sperm and both partners developing cocoons.

Metabolism

Xenobiotic metabolism has been examined but not thoroughly explored in earthworms. The subject has been reviewed by Stenersen (1984). Because of its size, *L. terestris* has been the species best studied. It has been shown to have cytochrome P-450-dependent mono-oxygenase activity, metabolizing aldrin to dieldrin, for example. This highest concentration has been found in the typhosole, the large fold in the earthworm's intestine. Glutathione S-transferase has also been described (Stenersen 1984). As in mammalian toxicology, it has long been recognized that species differences in toxicity in worms can be due to the differences in metabolism. For example, Gilman and Vardanis (1974) reported that the difference in sensitivity of *L. terrestris* compared with *E. foetida* to carbofuran is due to differences in the metabolic distribution of carbofuran.

Husbandry

Caring for earthworms is not difficult (Laird and Kroger 1981). They literally feed on decaying organic matter found in the soil. One needs only to keep them in moist soil in a cool (15°C–20°C) dark place. Many papers described supplementing this regimen with animal droppings for *E. foetida* (Roberts and Dorough 1984). It is important that distilled water be used to moisten the soil, as the earthworm can be quite sensitive to organochlorochemicals. For most studies, husbandry procedures are relatively simple. The worms can be kept in the supplier container in a cool dark place, and used within 48 hr. Prior to use, worms are rinsed off with distilled water, and left in a large Petri dish or beaker on a water-loaded filter paper for a few hours in a darkened room. Worms obtained from domestic bait shops might be of uneven quality and age, and should be sorted. Worms that are obviously smaller, larger, or not as active as the others should not be used.

Dosing Techniques

A variety of dosing techniques have been described in the literature. These include mixing the test article matrices (e.g., artisol), dipping in aqueous solutions, topical applications, microinjections, and contact on filter paper (Fisher 1984; Heimbach 1984; Serda and Furst 1987).

Microinjection techniques, where the small amounts of test article are injected into the hemocoel or peristrorn are quite time consuming and can be quite traumatizing (Roberts and Dorough 1985). Therefore, these techniques are not generally recommended.

Dips in which the worms are placed in a beaker containing a test article solution for 2 hr and then maintained in soil as usual for 1 week have been described (Dean-Ross 1983). The main disadvantage of this system is that the worms have to be manipulated several times.

Several papers (Heimbach 1984; Neuhauser, Loehr, et al. 1985) describe experiments in which the test article is mixed with artificial soils of various compositions in which the worms are then left to reside for 2 to 4 weeks. This is the method of choice for subchronic studies, and is essentially a cross between a dermal application study and a dietary admixture study, as the test article will not only be absorbed across the outer cuticle, but also ingested.

All of these routes have very real disadvantages for use in acute lethality testing. With soilborne tests, for example, dead worms will decay and disappear. Hence, one must be prepared to dig through the soil frequently to obtain time-to-death estimates. The technique recommended here for this purpose is the filter paper contact method, and this is the focus of the remainder of this discussion.

48-Hour Contact Test

The 48-hr contact test has proven to be a fast and resource-effective way of assessing acute toxicity of chemicals in earthworms. The fundamentals of this test are outlined in table 11.4. The standardized method, approved by the European Community (EC) is discussed by Neuhauser et al. (1986). This is for environmental impact assessment where cross-laboratory comparisons are important. If, however, one wishes to adopt this technology for the purposes of screening new chemicals, variants of this method are acceptable, as internal consistency is more important than interlaboratory comparisons. There are two important considerations. First, because of seasonal variation in the quality of earthworms obtained from suppliers, positive controls or comparator chemicals should be included on every assay run. Second, distilled water must be used, as worms are quite sensitive to contaminants that might occur in chlorinated water. The filter paper should completely cover the sides of the vessel; otherwise, the worms will simply crawl up the sides to escape the adverse stimulus the chemical contact might provide. General fundamental standards for toxicity testing in nonvertebrates are discussed elsewhere (Standard Practice 1980).

Using these techniques, Robert and Dorough (1984, 1985) and Neuhauser et al. (1986) have compared acute toxicity in a variety of organic chemicals in several earthworm species. A comparison

Table 11.4 Earthworm 48-Hr Contact Test Acute Lethality

1. Place filter paper of known size (9 cm or 12 cm ∞ 6.7 cm) in a Petri dish or standard scintillation vial.
2. Take test article up in acetone or some other volatile solvent.
3. Slowly and evenly deposit known amounts of test article solution onto filter paper.
4. Dry thoroughly with air or nitrogen gentle stream.
5. Add 1 ml of distilled water. Let wet filter paper.
6. Add worm *(L. rubellus)*. Keep in 400 mg to 500 mg range.
7. Use 10 replicate vials per concentration.
8. Store or incubate in the absence of light at 15ºC to 20ºC for 48 hr.
9. Examine for lethality (swollen, lack of movement on warming up to room temperature, lack of response to tactile stimulation).
10. Express dose as µg/cm^2 and mortality as usual. Calculate LC_{50} using standard techniques.
11. Always include negative and positive (benchmark) controls.

of the lethality of selected insecticides in *E. foetida* and *L. rubellus* is given in table 11.3. Although there are some obvious quantitative differences between worm species, in general the rank order of toxicity is about the same. All earthworms are very sensitive to carbofuran under the conditions of this test.

Neuhauser, Loehr et al. (1985) have proposed a toxicity rating scheme based on acute lethality in the earthworms that is similar to the more familiar scheme based on acute lethality in rodents (table 11.5). Roberts and Dorough (1985) and Neuhauser, Loehr et al. (1985) have published extensive compilations of acute lethality in worms and compared these to acute lethality in rats and mice. A selection of these is shown in table 11.6. According to the toxicity rating scheme of Neuhauser, most of these chemicals are in the same toxicity category whether based on results from either *E. foetida* or mice. This might suggest that replacing the LD_{50} with the LC_{50} for rating toxicity (e.g., for a Department of Transportation shipping permit) deserves serious consideration.

Table 11.5 Earthworm Toxicity Rating

Rating	Designation	Rat LD_{50} (mg/kg)	*Eisena foetida* LC_{50} ($\mu g/cm^2$)
1	Supertoxic	< 5	< 1.0
2	Extremely toxic	5–50	1.0–10.0
3	Very toxic	50–500	10–100
4	Moderately toxic	500–5,000	100–1,000
5	Relatively nontoxic	> 5,000	> 1,000

Source: From Neuhauser, Loehr et al. (1985).

Table 11.6 Earthworm Acute Lethality Comparative Values

Chemical	*Eisena foetida* (LC_{50})	Mouse (LD_{50})
2,4-dinitrophenol	0.6 (1)	45 (2)
Carbaryl	14 (3)	438 (3)
Benzene	75 (3)	4,700 (4)
1,1,1-trichloroethane	83 (3)	11,240 (5)
Dimethylphthalate	550 (4)	7,200 (5)

Source: From Roberts and Dorough (1985).

To the extent that the 48-hr contact test is artificial and that earthworms are in direct contact with a chemical in a closed system, it might not be truly reflective of toxicity of a chemical to earthworms in the environment. Van Leemput et al. (1989) compared the LC_{50} for enilconazole (a fungicide) in the 48-hr contact test to a 14-day artificial soil test (OECD guideline 207; protocol summarized in table 11.7). In the contact test, the LC_{50} was 12.8 $\mu g/cm^2$ (filter paper), whereas in the 14-day artificial soil test, the LC_{50} was 541 $\mu g/g$ (soil). Hence, even given the longer exposure period of the artificial soil test, worms were less sensitive to chemical toxicity in this milieu. The same phenomenon is true for other chemicals (Van Leemput et al. 1989) Thus, although the 48-hr contact test has utility as a predictive, screening, or ranking tool, it has limited value in direct environmental assessment. Additionally (as discussed by Van Gestel et al. 1989), lethality is a poor parameter to use in assessing the impact of environmental exposure of earthworms to chemicals. Reproductive function is of greater importance for the maintenance of populations. These authors recommended using cocoon production rather than lethality as a more sensitive endpoint in artificial soil tests. Their reasoning appears to be quite sound. In addition, cocoon counts are easily quantifiable, and they provide an alternative parameter for assessing toxicity in the artificial soil test where the animals are difficult to see and change in behavior, therefore, is difficult to assess.

Table 11.7 Earthworm 14-Day Toxicity Test in Artificial Soil (OECD Guideline 207)

1. Prepare artificial soil: 10% sphagnum peat, 20% kaolinite clay, 69% construction sand, and 1% calcium carbonate (all % by weight).
2. Obtain worms *(E. foetida)* from supplier. Only adults (400 mg–700 mg) with a well-developed clitellurn should be used. Keep for 14 days in shallow trays containing artificial soil before the start of the study.
3. Test article concentrations in test soil are in terms of micrograms per grams of dry weight. Stock solutions in distilled water can be diluted and mixed with soil in a household mixer. Hydrophobic substances can be taken up in a small amount of solvent and mixed with a small amount of test soil. After evaporation of the solvent, the treated soil can be thoroughly mixed with additional soil to obtain appropriate concentrations.
4. For each concentration, four 1-L beakers are filled with 750 g of treated soil. Moisture content is adjusted to 35g to 40 g H_20/100 g of soil. Ten worms are added to each beaker. Beakers are covered with perforated plastic.
5. Maintain at 20°C ± 2°C 12/12 hr light–dark cycle.
6. After 7 and 14 days, earthworms are removed from the test soil, counted, and sorted. Those not responding to mechanical stimuli are sorted as dead.
7. Mortality data can be analyzed by conventional means.

Source: From Van Leemput et al. (1989).

Advantages and Disadvantages

The main advantages of the 48-hr contact test are the savings of time and money. The cost savings fall into three categories. First, earthworms are cheap. One hundred *L. rubellus* will cost about $2. The 100 mice they could replace in toxicity screens, for example, would cost $125 to $175. Second, earthworms require no vivarium space, and their use could decrease the number of rodents used, resulting in a net decrease in vivarium use. Third, adapting the 48-hr contact test would require little capital investment other than a dedicated under-the-counter refrigerator set at 15°C to 20°C Otherwise, the assay can be easily performed in a standard biochemistry laboratory. With regard to time savings, the standard lethality test with rodents requires 7 to 14 days of postdosing observations. The 48-hr contact test is completed in 48 hr. Not only is the turnaround faster, but the amount of time that technical personnel will have to spend observing animals and recording observations is decreased. An incidental advantage to earthworms is that they are cold-blooded vertebrates, and thus exempt from the requirements of animal welfare laws.

There are two main disadvantages to the use of earthworms in acute toxicity testing. First, there are a limited number of endpoints. Other than death and a few behavioral abnormalities (Drewes et al. 1984; Stenersen 1979) the test does not yield much qualitative information. Second, there probably is some institutional bias. Because the test is not cutting-edge technology (no tissue culture) and uses a nonmammalian model, it has been easy to dismiss its utility .

FISH

Fish, like earthworms have historically been commonly used to assess potential environmental impact. Some of the more common species of fish used and their scientific names are listed in table 11.8. Most fish used in toxicity studies belong to the superclass *Gnathostoma,* class *Osteichthyes,* subclass *Actinoptergii,* infraclass *Teleostei,* division *Eutelaosti* (rainbow trout, zebrafish, and Japanese medaka). Rainbow trout in particular have been extensively used in carcinogenicity and mechanistic cancer research (Law 2003; Stoskopf 2001). They are, of course, cold-blooded vertebrates. In addition to environmental impact studies, the potential use of fish in carcinogenicity testing has been recognized since the early 1970s, when it was reported that some fish develop hepatic neoplasias to many of the same chemicals that rodents do. In fact, the possible use of fish in carcinogenicity testing was the subject of a symposium sponsored by the National Cancer Institute in 1981. This practice, however, has not gained wide acceptance, despite the data suggesting that fish might, in fact, make good models for carcinogenicity testing. Here, the use of the rainbow

trout (*Oncorhynchus mykiss,* formerly *Salmo gairdneri*) and the Japanese medaka (*Oryzias latipes*) in carcinogencity testing are reviewed.

Table 11.8 Toxicity Testing in Fish Species Studied

Common Name	Formal Name	Use[a]
Bluegill	*Leopomis macrochirus*	E
Carp	*Cyprinus carpio*	M
Channel catfish	*Ictalurus punctatus*	E
Cobra guppy	*Poecilia reticulata*	C
Cod	*Gadus morhau*	M
Fathead minnow	*Pimphales promelas*	E
Gulf killfish	*Fundulus grandis*	C
Inland silverside	*Menidia beryfflina*	C
Japanese medaka (rice fish)	*Oryzias latipes*	C
Rainbow trout	*Oncorhynchus mykiss (Salmo gairdneri)*	E, C, M
Toad fish	*Opsanus tau*	M
Trench	*Tinca tinca*	M
Sheepshead minnow	*Cyprinodon variegatus*	C, E

[a] E = environmental impact; C = carcinogenicity; M = metabolic studies.

Husbandry

Fish obviously require water and need to be kept in either glass aquaria or fiberglass or stainless steel tanks. Water should be of consistent quality from a consistent source. Oxygen content, hardness, and alkalinity should be monitored. As long as water quality is consistently monitored and maintained, both the rainbow trout and Japanese medaka are remarkably free of background diseases. Temperature needs to be maintained at 12°C to 17°C for trout and 22°C to 27°C for medaka. Rates of water turnover in flow-through systems have been described in terms such as 6 to 10 volume changes per day or 3 L per hr. Published papers describe using aerated well water or dechlorinated tap water. Frequent analysis of potential confounding contaminants should be routine. Lighting can be 12/12 hr cycles. Commercial diets are available for both trout and medaka.

Dosing Techniques

The dosing techniques most often mentioned in the literature include intraperitoneal injection, mixing with water for either static or flow-through exposure, and dietary admixture. The intraperitoneal technique is only suitable for treating larger fish and only for single exposures. Exposure in solution under static conditions requires relatively small amounts of material, is much easier to control, and has been shown to be effective for short-term exposure. Flow-through conditions require relatively large amounts of material (and generate large amounts of potentially contaminated water) and require extensive engineering safeguards to prevent the exposure of laboratory personnel to potential carcinogens. Flow-through systems allow for longer term exposure.

A variety of different systems have been described (see Walker et al. 1985, for an example). Water-based delivery requires a certain level of aqueous solubility that might not always be achievable. In that case, dietary admixture is the preferred dosing technique. In fact, dietary admixture has proven to be a reliable and effective method for dosing large numbers of fish over long dosing periods, and using considerably less test article than flow-through methods. Various methods have been described for preparing the diets, such as dissolving the test article in a highly volatile solvent and applying it by micropipette to individual pellets or mixing it up with salmon oil prior to dressing the basal diet prior to feeding. Exposures are described in terms of parts per million (of test article in the diet). Other dosing techniques have been described in papers in which the emphasis was on studying drug metabolism, and relatively small numbers of fish were used in

acute preparations. Most of these, such as intra-aortic injection, would be impractical to use for carcinogenicity studies.

Metabolism

Xenobiotic metabolism in medaka has not been extensively examined. This discussion, therefore, focuses on xenobiotic metabolism of the rainbow trout. Some parameters of xenobiotic metabolism are summarized in table 11.9. Microsomal mixed function oxidase (MMFO) activity has been studied in the rainbow trout in fairly extensive studies since mid-1970 (see Stegman and Kloepper-Sams 1987, for a review). Trouts have identifiable activity with most of the substrates that rats do, but the activity tends to be less. No sex- or age-related differences have been discussed in the literature. Gregus et al. (1983) have published comparisons of several of the key enzyme systems in trout to those of the rat (table 11.9). In most instances, the trout had comparable or even higher activity.

Table 11.9 Rainbow Trout Hepatic Xenobiotic Metabolism

Parameter	Trout	Rat
Liver/body weight ratio (%)	1.1	4.0
Microsomal protein (mg/g)	28.1	25.7
Cytosolic protein	74.5	93.7
Cytochrome P-450	0.34	0.51
MMFO activity		
Benzphetamine	0.2	2.2
Ethylmorphine	0.1	3.8
Ethoxyresorufin	0.4	0.1
Epoxide hydrolase		
styrene oxide	9.0	6.0
UDP-glucuronosyl transferase		
4-nitrophenol	1.0	5.0
testosterone	0.06	0.01
Glutathione S-transferase		
Dichloronitrobenzene	5	75
ethacrynic acid	30	40
N-acetyl transferase		
2-aminofluorene	4	0.1
4-aminobenzoate	0.03	0.06
Temperature	25°C	37°C

Note: Estimated enzyme activities: nmol/min/mg protein.
Source: From Gregus et al. (1983).

The remarkable aspect of the trout MMFO is the high degree with which it responds to 3-methcholanthrene (3-MC)-type inducing agents. For example, Voss et al. (1982) reported that 5 days of treatment by dietary admixture with polychlorinated biphenyl (PCB; (100 ppm), resulted in large increases in cytochrome P-450 content as well as increases in 7-ethoxyresorufin, 7-ethoxycoumarin, and benzo(a)pyrene metabolism in rainbow trout (table 11.10).

Further, Erickson et al. (1988) have reported that exposure of trout to as little as 1 ppm piperonyl butoxide for 3 weeks in a continuous flow-through system resulted a threefold increase in cytochrome P-450, a 17-fold increase in ethoxycoumarin demethylase, and a 36-fold increase in ethoxyresorufin demethylase, the latter considered a marker for cytochrome P-448 or 3-MC-type induction.

In fact, because of the extreme responsiveness of trout to this type of induction, Julkunen et al. (1986) have proposed using fish MMFO activity to monitor pollutant levels in a body of water.

Interestingly, trout are not responsive to phenobarbital-type induction (Stegman and Kloepper-Sams 1987). For example, Miyauchi (1984) reported that phenobarbital pretreatment did not

Table 11.10 Induction of Hepatic Xenobiotic Metabolism in Trout with Aroclor

Parameter	Control	+PCB [a]
Cytochrome P-450 (nmol/mg)	0.15 ± 0.04	0.29 ± 0.05
MMFO activity		
Ethoxyresorufin	0.06 ± 0.02	4.07 ± 0.44
Ethoxycoumarin	0.04 ± 0.0	0.49 ± 0.02
Benzo(a)pyrene	0.004 ± 0.006 (nmol/min/mg)	0.97 ± 0.04

[a] Following dietary exposure to 100 ppm Aroclor 1254 (polychlorinated biphenyl) for 5 weeks.

Source: From Voss et al. (1982).

increase the metabolism or the mutagenicity (in the Ames assay) of 2-acetylaminoflourene, whereas pretreatment with PCBs or 3-MC caused increases in both (table 11.11). In addition, α-napthaflavone, an inhibitor of cytochrome P-448 activity, inhibited revertant colony formation, whereas metyrapone, an inhibitor of cytochrome P-450 activity, had no effect on revertant colony formation. Thus, trout differ from rodents with regard to inducibility in both selectivity and degree of response.

Table 11.11 Inaction of Hepatic Xenobiotic Metabolism in Trout (Using the Ames Assay)

	Mutagenicity	
Treatment Group	BENZP[a]	2-AAF[b]
Untreated	20	300
3-Methylcholanthrene	140	600
+ alpha-naphthaflavone	20	90
+ metyrapone	130	500
Phenobarbital	20	290
+ alpha-naphthaflavone	20	20
+ metyrapone	20	220

Note: Approximate number of revertant colonies (test strain TA 98) per plate. S-9 fractions from fish treated with either 3-methylehoanthrene or phenobarbital. Inhibitors (α-naphthaflavone or metyrapone) used *in vitro* (0.1 mm).

[a] BENZP = benzo(a)pyrene.

[b] 2-AAF = 2-acetylaminofluorene.

Source: From Miyauchi (1984).

Trout microsomal enzymes are assayed at room temperature (25°C) or even lower. These are temperatures at which MMFO activities in the rat would be severely compromised. The fact that trout have comparable enzyme activities at lower temperature optima than rats is an example of species differences in adaptation. Trout, like all fish, are poikilothermic. How do trout control MMFO activity at different body temperatures? Would small temperature variations affect carcinogen activation? Egaas and Varanasi (1981) compared MMFO activity of rainbow trout kept at either 7°C or 16°C. When benzo(a)pyrene metabolism was examined *in vitro* at 29°C, the fish kept at the lower temperature had significantly higher activity. Blanck et al. (1989) obtained similar results. They compared trout held at 5°C and 20°C. They observed that both benzo(a)pyrene and ethoxycoumarin metabolism at 18°C *in vitro* was more rapid in the preparations from the fish maintained at the lower temperature (table 11.12). However, when *in vitro* incubations were conducted at the same temperatures at which the fish were kept (i.e., microsomes from fish maintained at 5°C were incubated *in vitro* at 5°C), there were no differences in enzyme activity. Hence, the rainbow trout has a mechanism for responding to changes in environmental temperature

to maintain constant MMFO activity. As noted also in table 11.12, the different ambient temperatures do not alter cytochrome P-450 content, but do change NADPH: cytochrome C reductase activity. Thus, it would appear that trout respond to changes in ambient temperature by varying the ratio of cytochrome P-450 to the reductase to maintain constant MMFO activity. Small variations (group to group in temperature) should not effect carcinogen variation.

Table 11.12 Hepatic Xenobiotic Metabolism in Trout

	Temperature Effects		
Parameter	**Ambient Temperature (°C)**	**Kept at 20°C**	**Kept at 5°C**
Cytochrome P-450 (nmol/mg)	18	0.10	0.11
NADPH:cytochrome C reductase (nmol/min/mg)	18	24.9	47.7
Benzo(a)pyrene (pmol/min/mg)	18	17	57
	20	21	—
	5	—	17
Ethoxycoumarin (pmol/min/mg)	18	27	8
	20	37	
	5	—	31

Note: Microsomal enzyme activities *in vivo* from mature rainbow trout. Water temperatures were recorded at the time of harvest. $N = 15–20$.

Source: From Blanck et al. (1989).

Most carcinogens, particularly hepatic carcinogens, require metabolic activation. There are two criteria that should be met to support a conclusion that reactive metabolites are being produced. First, irreversible binding to tissue macromolecules must occur. With regard to the trout, Darnerud et al. (1989) have reported that 1,2-dibromoethane and chloroform form irreversibly bound metabolites in trout exposed *in vivo*. Egaas and Varanasi (1981) demonstrated that trout had the capacity when assayed *in vitro* (with S-9 or postmitochondrial supernatant fractions) to catalyze the irreversible binding of benzo(a)pyrene to deoxyribonucleic acid (DNA). Loveland et al. (1988) demonstrated that hepatocytes isolated from rainbow trout were capable of producing metabolites of aflatoxin that irreversibly bound to native DNA. With regard to mutagenic activity, Miyauchi (1984) demonstrated that trout hepatic preparations could activate mutagens (2-aminoanthracene and 2-acetylaminofluorene) in the Ames *Salmonella* assay. Thus, it would appear that trout are quite capable of the metabolic activation of carcinogens.

Examples of Carcinogenicity in Fish

Numerous reports of chemical carcinogenicity in fish exist and only a few illustrative reports are presented here. Hendricks et al. (1985) studied the carcinogenicity of benzo(a)pyrene in rainbow trout. The study was initiated with 3-month-old trout fingerlings (3.3 g). They were continuously fed a dietary admixture containing 1,000 ppm benzapyrene for 18 months. The results are summarized in table 11.13. Although there was no difference in mortality, there were decreases in body weight, increases in liver to body weight ratio, and a significant increase in hepatic carcinomas. Thus, benzo(a)pyrene is a positive hepatic carcinogen in rainbow trout. There were no hepatic neoplasias in the control group, which is a common observation (e.g., very low tumor background rate) in carcinogenicity studies in fish.

Shelton et al. (1984) reported that diethylnitrosoamine (100 ppm by dietary admixture) produced liver tumors in trout with 12 months of treatment, and that cotreatment with polychlorinated biphenyls (MMFO-inducing agents) greatly enhanced the tumor yield (table 11.14).

Table 11.13 Rainbow Trout Carcinogenicity Study

	Control	BP
N	114	114
Deaths	5	3
Body weights	425 ± 153 g	364 ± 125 g
Liver/body weight	0.66 ± 0.16%	0.70 ± 0.13%
Liver neoplasia		
Altered hepatic foci	0	4.5%[a]
Carcinoma	0	21%

Note: Study started with 3-month-old trout fingerlings (3.3 g), fed 100 ppm benzo(a)pyrene (BP) by dietary admixture ad libitum for 18 months.

[a] Incidence of animals having the lesion.

Source: From Hendricks et al. (1985).

Table 11.14 Rainbow Trout Carcinogenicity Study

	Control	DEN	DEN + AC
N	120	120	120
Deaths	1	2	28
Liver row	0.77 ± 0.08	0.65 ± 0.11	0.89 ± 0.13
Liver neoplasia	0	10.2%	40.2%

Note: Trout fingerlings fed 1,100 ppm diethylnitrosamine (DEN), or DEN + 100 ppm Aroclor (AC) 1242, by dietary admixture for 12 months.

Source: From Shelton et al. (1984).

Hawkins and Hinton and their colleagues have reported on the carcinogenicity of methylazoxyethanol (Hinton et al. 1984) and benzo(a)pyrene (Hawkins et al. 1988) in medaka (table 11.15). They have also identified tumors in medaka in organs other than the liver. (For a more complete review of carcinogenicity testing with medaka and other aquarium fish, see Hawkins et al. 1985.) Benzo(a)pyrene, diethylnitrosamine, and methylazoxymethanol are presumed, if not confirmed, human carcinogens. Thus, it would appear that rainbow trout and Japanese medaka are viable species in which to test suspect human hepatic carcinogens.

Currently, fish toxicity testing is mainly based on *in vivo* tests. The *in vivo* tests are of ethical concern, and they suffer from technical limitations and low cost–benefit efficiency. Their ecotoxicological relevance is doubtful, as they are not able to detect a number of relevant toxic effects.

Table 11.15 Medaka Carcinogenicity Study

	Incidence of Hepatic Tumors	
Dosage Groups	**At 24 Weeks**	**At 36 Weeks**
Negative control	0/82	1/89 (~1.1%)
Solvent control	1/75	1/97 (~1.0%)
1–4 PPB	1/75	1/94 (~1.1%)
8–34 PPB	0/70	0/96 (0%)
200–220 PPB	8/76	26/73 (~36%)[a]

Note: Initiated with two 6-hr exposure periods to benz(a)pyrene, 6 days apart when fish were 6 to 10 days of age. Maintained for 36 weeks under standard aquarium conditions.

[a] Twenty had adenomas and six had carcinomas of the liver.

Source: From Hawkins et al. (1988).

To date, the use of fish cells in ecotoxicology has mainly been focused on the measurement of: (a) cytotoxicity, both basal and selective (cell specific), (b) genotoxicity, and (c) effects on cell-specific functions and parameters, including studies on biotransformation and the induction of biomarkers, or mechanisms of toxicity. *In vitro* systems based on fish cells have been found to be valuable in the following circumstances:

- *For the toxicity ranking and classification of chemicals, including quantitative structure–activity relationship studies:* These types of studies are largely based on the measurement of basal cytotoxicity, where fish cells perform as well as mammalian cell systems. The more practical handling of fish cells even favors the replacement of mammalian cells for some purposes.
- *For toxicity measurements on environmental samples:* For this type of sample, fish cells offer a number of technical advantages over mammalian cells, as well as over *in vivo* fish tests, particularly for bioassay-directed fractionation studies, which are of increasing importance in environmental toxicology. The rapid, differentiated effects assessments provided by fish cells, as well as their need for only small sample volumes, represent distinct advantages over *in vivo* assays.
- *For toxicity characterization of chemicals and environmental samples:* Fish cells *in vitro* provide a bioanalytical tool for the assessment of differentiated effects, and the possibility of screening in a rapid and cost-effective way, for a wide range of endpoints (e.g., cytotoxicity, genotoxicity, dioxin-like activity, endocrine disruptor activity). Fish cells can be used to develop relevant ecotoxicological endpoints (e.g., the prediction of genotoxic effects in terms of genetic diversity). The introduction of genomics and proteomics will further enhance the potential value of fish *in vitro* systems.
- *For studies on toxic modes of action:* Fish cells *in vitro* can address aspects of toxic mechanisms that are difficult to study *in vivo*; for example, chemical-induced receptor activation, attacks on the permeability properties of the gills, or temperature effects. A further advantage of the *in vitro* system is that it is based on a relatively uniform cell type. Compared to mammalian cells, fish cells are the models of choice for investigations on fish-specific toxicokinetic and toxicodynamic processes.

The reduction and complementation of *in vivo* ecotoxicity tests by *in vitro* assays could greatly improve the quality of ecotoxicological hazard assessments. The *in vitro* systems offer the possibility of the more differentiated assessment of effects, involving the use of more endpoints, and could provide the necessary mechanistic understanding for effect prediction and the systematic classification of chemicals.

However, a disadvantage of fish cell systems, which they share with other cell systems, is their lower absolute sensitivity compared to the fish used in *in vivo* tests. The absolute sensitivity of tests is of relevance, for example, in the determination of environmentally safe levels of chemicals. This, however, is usually not done on the basis of a single test, but the results from several tests. Extensive data are available to support this investigation (Castano et al. 2002).

Advantages and Disadvantages

There are three main advantages to using fish in carcinogenicity testing. First, they have an extremely low background tumor rate, which enhances the sensitivity of the assays. Second, fish are less expensive to purchase and maintain than rodents. Group sizes of 100 or more (particularly with medaka) become quite manageable. Third, a positive carcinogen will generally show up in fish within 1 year's time. Rodent studies generally last for 18 months (mice) to 30 months (rats). Medaka have certain advantages over trout that magnify the cost savings. For example, they will thrive in waters maintained at room temperature, whereas the cooler temperature optimum of the rainbow trout generally requires the use of energy-dependent cooling systems. The larger size of the trout requires greater aquarium space. The smaller size of the medaka is an advantage at necropsy, where Medaka are fixed and slide mounted in toto for histological examination, saving both time spent on dissection and other histology laboratory resources.

There are three main disadvantages in using fish for carcinogenicity testing. First, compared to rodents, only relatively few tumors, primarily carcinomas of the liver, have been described in fish. Hence, it would appear to be prudent to use carcinogenicity testing in fish as an adjunct to testing in at least one mammalian species. On the basis of current data, fish would be best used in confirming potential hepatic carcinogens. Second, the assays cannot be adapted without capital changes to the standard vivarium. Third, as with earthworms, there is an ill-defined institutionalized bias against nonmammalian animal models. One could argue, however, that a chemical shown to be carcinogenic across a broad phylogenetic spectrum of species is a stronger candidate to be a human carcinogen than one shown to be positive in only one sex or species.

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CHAPTER 12

Clinical Pathology of Laboratory Animals

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CONTENTS

In the context of conventional toxicology studies, clinical pathology usually consists of routine hematology, clinical chemistry, and urinalysis tests. The majority of tests are the same as those used in human or veterinary medicine to establish a minimum database. There are, of course, species differences for reference ranges, interpretation of changes, methodology, and the value or appropriateness of individual tests. The choice of tests for a given study depends on a number of factors, including objective of the study, test species, test materials, duration of the study, and regulatory requirements.

The results of clinical pathology tests are used to identify general metabolic and pathologic processes. Although specific "diagnoses" or toxicologic mechanisms are infrequently identified, test results are rarely the only evidence of biologically important adverse toxicologic effects. Clinical observations and anatomic pathology findings usually corroborate pathologically meaningful laboratory findings. On the other hand, it is relatively common to observe minor effects of a test material only in the clinical pathology results. For example, changes in homeostatic mechanisms to maintain normal fluid, electrolyte, and acid–base balance secondary to a test material that alters water consumption or acid load might be reflected only in urinalysis results.

The ability to identify subtle changes in a group of treated animals that are compared directly with a control group is much greater than in an individual animal compared with historical reference ranges. In safety assessment studies, this fact inevitably leads to important decisions concerning the nature of a subtle change. Is it simply an alteration in normal homeostatic mechanisms, or is it an early indication of a more significant adverse effect? This question can be difficult to answer. It is clearly not as simple as referring to a table of historical reference ranges.

Accurate interpretation of laboratory results requires not only an understanding of the tests themselves, but also knowledge of species differences, study design and procedures, the test material(s), clinical observations, and anatomic pathology findings. The interpretation of one test result (e.g., high serum urea nitrogen) is dependent on the results of another (i.e., urine specific gravity). The urine specific gravity result is the difference between an interpretation of prerenal azotemia, as with dehydration, and renal azotemia, as with renal failure.

With respect to regulated safety studies, required or recommended clinical pathology tests have important limitations in their ability to demonstrate toxicologic effects. Some of the limitations are due to timing of sample collection and analysis during the study, and some are related more to the actual tests. In fact, some of the recommended tests are outdated or inappropriate and should either be eliminated or used only in specific circumstances. To add to the confusion, recommended test lists vary among regulatory agencies, both inside and outside of the United States.

This chapter addresses (a) the use and potential misuse of clinical pathology reference ranges; (b) sources of variation in laboratory test results and their potential impact on data interpretation; (c) the characteristics and interpretation of routine hematology, clinical chemistry, and urinalysis tests used in toxicology studies; and (d) aspects of laboratory test results that are unique to individual laboratory animal species. For more in-depth discussions of the laboratory tests and their interpretation, the reader is referred to Sanderson and Phillips (1981), Duncan and Prasse (1994), Jain (2000), Kaneko (1997), and Loeb and Quimby (1999).

REFERENCE RANGES

The term *normal range* has been replaced by the more appropriate terms *reference range* and *reference interval*. The word *normal* occasionally caused confusion because it implied that values outside of the range were, by definition, abnormal. However, because most laboratory test reference intervals are statistically constructed to include the range of values found in 95% of a population of healthy, or "normal," individuals, it should not be surprising that approximately 1 out of 20 results for a specific test (e.g., serum glucose concentration) from a group of "normal" animals is outside of the historical reference range. Taking this idea a step further, and because of the fact that a minimum hematology and clinical chemistry database consists of approximately 30 individual test results, it should also not be surprising that a "normal" animal will often have at least one test result that falls outside of the historical reference ranges. In this light, it is easy to see that study designs requiring all animals have normal clinical pathology test results as a prerequisite for inclusion in the study are too restrictive if taken literally.

Another potentially confusing aspect of the term *normal range* is that it seems to imply that a test result within the range is, by definition, normal, and therefore the organ system or metabolic process being assessed by that test is also normal. Unfortunately, severely abnormal animals might have laboratory values well within the reference range. For example, a dog with advanced liver cirrhosis often has serum liver enzyme activity levels within the established reference ranges for those tests. An animal with leukemia might have a "normal" white blood cell count. Certain aspects of a disease condition can mask abnormalities in a laboratory test. Dehydration might mask anemia or hypoproteinemia. The decrease in plasma volume due to fluid loss will spuriously increase red blood cell count and serum protein concentration. Acidosis might mask total body potassium depletion as intracellular potassium ions exchange with extracellular hydrogen ions, resulting in a "normal" serum potassium concentration.

Reference ranges are influenced by many variables, and a number of these are discussed in the following section. Of particular importance are the individual laboratory performing the tests and the specific methodology employed. For instance, the temperature at which enzyme assays are conducted will greatly influence results; the higher the temperature, the higher the reference range. It is difficult and potentially misleading to use reference ranges found in the literature for

the interpretation of study data. Whenever possible, investigators should establish in-house reference ranges. Because of a number of limitations, not the least of which are economic, this is not always feasible. For this reason, reference ranges for common hematology and clinical chemistry tests for the eight laboratory animal species described in this book have been included with this chapter (tables 12.1–12.8). These ranges are presented to serve only as general guidelines and to show the differences among the species. They represent a composite of ranges found in the literature and at Covance Laboratories, Inc. When reference ranges are established in house, it is important that they be periodically updated. Changes in test methodology, animal suppliers, and animal husbandry or handling practices are examples of variables that can cause earlier reference ranges to become obsolete.

Reference ranges do not replace the need for control animals in large studies designed to assess the toxic potential of a test material. Because it is not possible to repeatedly duplicate a standard set of study conditions, there is no substitute for age- and sex-matched control animals from the same supplier undergoing the same procedures as the treated animals. For instance, test results from animals receiving a purified diet cannot be compared directly with reference ranges established for animals fed a conventional diet. In small investigational studies with few or no control animals, however, reference ranges might help identify potential toxic effects.

Reference ranges should never be relied on as the sole means of making judgments concerning the biological importance of a test material-related effect. Values outside of the reference range do not necessarily indicate an abnormal condition and values within the reference range do not necessarily signify a normal condition. Monkeys tend to have wide reference ranges for many parameters. The finding that the mean serum alanine aminotransferase activity of a treated group is significantly higher than that of the control group should not be dismissed as toxicologically unimportant simply because the mean activity of the treated group falls within the upper reference limit. On the other hand, reference ranges for serum urea nitrogen concentration in rats are generally very narrow. If addition of test material to the drinking water of rats caused reduced water consumption, it is quite possible that the mean serum urea nitrogen concentration for these rats would be significantly higher than for the control animals and would exceed the upper reference limit without a meaningful toxic effect on the kidney.

Although limited in conventional toxicology studies, reference ranges do have value. Proper assessment of the significance of laboratory findings is only possible with a firm understanding of what is typical for healthy animals under similar conditions. Reference ranges can serve as the starting point, a point of reference, from which one can move toward an appropriate conclusion based on all of the variables that might have impacted a particular study.

SOURCES OF VARIATION IN LABORATORY MEASUREMENTS

The need for developing in-house reference ranges is based on the fact that many variables, such as methodology, affect test results, and that controlling these variables is best accomplished within one's own laboratory. In addition, however, there are sources of variation that can affect the results of individual animals within a group and thereby complicate data interpretation. Most variables can be categorized as either physiological, procedural, or artifact. Examples of these are discussed in the following paragraphs.

Age-Related Changes

Age-related changes must always be considered, and reference ranges should be established in accordance with the age of animals used most often by the individual laboratory. As young animals mature, typical changes in most species include decreasing reticulocyte count, mean corpuscular volume, serum alkaline phosphatase activity, and serum inorganic phosphorus con-

centration, as well as increasing red blood cell count, hematocrit, hemoglobin concentration, total serum protein concentration, and serum globulin concentration. In many species, neutrophil count will increase and lymphocyte count will decrease with age. As a population of animals becomes older, test results exhibit greater variability because of subclinical disease conditions such as progressive nephropathy in rats. Reference ranges therefore become wider, and identification of mild effects becomes more difficult.

Strain and Gender Differences

Strain differences can be important, especially in mice and rats, just as species differences are important in nonhuman primates. Gender-related differences also occur, but they are often subtle. Depending on the laboratory's need, it might be desirable to combine the data from both sexes into a single reference range. On the other hand, the effects of estrus might be extremely important, as in ferrets with estrus-induced bone marrow hypoplasia. Axenic animals might have significant differences from others of their species, especially with respect to leukocyte counts and serum globulins.

Effect of Diet

Diet has an effect on laboratory data. Changes in cholesterol are obvious in some species fed atherogenic diets, but less obvious changes can occur in tests such as serum urea nitrogen concentration in animals fed diets containing different types or amounts of protein. Data from animals fed purified diets should always be carefully examined. Small errors in the formulation of these diets can have a significant effect on health. For most species, fasting is a generally accepted practice designed to help stabilize test results. However, fasting could actually be a detriment in rabbits and mice.

Excitement and Stress

Excitement and stress can have pronounced effects on laboratory data. Excitement is associated with endogenous catecholamine release and stress with endogenous corticosteroid release. The effects of catecholamines on test results are immediate, whereas those of corticosteroids take longer. The most obvious changes observed in excited or frightened animals (e.g., untrained, unanesthetized monkeys) affect leukocytes and serum glucose concentration. Endogenous corticosteroid release also affects these parameters, but somewhat differently. If possible, clinical pathology testing should be delayed for at least a week following shipping to avoid stress-related changes.

Choice of Collection Site and Use of Anesthesia

The most familiar procedural influences on laboratory test results are those associated with sample collection site and the use of anesthesia. Many investigators have analyzed the differences in data resulting from choice of collection site and anesthesia. This is especially true for the rat in which variety of bleeding techniques have been used (Neptun et al. 1985; Suber and Kodell 1985). The principle message of these works is that researchers should use the technique with which they have had good success and with which they are comfortable. Although specific study objectives might occasionally dictate which bleeding technique is most appropriate, it is very difficult to consistently obtain high-quality specimens if the techniques are not used routinely. Furthermore, few research facilities have the resources to establish reference ranges for more than one or two bleeding techniques. Choice of bleeding site and anesthesia appear to have their greatest effect on peripheral blood cell counts. Serum enzyme activities, especially for enzymes derived from muscle, can also be affected by bleeding site and anesthesia.

Artifacts

Due to Hemolysis

One of the most common causes of variation due to artifact is hemolysis. Free hemoglobin could interfere with a variety of assays depending on the method and instrumentation used. In addition, lysis of erythrocytes releases intracellular constituents such as aspartate aminotranferase, lactate dehydrogenase, inorganic phosphorus, and potassium, that cause spuriously high serum activities and concentrations. There are species differences for erythrocyte intracellular potassium concentration that are discussed later in the chapter.

Whereas hemolysis is generally obvious and can, therefore, be considered when interpreting data, poor laboratory procedures can have effects that are not grossly evident. Intracellular erythrocyte constituents will leach into serum if clotted blood samples are not centrifuged and separated quickly. In addition, serum glucose concentration will decrease at a rate of about 7 to 10 mg/dl each hour due to erythrocyte metabolism. These changes, along with the potential of analytical drift by laboratory instrumentation, are some of the reasons why randomization of animals for blood collection is extremely important. For example, if control animals in a large study are bled first and high-dose animals last, and the serum is not separated from the clotted blood until after the last animal is bled, serum lactate dehydrogenase activity and inorganic phosphorus concentration might be statistically lower in the high-dose group simply because their blood did not sit as long before separation.

Due to Multiple Bleeding Intervals

Sometimes overlooked are the effects of multiple bleeding intervals. It is relatively easy to create iatrogenic, blood-loss anemia and hypoproteinemia in animals that are bled frequently for pharmacokinetic studies or for serial test determinations such as plasma cholinesterase activity. In the absence of control animals undergoing the same procedures, it might be difficult to separate test material effects from those of the multiple blood collections.

Due to Urine Collection Procedures

Timed urine collection procedures, as currently practiced in most toxicology laboratories, cannot avoid a number of artifacts in the urinalysis results. It is virtually impossible to make long-term collection (16–24 hr) in such a way as to prevent bacterial contamination and proliferation. Preservatives have disadvantages, and it is very difficult to keep samples constantly chilled. Because of these problems, the list of artifacts is long: bacteria proliferate; urine pH increases because urease-producing bacteria result in ammonia formation; highly alkaline urine can cause false-positive proteinuria with reagent strip methods; glucose is consumed by proliferating bacteria; volatile ketones leave solution; bilirubin is oxidized by light to biliverdin, which does not react with reagent strips; cells and casts disintegrate over time; and crystals form, especially those that occur in alkaline urine.

Well-conceived standard operating procedures and careful study design can help to eliminate or reduce many potential sources of variation. However, source variation must always be considered when interpreting clinical pathology data.

CLINICAL PATHOLOGY TESTS AND INTERPRETATIONS

Hematology

The hematology tests routinely performed during toxicology studies evaluate erythrocytes, leukocytes, platelets, and coagulation. Many automated cell counters can determine red blood cell

(RBC) count, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell (WBC) count, and platelet count from as little as 100 µl of anticoagulated whole blood. Because erythrocytes of the common laboratory species are smaller than human erythrocytes, the cell counters must be adjusted to ensure accuracy. In addition, platelet counts from rodents are often higher than the upper limit for some analyzers (1 million/µl) and adjustments are sometimes necessary for the instrument to report the high counts. Cell counters can now perform WBC differential counts on animal blood, but microscopic examination of a stained blood film is often necessary to confirm results when abnormalities occur. If an automated cell counter is unavailable, a considerable amount of information can be acquired from a packed cell volume (PCV; essentially the same as hematocrit) obtained by microhematocrit tube centrifugation, a WBC count performed on a hemocytometer, and examination of the blood film for cell morphology, estimation of platelet count, and WBC differential count. The common coagulation tests, prothrombin time (PT) and activated partial thromboplastin time (APTT), can be done manually but are usually determined by automated coagulation analyzers. Like the cell counters, some of the analyzers must be specifically set up for animal specimens.

Erythrocytes

The most common hematology findings in toxicology studies are mild decreases (e.g., < 10% from control animal values) in RBC count, hemoglobin concentration, and hematocrit (the percentage of whole blood made up of erythrocytes). The affected animals often exhibit additional findings such as mild reduction in body weight, body weight gain, or food consumption and clinical observations such as dull haircoat, poor grooming habits, and decreased activity. Although specific mechanisms for the erythrocyte effects are typically not identified, there appears to be a generalized reduction of anabolic processes, including erythropoiesis. Considering the normally brisk pace of RBC production (in humans, approximately 100 billion new cells per day), it is not surprising that erythrocyte mass is ultimately affected. Decreased physical activity and correspondingly decreased tissue oxygen demand can also contribute to reduced erythropoiesis. These relatively mild, nonspecific findings for circulating erythrocyte mass are identified most frequently in rat studies where the number of animals/sex/group is usually high (e.g., 10 animals/sex/group), the dose levels used are typically higher than those for dog or monkey studies, and the normal interanimal variability of hematology data is relatively low. In addition, because the circulating life spans of mouse and rat erythrocytes are shorter than those for dogs and nonhuman primates, similar reductions in erythropoiesis will become apparent for rodents before larger animals. Anemia is usually an inappropriate term for these mild effects on RBC count, hemoglobin concentration, and hematocrit and should be avoided so as not to imply a toxicologically adverse condition.

Anemia

Anemia is defined clinically as the condition characterized by a hemoglobin concentration below the lower reference limit. RBC count and hematocrit might or might not be proportionately lower, depending on the cause of the anemia and whether or not cell size and hemoglobin content are affected. The erythrocyte indexes, MCV and MCHC, measure cell size and hemoglobin concentration. Reticulocyte count and erythrocyte morphology are critical pieces of information for determining the potential causes of anemia. The general diagnostic approach to anemia is simple. The first step is to determine whether the anemia is regenerative or nonregenerative.

Regenerative Anemia

In regenerative anemia, the hematopoietic tissues are actively trying to replenish the lost erythrocytes by increasing production and release of new erythrocytes into circulation. Following

acute blood loss or hemolysis, it takes approximately 3 to 4 days for the new erythrocytes, or reticulocytes, to appear in peripheral blood. Reticulocytes can be counted manually as a percentage of erythrocytes on a peripheral blood film stained with a vital stain such as new methylene blue. Reticulocyte counts can also be done automatically by some of the newer hematology analyzers. It is best to determine the absolute reticulocyte count by multiplying the reticulocyte count that is no greater than a normal animal. In this case, the animal would not be showing an appropriate regenerative response for its degree of anemia. With the typical Romanowsky stains used for WBC differential counts, reticulocytes are larger and stain slightly more basophilic than the other erythrocytes. During a regenerative response, the erythrocyte morphology would be described by the terms anisocytosis (variable size) and polychromaisia (variable color). Nucleated red blood cells and Howell-Jolly bodies are often more numerous in peripheral blood films of animals with regenerative anemaia, but they also occur in some forms of nonregenerative anemia. Basophilic stippling of erythrocytes is a seldom seen change that can occur during regeneration; it is primarily associated with lead toxicity, however. During a regenerative response, the erythrocyte indexes usually show a higher MCV because of the influx of larger young cells and a lower MCHC because these large cells have a lower hemoglobin concentration. In toxicology studies, increased MCV is more common than decreased MCHC.

The two primary causes of regenerative anemias are blood loss and hemolysis. In addition to the erythrocyte changes, blood loss is usually characterized by lower serum protein concentrations. The source of the blood loss can be identified by clinical observations, necropsy findings, or tests such as fecal and urine occult blood. Hemolytic conditions do not generally cause changes in serum protein concentrations. Hepatosplenomegaly, icterus, bilirubinemia, and bilirubinuria might be associated findings. When hemolysis is suspected, careful examination of the peripheral blood films can sometimes identify the mechanism.

Heinz Body Anemia. Oxidizing agents can cause Heinz bodies, which are irreversibly precipitated hemoglobin attached to the internal surface of the erythrocyte membrane. Affected cells are removed from circulation by the mononuclear phagocyte system; the process is called extravascular hemolysis. Although Heinz bodies can usually be seen with Romanowsky stains, they are more readily identified with vital stains such as those used to count reticulocytes. The size and number of Heinz bodies observed in chronic low-level exposures might be small and difficult to see. Acute, high-dose exposures cause large, prominent Heinz bodies and a variety of other morphologic changes such as ghost cells (remnants of cell membrane without associated hemoglobin). Agents that produce Heinz bodies have the potential to cause methemoglobinemia and vice versa. Whenever one is observed, the other should be evaluated.

Immune-Mediated Hemolytic Anemia. Many agents can induce immune-mediated hemolytic anemia, although this tends to be an idiosyncratic reaction. There are three general mechanisms by which this occurs: the agent acts as a hapten bound to the erythrocyte membrane; the agent elicits an antibody response and the antigen–antibody complex binds to the erythrocyte membrane; or the agent causes the immune system to mistakenly recognize normal erythrocyte antigens as foreign. Immune-mediated hemolysis is also extravascular. Macrophages of the mononuclear phagocyte system might phagocytize entire erythrocytes or just portions of the membrane coated with immunoglobulin. In the latter case, spherocytes are formed, which can be identified microscopically on a peripheral blood film. Animals with immune-mediated hemolytic anemia might be severely anemic, but they usually exhibit a pronounced reticulocytosis. The erythrocytes are less osmotically resistant, and the osmotic fragility test has been performed to support the diagnosis. Unfortunately, this test is nonspecific and labor intensive. Antiglobulin, or Coombs' tests, might help to confirm the diagnosis by identifying immunoglobulin and complement on the cell surfaces. This test requires species-specific anti-immunoglobulin or anticomplement. Agglutination indicates a positive test. Occasionally, an animal's fresh whole blood will exhibit autoagglutination in the test tube or on a

wet mount of the blood; this is provisional evidence that the animal might have immune-mediated disease. Perhaps the most dependable way to prove that a test material caused immune-mediated hemolysis is to discontinue its administration until the animal exhibits recovery and then rechallenge the animal with the test material.

Hemolytic Anemia Due to Parasites. Hemotropic parasites can cause hemolytic anemia, but with the exception of malaria, caused by *Plasmodium* organisms in nonhuman primates, this is a rare cause of anemia in toxicology studies. Malarial organisms are intracellular and cause hemolysis via multiplication and rupture of the cell. They are readily observed during microscopic examination of blood films, but their appearance in blood fluctuates and it might be necessary to examine blood specimens at multiple intervals. Extracellular organisms that attach to the surface of the cell, such as *Eperythrozoon coccoides* in mice and *Hemobartonella* species in rodents and dogs, are rarely a problem with today's laboratory-reared animals. The infections are generally subclinical, but manifestations of hemolytic anemia can be induced by splenectomy or immunosuppression. Interestingly, subclinical infections in mice can alter certain responses in immune function tests and lead to erroneous conclusions.

Fragmentation Anemia. An unusual form of hemolysis that is also rarely observed in toxicology studies is fragmentation anemia. Injury to highly vascular tissue such as the lung, liver, or intestine with the formation of fibrin strands across small vessels can lead to intravascular lysis of erythrocytes as they are "clothes-lined" by the fibrin during passage through the vessels. The observation of schistocytes (helmet cells) on peripheral blood films is evidence of the process. Fragmentation anemia can also occur with vascular neoplasms such as hemangiosarcoma. Disseminated intravascular coagulation (DIC) is characterized by, among other things, schistocyte formation. Unfortunately, this condition is usually so severe that the animals bleed to death or die from major organ dysfunction before regeneration is possible.

Nonregenerative Anemia

In nonregenerative anemia, the hematopoietic tissues are unable to respond appropriately to the reduced circulating erythrocyte mass. The anemia is characterized by the absence of polychromasia and reticulocytosis. Although the erythrocytes often appear normal in color (normochromic) and size (normocytic), some types of nonregenerative anemia are distinguished by morphologically distinct cells. The severity of nonregenerative anemias varies according to the etiology.

Aplastic Anemia. If hematopoietic pluripotent stem cells are injured, as in irradiation or benzene toxicity, the anemia becomes progressively worse because senescent cells are not replaced. This form of anemia is called aplastic anemia. Typically, however, the animal will die from the consequences of severely decreased WBC count (infections) or platelet count (hemorrhage) long before it becomes it becomes severely anemic. This is because the circulating survival time of erythrocytes is several weeks, whereas it is only about a week for platelets and about a day for most leukocytes. Agents that damage the microenvironment necessary for hematopoiesis can have a similar effect. In addition, it is possible for a single cell line to be severely depleted without an effect on the others.

Anemia of Chronic Disease and Iron Deficiency Anemia. Chronic inflammatory lesions are associated with mild to moderate nonregenerative anemia. This is not unusual in chronic studies. The putative mechanism is a decrease in the transfer of iron to developing erythrocytes. The cells are normochromic and normocytic. In contrast, iron deficiency, most commonly associated with chronic blood loss or inadequate dietary iron, is characterized by cells that are hypochromic (inadequate hemoglobin) and microcytic. Animals with long-standing iron deficiency anemia have a low MCV and MCHC.

Megaloblastic Anemia. Humans who have folate or vitamin B_{12} deficiency have macrocytic erythrocytes because these nutrients are necessary for DNA synthesis and developing erythrocytes undergo fewer divisions before maturation. The anemias that result are referred to as megaloblastic, and asynchronous development of erythrocyte precursors is observed in bone marrow smears. Although folate and vitamin B_{12} deficiency are not problems in animals, agents such as methotrexate that block folate synthesis, or cyclophophamide, an alkylating agent that inhibits DNA synthesis, can cause megaloblastic anemia in animals.

Nonregenerative Anemia Associated with Chronic Renal or Liver Failure. Chronic renal failure is associated with a moderate to severe nonregenerative, normochromic, normocytic anemia. Although the mechanism is usually decreased production of erythropoietin, a number of uremic toxins have also been implicated in the anemia of chronic renal disease. Chronic liver failure is associated with moderate anemia. Acanthocytosis, an erythrocyte morphologic abnormality characterized by several blunt cytoplasmic projections resembling pseudopodia, is sometimes a feature of this anemia. The mechanism is believed to be an imbalance in the cholesterol:phospholipid ratio in the cell membrane. As with anemia of renal failure, the anemia of liver failure is not nearly as significant to the animal as the primary organ dysfunction. Endocrine disorders such as hypothyroidism and hypoadrenocorticism can also have mild nonregenerative anemia as part of the disease syndrome.

Nonregenerative Anemia Associated with Leukemia. Finally, nonregenerative anemia is typically a feature of leukemia. The cells are generally normochromic, normocytic unless it is the erythrocytic line that is neoplastic. The principle mechanisms of the anemia are the "crowding out" of normal hematopoietic tissue by the neoplastic cell proliferation and competition for nutrients. An interesting exception is large granular lymphocyte leukemia of Fischer-344 rats. This form of leukemia is associated with a predictable immune-mediated hemolytic anemia that is regenerative.

Polycythemia

Increased RBC count, polycythemia, is fairly unusual in toxicology studies. The most common cause of relative polycythemia is fairly unusual in toxicology studies. The most common cause of relative polycythemia is simple dehydration. Relative polycythemia can also be observed secondary to lung disease, which causes systemic hypoxia and trigger erythropoietin production. A similar mechanism occurs with systemic alkalosis. By increasing the affinity of hemoglobin for oxygen, alkalosis causes the renal tissue sensors to detect hypoxia, triggering erythropoietin production.

Leukocytes

The examination of leukocytes is part of the minimum hematologic database. It includes the quantitative determination of total and differential WBC counts and the qualitative assessment of cellular morphological abnormalities. The differential WBC count enumerates granulocytes (neutrophils, eosinophils, and basophils), lymphocytes, and monocytes. Increased numbers of these cells are called neutrophilia, eosinophilia, basophilia, lymphocytosis, and monocytosis, respectively. Neutropenia, eosinopenia, and lymphopenia refer to decreases. The normal cell counts for basophils and monocytes are so low that decreases are difficult to recognize. Relative counts (percentages) for the different types of leukocytes, obtained by doing the differential count, are of little or no value without knowledge of the total WBC count. A dog with 70% neutrophils could be normal or have neutrophilia or neutropenia depending on the total WBC count. Unfortunately, some of the regulatory guidelines for carcinogenicity studies require differential counts but do not require total WBC counts. By looking only at the relative count data, a 90% lymphocyte count with 100,000 lymphocytes/μl cannot be distinguished from a 90% lymphocyte count with 5,000 lymphocytes/μl.

Because it is not always possible to microscopically distinguish neoplastic lymphocytes from normal lymphocytes, nothing in the reported data would indicate the possibility of lymphocytic leukemia in the first case. Differential WBC counts should always be reported as absolute numbers.

Neutrophils and lymphocytes are the principle cell types found in peripheral blood, and toxicologic effects on leukocytes usually involve one or both of these cell lines. Although primary effects occur, the changes observed are most commonly secondary changes in response to primary toxicity of other tissues or organ systems.

Excited or frightened animals might have a physiological leukocytosis (increased WBC count) due to endogenous catecholamine release. Increased heart rate, blood pressure, or muscular activity mobilizes cells that normally marginate along the endothelium of smaller vessels. The addition of these cells can more than double the number flowing freely in circulation and consequently increase the WBC count proportionately. Neutrophilia and lymphocytosis can both occur.

A steroid or stress-induced leukocyte response refers to a combination of changes observed in animals receiving corticosteroids or producing increased endogenous corticosteroids because of some stressful condition. It generally consists of a mature neutrophilia (no immature neutrophils such as bands or metamyelocytes), lymphopenia, and sometimes monocytosis depending on the animal species. The mature neutrophilia develops as a consequence of increased release of segmented cells from the bone marrow storage pool, decreased margination of cells, decreased movement of cells into tissues, and increased stability of lysosomal membranes. Lymphopenia results from steroid-induced lysis and cell redistribution. Eosinopenia develops as a result of decreased production and release from the bone marrow. Monocytosis, when it occurs, is thought to result from mobilization of marginated cells. It is interesting that the stress-induced leukocyte response is a relatively infrequent finding in toxicology studies even though the study design or the test material often creates physical conditions that appear to be quite stressful.

Neutrophils

The primary function of the neutrophil is phagocytosis of small particulate matter (e.g., bacteria). The neutrophil is also an integral cellular component of inflammation. It is therefore not unusual to observe neutrophilia secondary to nearly any inflammatory lesion caused by a test material. Unless moderately severe, however, dermal inflammation might not induce increases in neutrophil count. The term *left shift* indicates an increased number of immature neutrophils in circulation. A left shift can occur whenever an inflammatory lesion has a significant demand for neutrophils and immature cells are released from the bone marrow. Lesions that cause a left shift are almost always easily identified, if not by physical examination and the evaluation of other laboratory data, then certainly at necropsy. They frequently involve infectious organisms that have invaded tissue damaged by the test material. A “degenerative” left shift describes the situation of a normal or decreased neutrophil count with more immature than mature neutrophils. It generally indicates a severe infection such as might occur with aspiration pneumonia or during a study using indwelling IV catheters that become contaminated, leading to systemic bacterial infection. In severe conditions such as these, when the demand for neutrophils is extreme, so-called toxic neutrophils might be observed. These are neutrophils with morphologic changes such as cytoplasmic basophilia, vacuolation, or granulation and Dohle bodies (small, bluish-gray cytoplasmic inclusions that represent aggregated rough endoplasmic reticulum).

Neutropenia occurs for three primary reasons. There might be extreme demand for and consumption of neutrophils as described earlier. There might be sequestration of neutrophils along the endothelium in capillary beds as occurs in endotoxic shock. And there might be decreased production of neutrophils. Irradiation, chemotherapeutic drugs, inorganic solvents, and estrogens are examples of agents that cause damage to myeloid precursors. It is very common for these agents to also affect erythrocyte and platelet production, but neutropenia and increased susceptibility to

bacterial infection will be the hematologic problem first encountered. On rare occasion, drugs might induce immune-mediated neutropenia.

Lymphocytes

These cells are responsible for a wide variety of immune system functions. Although there are many lymphocyte subpopulations, it is not possible to distinguish them by light microscopic examination. Lymphocytes are unique among leukocytes in that they recirculate; that is, lymphocytes leave the vascular system through venules in lymph nodes and ultimately return to the blood through the thoracic duct. They are long-lived cells compared with other leukocytes. The most common cause of lymphocytosis is the physiological lymphocytosis associated with excitement, as previously described. Occasionally, increased lymphocyte counts are observed with chronic infections (especially in rodents), and much less frequently with hypersensitivity syndromes and immune-mediated diseases.

Much more common than lymphocytosis is the presence of morphologically distinct lymphocytes sometimes called "reactive" lymphocytes or immunocytes. They can be seen in low numbers secondary to any type of antigenic stimulation and are so common that no mention is usually made of their presence.

Lymphopenia occurs most frequently as a part of the steroid or stress-induced leukocyte response. Agents that cause neutropenia, such as chemotherapeutic agents, will usually cause lymphopenia as well. Because of the many subpopulations of lymphocytes, it is difficult to know the biological significance of a small change in lymphocyte number. It is possible that a small change could represent an effect on a specific subpopulation of cells. This is the case with HIV infection in humans.

Eosinophils, Basophils, and Monocytes

Absolute eosinophil, basophil, and monocyte counts are normally very low (usually < 1,000/μl) and quite variable. It is very unusual, therefore, to be able to detect toxicologic effects on these cell types. Eosinophilia might occur secondary to some hypersensitivity syndromes. Eosinophilia can result from the steroid or stress-induced leukocyte response. The primary function of the monocyte is phagocytosis and digestion of large particulate matter such as senescent cells, necrotic cellular debris, and large micro-organisms. Monocytes process antigens and present them to lymphocytes in a more antigenic form. Monocytosis can occur secondary to lesions involving extensive tissue destruction such as neoplasms with associated necrosis or hemolytic anemia.

Leukemia

It is not unusual for a small percentage of rodents (control and treated animals) to develop leukemia in a carcinogenicity study. Animals with leukemia do not always have elevated WBC counts, and neoplastic cells might be difficult to find on peripheral blood films. Unfortunately, when neoplastic cells are present it large numbers, it is often difficult to identify the specific cell type using routine staining procedures and light microscopic examination. Diagnosis of leukemia is more easily accomplished by the histopathologic examination of study animals than by periodic examination of the blood as required by regulatory guidelines.

Platelets

Almost immediately following vascular injury, platelets adhere to exposed collagen and begin to aggregate, forming a primary platelet plug that is sufficient to control bleeding from minor injuries of very small vessels. The aggregated platelets release a variety of substances that stimulate

vasoconstriction and fibrin formation. The fibrin acts to cement the mass of platelets into a stable hemostatic plug. Signs of thrombocytopenia (decreased platelet count) include petechial and ecchymotic hemorrhages (most easily observed in mucous membranes), melena, and prolonged bleeding from small wounds such as venipuncture sites. Signs are generally not apparent until the count is less than 50,000/μl. Platelet function defects, such as that caused by aspirin, might cause identical signs, but the tendency to do so is much less. In addition to platelet count, platelet function studies measuring adhesion and aggregation are available but impractical as standard tests. Bleeding time is an *in vivo* test that measures the functional ability of platelets to stop the bleeding from a controlled superficial wound. Although commonly used in human medicine, this test is difficult to standardize in animals. It should be reversed for investigational purposes.

Thrombocytopenia

This occurs as a result of either decreased production or increased consumption of platelets. Toxins that affect erythroid and myeloid progenitor cells often cause injury to megakaryocytes (platelet precursors). Because the circulating life span of platelets is about 7 to 10 days, acute toxicity of megakaryocytes leads to decreased platelet count in about a week. Increased consumption of platelets might be caused by immune-mediated phenomena or disseminated intravascular coagulation. In the latter, prolonged coagulation times, decreased plasma fibrinogen concentration, and increased fibrin and fibrinogen degradation products (FDPs) are associated findings. One clue for differentiating decreased production from increased consumption is the presence of large macroplatelets in peripheral blood. These generally indicate increased production of young platelets that are larger than normal.

Thrombocytosis

Increased platelet count, thrombocytosis, is rarely a primary effect of a test material. It can be observed as a secondary effect in conjunction with generalized bone marrow stimulation as in hemolytic anemia or some inflammatory diseases. Thrombocytosis is also associated with iron deficiency. The increases in platelet numbers that occur in toxicology studies are generally small and not likely to have any biological significance. If platelet counts are markedly elevated, however, the potential for thromboembolic events is increased.

Coagulation

The coagulation mechanism is traditionally divided into two pathways. The intrinsic pathway, routinely evaluated by the APTT or the activated coagulation time (ACT), begins with exposure of factor XII to subendothelial collagen or other abnormal surfaces. The extrinsic pathway, evaluated by the one-stage PT, is initiated by exposure of factor VII to tissue thromboplastin. Both mechanisms share the terminal sequence of events, including conversion of prothrombin to thrombin, which in turn converts fibrinogen to fibrin. The coagulation assays can be compromised in smaller laboratory animal species by sample collection problems.

The assays are not particularly sensitive to small changes in the concentration of clotting factors. In general, the activity of a single factor must be reduced below approximately 30% of normal before coagulation times are prolonged. Given the conditions in most toxicology studies, where animals are exposed to the test material for a prolonged period of time, if there were a significant effect on the production of a clotting factor, it is likely that the animals would exhibit some form of bleeding diathesis. These include severe hemorrhage externally (e.g., epistaxis or hematochezia) or internally (e.g., subcutaneous hematoma, hemothorasx, or hemarthrosis).

The liver synthesizes the majority of clotting factors. Liver injury and dysfunction can cause depletion of clotting factors sufficient to prolong the coagulation assays. The liver requires vitamin

K for the production of functional forms of factors II, VII, IX, and X. Vitamin K antagonists, such as warfarin and diphacinone, cause prolongation of both the intrinsic and extrinsic mechanism assays. Because factor VII has the shortest half-life and is part of the extrinsic mechanism, prolonged PT occurs before prolonged APTT. Because vitamin K is a fat-soluble vitamin, coagulation assays are indicated when test materials are administered that have the potential of depleting fat-soluble vitamins. This is true of synthetic fats that are not absorbed by the intestine.

Depletion of all clotting factors with subsequently prolonged coagulation times is a feature of DIC. Fibrinogen concentrations and FDP assays are used to help confirm the diagnosis.

Small, statistically significant differences in mean PT and APTT between control and treated animals (e.g., 2 sec) are occasionally observed in toxicology studies. This degree of change has little or no biological impact on the individual animals. However, it should not be casually dismissed because it could be an early indication of a potential problem. Depending on the test material, it might be necessary to design longer studies or increase the dose levels to see if the effect is repeatable and meaningful.

Clinical Chemistry

The clinical chemistry tests routinely performed during toxicology studies generate information concerning carbohydrate, lipid, and protein metabolism, renal function, liver function, hepatocyte injury, and electrolyte balance. Advances in clinical chemistry instrumentation have greatly reduced sample volume requirements and have therefore enabled the laboratory to produce complete biochemical profiles on animals as small as rats without compromising the study because of excessive blood collection. Most instruments can use 250 µl of serum or less to run a 17- or 18-test panel. The vast majority of common tests do not require modification of the methods used for testing human samples. There are, however, many differences in the expected ranges of results for individual species. When purchasing an instrument and reagents for work with animal specimens, volume requirements (including dead space) and ranges of linearity are important considerations.

Carbohydrate, Lipids, and Proteins

Glucose

Serum glucose concentration depends on intestinal absorption, hepatic production, and tissue uptake of glucose. The balance between hepatic production and tissue uptake is influenced by a variety of hormones including insulin, glucagons, corticosteroids, adrenocorticotropic hormone (ACTH), growth hormone, and catecholamines. Insulin is the primary factor responsible for the uptake of glucose by tissues. Corticosteroids, catecholamines, and growth hormone are called insulin antagonists because they interfere with insulin's action on cells. Further more, glucagons and glucocorticoids stimulate hepatic gluconeogenesis, and glucagons and catecholamines glycogenolysis. These actions tend to increase serum glucose concentration. The practice of fasting animals prior to blood collection decreases the variability that accompanies postprandial intestinal absorption of glucose. Another procedural consideration for glucose analysis is prompt separation of the serum from clotted blood. As mentioned previously, erythrocyte, and to a lesser degree leukocyte, glycolysis will reduce serum glucose concentration by approximately 7 to 10 mg/dl every hour that the blood cells remain in contact with the serum at room temperature.

Hyperglycemia and Hypoglycemia. The most frequently encountered causes of hyperglycemia are failure to fast an animal and catecholamine release secondary to excitement or fear. Animals that become moribund occasionally develop hyperglycemia. Less frequently encountered causes, especially in toxicology studies, include insufficient insulin (diabetes mellitus and pancreatitis) and increased glucocorticoids (hyperadrenocorticism and steroid therapy). Hypoglycemia can result

from improper handling of the specimen, malnutrition, malabsorption, severe hepatic disease, endotoxemia, and some tumors, in particular, insulinomas and hepatomas. Occasionally in toxicology studies, treated animals that fail to thrive and gain body weight will also have mildly lower serum glucose concentration (e.g., 10–15 mg/dl) than the control animals even though there are no differences in food consumption. Although the mechanism for this phenomenon is not clear, two possibilities are poor assimilation of the food and alteration of the body's "set-point" for serum glucose. Regardless of the cause, the reduction is probably of little biological importance and is simply a reflection of the overall process that has caused the animals to do poorly.

Cholesterol and Triglycerides

Cholesterol is required for the biosynthesis of bile acids, corticosteroids, and sex steroids. Triglycerides serve as an important source of energy. Serum cholesterol and triglycerides are derived from dietary intake and endogenous synthesis, primarily by the liver. The liver, via the biliary system, is the major excretory pathway for cholesterol. In circulation, cholesterol and triglycerides are components of chylomicrons and the lipoproteins: very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). Chylomicrons, which cause serum to appear lipemic, are produced by intestinal cells after a fatty meal and are rich in triglycerides. Hepatocytes synthesize VLDL, which has less triglyceride than chylomicrons but more cholesterol. The triglycerides in chylomicrons and VLDLs are broken down to free fatty acids and monoglycerides by lipoprotein lipase attached to the surface of endothelial cells, especially in the capillaries of adipose tissue and muscle. Adipocytes tend to re-esterify the fatty acids for storage as triglycerides. Muscles tend to oxidize the fatty acids for energy. The loss of triglyceride causes VLDL to become LDL. In humans, about two-thirds of serum cholesterol is transported by LDL. In contrast, HDL, generated by the liver and other tissues, is the principal lipoprotein responsible for cholesterol transport in several animal species. Species differences in lipid metabolism make it quite difficult to correlate effects in animal models with those in humans.

Serum triglyceride concentration is elevated postprandially, whereas serum cholesterol concentration is relatively stable. Both are elevated in hypothyroidism and diabetes mellitus. Cholesterol is the predominant lipid in hypothyroidism, and triglycerides predominate in diabetes mellitus. In both cases, lipoprotein lipase activity is reduced.

Biliary stasis, whether intrahepatic or extrahepatic, and other forms of liver disease can increase serum cholesterol concentration, but severe liver disease is associated with hypocholesterolemia.

Nephrotic syndrome, which is almost always the result of glomerular injury, is characterized by increased urinary protein excretion, hypoalbuminemia, and hypercholesterolemia. Steroid therapy, and perhaps hyperadrenocorticism, are also associated with increased serum cholesterol concentration. As might be expected, nutrient deficiency and malassimilation can cause decreased serum cholesterol concentration.

Effects on serum cholesterol concentration are relatively frequent findings in toxicology studies. Both increases and decreases are observed. The changes are usually small and generally believed to represent minor alterations in lipid metabolism, and the exact mechanisms involved are rarely identified. Many factors are probably involved, including food consumption and assimilation, body weight and composition, activity, and hormone balance.

Protein

Total serum protein concentration is a measure of all of the different proteins in plasma with the exception of those that are consumed in clot formation such as fibrinogen and the clotting factors. For this reason, plasma protein concentration is generally about 0.3 to 0.5 g/dl higher than serum protein concentration.

Albumin. Albumin is the most abundant individual protein and is largely responsible for maintaining intravascular oncotic pressure. Albumin also serves as a storage reservoir of amino acids and as a transport protein, binding most plasma constituents that do not have a specific transport protein.

Globulins. The globulins constitute a heterogeneous population of proteins, including specific transport proteins (e.g., transferring for iron, lipoproteins for lipids, haptoglobin for hemoglobin, and thyroxine-binding globulin for thyroxine), mediators of inflammation (e.g., complement and C-reactive protein), clotting factors (e.g., fibrinogen, thrombin, and factor VIII), catalysts and inhibitors of biochemical reactions (e.g., enzymes), and immunoglobulins (e.g., IgG, IgM, and IgA). Globulins are nonspecifically categorized by their electrophoretic migrations pattern as α-, ß-, and γ- globulins. Depending on the animal species, these regions can be further subdivided; most species have two α regions, $alpha_1$ and $alpha_2$. Immunoglobulins are generally found in the γ regions of the electrophoretogram but some, IgM in particular, also extend into the ß regions. The live synthesizes albumin and most of the globulins, with the major exception of immunoglobulins.

Serum Protein Electrophoresis. In most laboratories, total serum protein and albumin concentrations are measured directly, and serum globulin concentration is calculated by subtraction. Serum protein electrophoresis is sometimes valuable for determining the cause of increased or decreased globulin concentration, and its use should be reserved for that purpose. A broad-based increase in γ-globulins, generally the result of antigenic stimulation, is termed a polyclonal gammopathy because plasma cells have produced a heterogeneous population of immunoglobulins in response to the antigenic challenge. A narrow-based increase in γ-globulins is termed a monoclonal gammopathy and is usually the result of a single immunoglobulin class being produced in excess by a single clone of lymphocytes or plasma cells. Most frequently, but not always, the clone of cells is neoplastic (e.g., multiple myeloma). A broad-based increase of the α-globulin regions, especially $alpha_2$, frequently occurs secondary to inflammation and the production of acute-phase reactant proteins. These acute-phase proteins (e.g., $alpha_2$-macroglobulin, haptoglobin, and ceruloplasmin) are part of the general response to inflammation.

The cost:benefit ratio of serum protein electrophoresis is high, and it should not be routinely run as part of the minimum database. Unfortunately, some regulatory guidelines recommend routine serum protein electrophoresis in safety assessment studies. If the production of a single, specific protein in the α or ß regions is affected by a test material, the absence of change in the other proteins of the same region will probably be sufficient to mask any effect on the electrophoretogram. On the other hand, if the test material does affect a particular region, further identification procedures are necessary because electrophoresis does not identify specific globulin proteins.

Hyperproteinemia. The most frequent causes of hyperproteinemia are dehydration and polyclonal gammopathy secondary to antigenic stimulation. Monoclonal gammopathies are rare in toxicology studies. When uncomplicated dehydration is the cause of hyperproteinemia, both serum albumin and serum globulin concentrations are increased proportionately.

Hypoproteinemia. This results from either decreased production or increased loss of protein. In dietary toxicity studies, decreased protein production can result from effects on food consumption, digestion, or absorption. Because of the reserve capacity of the liver, hepatic injury must be fairly severe before protein synthesis is notably diminished. However, in large studies, small differences between the control and treated groups might be apparent with mild to moderate hepatotoxicity. Loss of protein, both albumin and globulin, occurs with hemorrhage and exudative lesions such as burns. Albumin is the principle protein lost as a result of enteropathies and glomerulopathies. The half-life of albumin is shorter in smaller species, at approximately 2 days for mice and approximately 8 days for dogs (Kaneko 1997). Theoretically, impaired albumin synthesis or albumin loss can be

detected earlier in the smaller species. Hydration status of the animal is always an important factor for proper interpretation of changes in serum protein concentrations. Hypoproteinemia, like anemia, can be masked by dehydration. A small, statistically significant decrease in serum albumin concentration is one of the most frequent findings in toxicology studies. The exact mechanism is usually not apparent but a combination of factors, similar to those causing mildly lower glucose, are probably responsible.

Renal Function

Serum urea nitrogen concentration and serum creatinine concentration are used in conjunction with urine specific gravity or osmolality to evaluate renal function. These tests are relatively insensitive to small effects on the kidney, and a number of nonrenal causes for serum elevations must be considered.

Urea Nitrogen

Urea is synthesized by the liver from ammonia that is absorbed from the intestine or generated by endogenous protein catabolism. It is freely filtered through the glomerulus and excreted in urine. Some urea is passively reabsorbed with water in the proximal tubule; the amount that is reabsorbed is inversely related to rate of urine flow through the tubules. Decreased glomerular filtration rate (GFR) causes serum urea nitrogen concentration to increase. However, because urea production can vary with diet or protein catabolism, serum urea nitrogen concentration, termed azotemia, can be categorized as prerenal, renal, or postrenal.

Prerenal Azotemia. This type of azotemia develops as a result of increased hepatic urea synthesis or decreased renal blood flow. The former can result from high-protein diets or conditions that increase protein catabolism such as starvation, fever, infection, tissue necrosis, and high GI hemorrhage. Decreased renal blood flow, with subsequently decreased GFR, can result from dehydration, shock (hemorrhagic or circulatory), or cardiovascular disease. The changes in serum urea nitrogen concentration caused by increased urea synthesis are typically small, but those caused by decreased renal perfusion are dependent on the degree of GFR reduction and can be quite large. The concentrating ability of the kidney is not affected by these conditions. In the case of prerenal azotemia due to dehydration, the kidneys attempt to conserve body water and urine specific gravity is elevated.

Renal Azotemia. This type of azotemia develops as a result of primary renal disease or toxicity. The renal lesions can be acute or chronic. Because of the sizable reserve capacity of the kidney, serum urea nitrogen concentration does not increase noticeably until approximately 75% of the kidneys' nephrons are nonfunctional. By this time, renal concentrating ability is usually impaired and urine specific gravity is isosthenuric (i.e., the same as the glomerular filtrate: 1.008–1.012). An indication of the chronicity of the renal lesions can be gleaned from the hematology data. Concurrent nonregenerative anemia suggests that the lesions are chronic.

Postrenal Azotemia. This type of azotemia results from obstruction of the urinary outflow tract. This is rarely observed in toxicology studies, but test materials that promote urinary calculi formation might cause this condition.

Creatinine

This is a nonprotein nitrogenous waste material that is freely filtered by the glomerulus and, unlike urea, is not reabsorbed by the tubules. It is formed at a fairly constant rate by the breakdown of creatine, a molecule that stores energy in muscle as phosphocreatine. Serum creatinine concentration

is influenced by muscle mass and conditioning but is relatively independent of dietary influences and protein catabolism. Although it tends to rise and fall more slowly, serum creatinine concentration parallels changes in serum urea nitrogen concentration caused by alterations in renal blood flow, renal function, or urinary outflow.

Endogenous creatinine clearance is sometimes used as a measure of GFR because blood levels of creatinine are relatively stable over short intervals, creatinine is freely filtered, and creatinine is not significantly secreted or reabsorbed. Endogenous creatinine clearance might underestimate the true GFR because of the presence of noncreatinine chromagens that spuriously increase the measure of serum creatinine concentration but not urinary creatinine concentration.

Liver Function and Hepatocyte Injury

The critical metabolic, synthetic, and excretory roles of the liver and the abundant enzymatic machinery needed to perform these functions result in a large number of biochemical parameters that could be altered due to toxicity. On the other hand, the large functional reserve of the liver makes possible a significant loss of tissue with minimal or no detectable change in routine laboratory tests. Although no single test is superior for detecting liver toxicity, the pattern of abnormal findings in a battery of tests could help to determine the location and severity of liver lesions.

Many enzymes have been identified that have increased serum activity when hepatocellular damage is present. Although these enzyme activities are not measures of liver function, they can detect cellular degeneration or necrosis. The utility of a particular enzyme depends on a number of factors, including relative specificity to liver; intrahepatic location; intracellular location; concentration gradient between cell and serum; serum half-life; *in vitro* stability; and the ease, accuracy, and economy of measurement. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), sorbitol dehydrogenase (SDH), lactate dehydrogenase (LDH), and glutamate dehyrogenase (GDH) are some of the enzymes that have been used to evaluate hepatocellular damage. Each has advantages and disadvantages, some of which depend on the laboratory animal species being tested.

Serum ALT Activity

In general, serum ALT activity, formerly known as serum glutamic pyruvic transaminase (SGPT), is the most useful enzyme activity for identifying the presence of hepatocellular damage. The enzyme is found in many tissues, but its greatest concentration in most species is within hepatocytes. For practical purposes and in the absence of severe muscle necrosis, significant elevations of serum activity result only from hepatocyte ALT. The enzyme is primarily cytosolic, and its concentration within the cell is up to 10,000 times greater than that in the serum. Therefore, ALT can "leak" into serum in any condition that alters membrane permeability to a sufficient degree. This does not require cell death; elevated activity does not imply necrosis. The magnitude of serum activity elevation is proportional to the number of affected hepatocytes and is not indicative of the reversibility of the lesion. For example, it is possible to have higher serum ALT activity following reversibility of the lesion. It is also possible to have higher serum ALT activity following reversible cellular hypoxia secondary to hypoolemic shock than might occur with focal necrosis caused by localized hepatic abscess. Of course, the greatest elevations result from severe lesions that affect a large portion of the liver tissue.

Following an acute hepatotoxic episode, serum ALT activity will rise within 1 hr. Depending on the injury, the activity will peak in 1 to 2 days and then decline. Prolonged elevations might reflect increased production of ALT in regenerative liver tissue or continued "leakage" from cells with close proximity to the primary lesion that has resulted in degenerative changes secondary to the altered microenvironment. However, acute studies designed so that clinical pathology tests are run 14 days after a single administration of the test material are likely to miss changes in serum ALT activity even if the test material causes marked hepatotoxicity (e.g., carbon tetrachloride).

Increased serum ALT activity is not specific for primary hepatocellular disease. Bile duct obstruction also causes increased activity. The probable mechanism is that retained bile salts physically damage the cell membranes of surrounding hepatocytes. Drugs such as corticosteroids and anticonvulsants appear to induce ALT production. Because these drugs can also cause pathological changes that result in high serum enzyme activity, it is difficult to determine whether an elevation is due to enzyme induction or drug-induced disease without histopathological examination.

Serum AST and LDH Activities

Serum AST (formerly serum glutamic oxaloacetic transaminase; SGOT) and LDH activities tend to parallel serum ALT activity with respect to liver damage. Unfortunately, they are not liver specific because of high concentrations in other tissues, especially muscle. In small species, blood collection techniques sometimes cause increased variability, reflected in wider reference ranges. There appears to be no advantage in determining both of theses enzyme activities, and AST is generally preferred. In most species, elevations in serum AST activity due to hepatotoxicity are not as pronounced as the elevations in serum ALT activity; this might occur because a portion of AST is mitochondrial. Corticosteroids and anticonvulsants affect AST in a similar manner as ALT. Decreased serum activities of AST and ALT are occasionally observed in toxicology studies and might indicate decreased hepatocellular production or release, inhibition of the enzymes' activity, interference with the enzyme assay, or an effect on the coenzyme, pyroidoxal 5'-phosphate (vitamin B_6). Decreased serum activity of these enzymes has not been shown to be a pathologically important phenomenon.

Serum SDH and GDH Activities

Serum SDH and GDH activities, neither of which are used in human medicine in the United States, have occasionally been recommended as good indicators of hepatic toxicity in laboratory animal species. Increased serum activity of each enzyme is liver specific; SDH is a cytosolic enzyme and GDH is mitochondrial. Theoretically, for serum GDH activity to increase, the cell damage must be relatively severe. Elevations in serum SDH activity return to baseline levels faster than they do for other liver enzymes because of a short serum half-life. The major drawback for both of these enzymes is the assay. Their lack of popularity in human medicine is responsible for the absence of standard automated procedures.

There are few serum enzymes, originating from hepatocytes and biliary epithelial cells, that increase as a result of increased production secondary to intrahepatic or extrahepatic cholestasis and biliary proliferation. These include serum alkaline phosphatase (AP), γ-glutamyl tranferase (GGT), leucine aminopeptidase (LAP), and 5'-nucleotidase (5'N). The most commonly used are AP and GGT. Both are bound to cytoplasmic and microsomal membranes. The mechanism for the cholestasis-induced production is uncertain, but bile acids are thought to stimulate enzyme synthesis.

Serum AP Activity

In the dog, serum AP activity is the most sensitive test for detecting cholestasis. It lacks specificity, however. Isoenzymes or isoforms of AP are produced by cells of the intestine, kidney cortex, liver, bone, placenta, and myeloid series. Normal serum AP activity in most adult animals is primarily the liver isoenzyme. Young, growing animals have an increased amount of the bone isoenzyme in serum secondary to increased osteoblast activity. In humans, but not dogs, placental isoenzyme is increased during pregnancy. The isoenzymes of liver and bone have half-lives of about 3 days in the dog, whereas those of the intestine, kidney, and placenta are only 3min to 6 min. It is very unlikely that any of the latter three isoenzymes will produce serum elevations. Elevations in the bone isoenzyme rarely exceed three to five times the normal adult levels, even in young

animals and animals with metabolic bone diseases. Corticosteroids and anticonvulsants can induce liver isoenzyme production. Knowledge of the test material characteristics might help to identify probable drug-induced increases. In some dogs, a unique steroid-included isoenzyme can cause extremely high serum AP activity.

Because of cell swelling and pressure obstruction of small bile ductules, primary hepatocellular toxicities can and do cause enough intrahepatic cholestasis to elevate serum AP activity. Periportal lesions induce greater increases than do centrilobular lesions. Extrahepatic cholestasis, as might be seen with pancreatitis or biliary calculi, stimulates higher serum AP activity than intrahepatic cholestasis. Unfortunately, the degree of elevation is rarely sufficient to differentiate primary hepatocellular toxicity from primary biliary toxicity.

Serum GGT Activity

Serum GGT activity became popular because it purportedly had fewer interpretation problems than serum AP activity. Although its highest tissue concentrations are in the kidney and pancreas, serum elevations are reported to occur only with hepatobiliary lesions and certain drug therapies. Like AP, it can be induced by steroids and other drugs. Unlike AP, it is not affected by bone growth or disease. Furthermore, its serum activity is less likely to increase secondary to primary hepatocellular toxicity or intrahepatic cholestasis due to hepatocellular swelling. In laboratory animal species, GGT appears somewhat less sensitive to hepatobiliary disease than AP; however, there are some exceptions (Ghys et al. 1975; Leonard et al. 1984).

Serum LAP and 5'N activities have been investigated as alternatives to serum AP activity but have not found general acceptance. In some models of liver toxicity, 5'N appears to be more sensitive than AP.

The absence of change in serum hepatic enzyme activities does not signify an absence of hepatic toxicity. The liver can be severely dysfunctional and yet have little or no ongoing cellular degeneration. Elevations can be missed by poor timing of clinical pathology testing.

Bilirubin

Heme breakdown by cells of the mononuclear phagocyte system produces bilirubin. Hemoglobin from senescent erythrocytes provides about 85% of all bilirubin; hemoglobin from ineffective erythropoiesis provides another major source. Macrophage enzymes split hemoglobin into heme and globin, and heme is broken down into biliverdin and iron. Biliverdin reductase converts biliverdin to bilirubin, which is then released into circulation. At this point, the bilirubin is known as free, unconjugated, prehepatic, or indirect-reacting bilirubin. It is not water soluble and circulates bound to albumin. Hepatocytes remove unconjugated bilirubin from plasma and prepare it for removal from the body by a four-step process that includes uptake, conjugation, secretion, and excretion. Secretion of conjugated bilirubin across the canalicular membrane is the rate-limiting step in the process, and small amounts of conjugated or direct-reacting bilirubin escape into plasma, not bound to albumin. Conjugated bilirubin is freely filtered through the glomerulus. In most species, it is reabsorbed by the renal tubular epithelium, but in the dog the renal threshold is low and traces of bilirubin are normal in concentrated urine.

The van den Bergh test differentiates unconjugated (or indirect) bilirubin from conjugated (or direct) bilirubin. The test is not very sensitive and should not be run unless the total bilirubin concentration is greater than 2 to 3 mg/dL. It was designed to help distinguish prehepatic causes of hyperbilirubinemia from hepatic or posthepatic causes in clinical cases. In toxicology studies, however, the combination of clinical observations, other laboratory data (e.g., hematocrit and liver enzyme activities), and anatomical pathology findings is usually more than sufficient to determine the mechanism of hyperbilirubinemia. Laboratory determination of direct and indirect bilirubin is generally of little value.

Hyperbilirubinemia. Prehepatic or unconjugated hyperbilirubinemia is a very uncommon finding in toxicology studies. It occurs almost exclusively as a result of acute, moderate to severe hemolysis. If hepatocytes are unable to process the large amount of unconjugated bilirubin produced by the mononuclear phagocyte system during a hemolytic episode, there is an increase in total serum bilirubin concentration consisting primarily of the free form. It has been estimated that the healthy liver has the capacity to metabolize up to 30 times more free bilirubin than normal before hyperbilirubinemia results. A hemolytic even sufficient to overload a normal liver and cause unconjugated hyperbilirubinemia can be expected to cause other evidence of hemolysis. Unconjugated hyperbilirubinemia can occur with less severe hemolysis unconjugated hyperbilirubinemia syndromes in humans that result from a biochemical defect in the uptake and conjugation of free bilirubin. The Gunn rat is an animal model of defective conjugation.

Conjugated hyperbilirubinemia occurs as a result of impaired secretion of bilirubin, obstruction of bile outflow, or both. Because bilirubin secretion is the rate-limited step, any disease that damages the hepatocyte can potentially increase serum conjugated bilirubin concentration. Obstruction to bile flow can be either intrahepatic or extrahepatic in origin. As with serum AP activity, periportal lesions induce greater hyperbilirubinemia than centrilobular lesions, and extrahepatic obstructions cause higher serum bilirubin concentration than do intrahepatic obstructions. In the dog, when hyperbilirubinemia is the result of a cholestatic process, serum AP activity is elevated.

Hyperbilirubinemia is most commonly due to increases in both conjugated and unconjugated bilirubin. For instance, hemolytic disease can cause secondary hepatocyte dysfunction due to hypoxia. The result is swollen hepatocytes that have impaired ability to secrete conjugated bilirubin. Alternately, biliary obstruction can cause secondary hepatocyte damage that might decrease the uptake and conjugation of free bilirubin.

Unlike hepatic enzyme activities, serum bilirubin concentration is a measure of liver function. In the absence of hemolysis, hyperbilirubinemia is an indication that the liver is not functioning properly. In most examples of hepatotoxicity, however, there is normal total serum bilirubin because of the tremendous functional reserve of the liver. In the dog, where some conjugated bilirubin is excreted in urine, a 70% hepatectomy does not elevate serum bilirubin concentration.

It is interesting to note that human patients receiving anticonvulsant therapy have lower serum bilirubin levels than the population as a whole. Enzyme induction enhances the metabolism and excretion of bilirubin and can potentially mask an otherwise elevated bilirubin level.

Bile Acids

These are synthesized from cholesterol by hepatocytes, conjugated to amino acids, secreted into the biliary system, and eventually excreted into the intestine. Bacteria transform some of the primary bile acids to secondary bile acids in the intestine. There is a very efficient enterohepatic circulation of bile acids, with the greatest part of the reabsorption occurring from the ileum. Portal blood returns the bile acids to the liver for uptake, reconjugation, and resecretion. Any toxicity that affects the liver can potentially alter one of the steps in the metabolism of bile acids and cause increased levels in serum. Although not commonly used in toxicology studies, total serum bile acid concentration is sensitive and specific for hepatobiliary toxicity. By itself, however, total serum bile acid concentration does not provide enough information to discriminate between different types of hepatic lesions. Like serum bilirubin concentration, serum bile acids are a measure of a hepatic function.

The synthetic role of the liver has been previously mentioned concerning glucose, cholesterol, urea nitrogen, and a variety of proteins. Severe hepatocellular dysfunction might cause decreased serum urea nitrogen concentration, hypoglycemia, hypocholesterolemia, hypoproteinemia (especially hypoalbuminemia), and prolonged coagulation times. On the other hand, liver disease can result in hypercholesterolemia and hyperglobulinemia. The pattern of changes elicited by liver

toxicity is multifaceted. Examination of the entire biochemical profile, along with hematological and urinalysis findings, is necessary to properly evaluate liver toxicity.

Calcium and Inorganic Phosphorus

Calcium

Serum calcium concentration is affected by parathyroid hormone, calcitonin, and vitamin D and represents a balance between intestinal absorption, bone formation and reabsorption, and urinary excretion. Serum inorganic phosphorus concentration is affected by the same hormones, but is more sensitive to dietary intake and urinary excretion. To interpret the changes in either of these parameters, it is helpful to know the results of the other.

Approximately 50% of serum calcium is ionized. As such, it is biologically active and participates in neuromuscular activity, bone formation, coagulation, and other biochemical mechanisms. Approximately 40% of serum calcium is bound to albumin in an inactive, un-ionized state. The remaining serum calcium is complexed to anions such as phosphate and citrate.

Hypercalcemia. This is relatively uncommon in toxicology studies unless the test material, like some plant toxins, has properties of vitamin D. Miscellaneous causes of hypercalcemia include hypervitaminosis D, primary hyperparathyroidism, pseudohyperparathyroidism (i.e., secondary to some types of neoplasia), and occasionally renal disease.

Hypocalcemia. Mildly decreased serum calcium concentration, secondary to hypoalbuminemia, is a frequent finding in toxicology studies. Signs of hypocalcemia do not occur because ionized calcium is relatively unaffected. Other miscellaneous and much less frequent causes of hypocalcemia include hypoparathyroidism, nutritional hyperparathyroidism, acute pancreatitis, puerperal tetany in the bitch, and renal disease.

Hyperphosphatemia and Hypophosphatemia

Increased serum inorganic phosphorus concentration is a normal finding in young animals. It might be as high or higher than serum calcium concentration. Serum inorganic phosphorus concentration is very sensitive to GFR and can be increased with prerenal, renal, or postrenal azotemia. Other less frequent causes of hyperphosphatemia include hypervitaminosis D, hypoparathyroidism, and nutritional hyperparathyroidsm due to excess dietary phosphorus. Greatly decreased food consumption ca cause hypophosphatemia, and this is sometimes observed in dietary toxicity studies if the animals refuse to eat.

Sodium, Potassium, and Chloride

Sodium is the major cation in serum and is the principal determinant of extracellular fluid volume (i.e., hydration status). Potassium is the major intracellular cation. Serum potassium concentration is maintained within narrow limits because of its critical role in neuromuscular and cardiac excitability. Chloride is the major anion in serum and serves to support fluid homeostasis and balance cation secretion.

Although reference ranges for these parameters might appear fairly wide, the range of results in a well-controlled study is often quite narrow. Occasionally, very small statistically significant differences occur between control and treated groups in toxicology studies. Unfortunately, the mechanism for the difference is usually not apparent.

In most instances, serum sodium and chloride and concentrations tend to parallel each other. Hypernatremia is relatively rare, but hyperchloremia can sometimes occur secondary to secretory diarrhea with metabolic acidosis. In this condition, renal tubular reabsorption of chloride is increased

because of decreased availability of bicarbonate. Decreased serum sodium and chloride concentrations can occur with GI losses (e.g., vomiting or diarrhea), polyuric renal losses (e.g., chronic renal failure or diabetes mellitus), diuretics, and hypoadrenocorticism (a rare finding in toxicology studies). Vomiting can cause hypochloremia and normonatremia because chloride, as hydrochloric acid, can be lost in excess of sodium.

Serum potassium concentration is a relatively poor indicator of total body potassium because of shifts between intracellular and extracellular compartments. Increased serum potassium concentration occurs with acidosis due to the exchange of extracellular hydrogen ions for intracellular potassium ions. Severe tissue necrosis and anuric or oliguric renal disease are infrequent causes of hyperkalemia. Decreased serum potassium concentration, like the changes in sodium and chloride, can be associated with GI losses and polyuric renal losses. Potassium is also sensitive to decreased dietary intake and serum concentrations might be decreased along with inorganic phosphorus in animals that refuse to eat. Finally, an infrequent cause of hypokalemia is alkalosis.

Urinalysis

Urinalysis is part of the minimum laboratory database for clinically ill patients. It provides a specific evaluation of the urogenital tract as well as information concerning more generalized conditions. As a general observation, however, urinalysis is utilized improperly in many toxicology studies. For the most part, this is due to the technical difficulties associated with collecting a large number of urine specimens from small laboratory animals. The method by which urine is collected greatly influences the value and interpretation of the data obtained. If a toxin is known or suspected to affect the urinary system, measures can be taken to provide appropriate specimens for urinalysis (i.e., by catheterization, cystocentesis, or carefully collected voided samples). Usually, however, when a large number of animals is being tested with a test material of unknown toxic potential, and regulatory guidelines require urinalysis, the most efficient method of urine collection (i.e., in a collection vessel at the bottom of a metabolic cage) produces a multitude of artifacts that diminish the value of the test results and might make interpretation impossible. Voided urine transverses the urethra, vagina or prepuce, and perineum or preputial hairs, where it can acquire both cells and bacteria. Added to the environmental contaminants in the bottom of a cage (e.g., cleaning chemicals, feces, bacteria, food, and hair) and given time (16–24 hrs) to incubate, it is little wonder that urinalysis data from timed collections in a metabolic cage are less than desirable. Furthermore, it is nearly impossible to keep the collection vessel chilled during a prolonged collection and preservatives such as toluene have disadvantages that preclude their use. The following discussion of the various parts of the urinalysis addresses the major issues of interpretation with respect to potential false-positive and false-negative results.

The urinalysis consists of two parts: physiochemical properties and sediment evaluation. The physiochemical properties include color, clarity, volume (for timed collections), specific gravity, and the reagent strip tests (pH, protein, glucose, ketones, bilirubin, urobilinogen, and occult blood). Some reagent strips have additional tests for nitrite (indicates presence of nitrite-producing bacteria) and leukocyte esterase, but these are not particularly valuable for animal specimens, especially when the urine sediment is examined microscopically. Urinary enzyme activities, for the identification of renal tubule toxicity, are sometimes determined in investigational studies on test materials that are known or potential renal toxins.

Urinary sediment evaluation is a semiquantitative microscopic measure of the presence of cells, casts, bacteria, and crystals. To properly evaluate changes in the physiochemical properties of urine, it is important to know the urine sediment results. The reverse is also true.

Urine Volume and Specific Gravity

Timed urine volume and urine specific gravity are the only measure of renal function in the urinalysis. They demonstrate the ability of the kidneys to perform work. The loss of urine concentrating

ability generally precedes the development of azotemia as a consequence of primary renal disease. Urine specific gravity, as determined by refractometry, is an approximation of the solute concentration, but it is dependent on molecular size and weight as well as the total number of solute molecules. Urine specific gravity usually varies inversely with urine volume. Animals that have lost the ability to concentrate their urine have decreased urine specific gravity and increased urine volume. However, because urine specific gravity is a function of fluid intake, solute intake, glomerular filtration, renal tubular cell health, and other factors, the range of values that can be considered physiologically normal (e.g., 1.001–1.075) is quite large.

Hyposthenuria (i.e., urine specific gravity = 1.001–1.007) is sometimes observed in toxicology studies. Perhaps the most common cause is a leaky water bottle or automatic water system. More important, however, some nonhuman primates develop psychogenic polydipsia that results in excretion of very dilute urine. Because of high urine flow through the kidney, these animals might develop medullary washout and lose the ability to concentrate urine even if water is withheld. If water is unavailable, they can rapidly become dangerously dehydrated. For this and other reasons, it is probably inappropriate to withhold water from nonhuman primates. Unfortunately, some monkeys play with their water source habitually and make urinalysis results from cage-bottom specimens meaningless. There are many diseases that cause hyposthenuria, but these rarely occur in toxicology studies.

Isosthenuria (i.e., urine specific gravity = 1.008–1.012), also referred to as "fixed" specific gravity, occurs with advanced renal disease. Isosthenuria and hyposthenia are particularly meaningful when serum urea nitrogen concentration is elevated or the animal is dehydrated. If the urine sample is free of water contamination, this combination of findings indicates primary renal disease. Isosthenuria and hyposthenuria can also occur if the test material has diuretic activity.

When an animal is dehydrated and its kidneys are functioning properly, urine should be more concentrated than plasma. In toxicology studies, urine specific gravity is sometimes higher in treated groups than in control groups because the treated groups are not eating and drinking normally. Occasionally, test materials cause excessive GI fluid losses that result in relative dehydration.

If water contamination of the urine sample is avoided, timed urine volume (e.g., 16 or 24 hr) and urine specific gravity are probably the most valuable urinalysis tests routinely performed. The other tests have too many complicating factors given the typical procedure for urine collection.

Reagent Strip Tests

Urine pH varies with diet; high-protein meat diets usually produce acid urine and cereal or vegetable diets usually produce alkaline urine. Although test materials might alter urine pH, it is not a good indicator of acid–base balance. As urease-producing bacteria multiply in a standing urine sample, ammonia is produced and urine pH becomes more alkaline. Loss of carbon dioxide from specimens in open containers also causes urine pH to rise.

A small amount of urine protein, as measured with reagent strips, is a normal finding in most animals, especially if the urine is concentrated. A large number of proteins, especially in dilute urine, is abnormal. Increased protein excretion might be due to glomerular injury, defective tubular reabsorption, hemorrhage, inflammation, or the presence of proteinaceous material from the lower urogenital tract in voided specimens. The sediment findings might help to interpret the cause of proteinuria. The reagent strip reactions measure albumin better than globulin so a false negative is possible in rare instances. The most common spurious findings are false positives owing to the effect of highly alkaline urine on the reagent strip reaction or contamination of the urine with quarternary ammonium compounds commonly used as disinfectants. Alternative methods of urine protein determination are sometimes used if these problems are suspected or if the urine protein result is expected to be an important piece of information.

Urine glucose is normally negative. Glucosuria is most commonly observed as a result of hyperglycemia (e.g., greater than 180 mg/dl in the dog) and the failure of the renal tubules to

reabsorb the increased glucose load entering the glomerular filtrate. Diabetes mellitus is the most frequent disease associated with glucosuria. In toxicology studies, glucosuria is very rare, but it can occur with test materials that affect proximal tubular cells, decreasing their ability to reabsorb filtered glucose. False-negative findings for urine glucose can occur as a result of bacterial proliferation and consumption of glucose.

Ketonuria is occasionally observed in debilitated, anorectic animals and animals that have been fasted for a prolonged period of time. Ketonuria indicates that energy metabolism has shifted to incomplete oxidation of fatty acids. As might be expected, diabetic animals often have ketonuria. False-negative findings for urine ketones can occur as a result of bacterial degradation and the loss of volatile ketones from open containers.

Bilirubinuria, especially in concentrated urine, is a normal finding in the dog, but an abnormal finding in other species. Increased urine bilirubin occurs as a result of the same conditions that cause hyperbilirubinemia, but it often precedes the change in blood. False-negative findings for urine bilirubin can occur from prolonged exposure of the urine specimen to light, which oxidizes the bilirubin to biliverdin.

Theoretically, urine urobilingen tests the patency of the bile duct. Once conjugated bilirubin reaches the intestine, bacterial action converts some of it to urobilinogen, a portion of which is reabsorbed by the intestine. Most of the reabsorbed urobilinogen is removed by the liver, but a small amount is normally excreted in the urine. Therefore, a negative urine urobilinogen is supposed to indicate an obstructed bile duct. There are a multitude of problems with this test, and its value is questionable in any circumstance. It generally is determined simply because it exists on the same reagent strip as the other tests.

Positive findings for urine occult blood frequently occur in normal animals. The origin of the blood is generally not known, but estrus in females is a common source. The reagent strips do not discriminate among erythrocytes, hemoglobin, and myoglobin. Results of this test must be correlated with the microscopic finding for proper interpretation. Hematuria occurs secondary to inflammation, trauma, or neoplasia of the urogenital tract and bleeding disorders.

Urine Sediment Evaluation

Small numbers of erythrocytes, leukocytes, and epithelial cells are normal findings in urine sediment. Large numbers, however, are abnormal. A variety of lesions can be responsible for hematuria and pyuria, at any level of the urogenital tract. Large epithelial cells (i.e., squamous and transitional cells) generally do not indicate serious abnormalities, but the presence of many small epithelial cells (i.e., renal tubular cells) is indicative of renal tubular lesions. Unfortunately, unless associated with granular or cellular casts, it is often difficult to distinguish renal tubular cells from other cells. Furthermore, prolonged exposure of all cells to the somewhat hostile environment of urine leads to degenerative changes or cell lysis. This is one of the major problems with prolonged, timed urine collections. If sediment detail is important, other means of urine collection should be used.

Casts are cylindrical molds of protein or cells that form within the lumens of renal tubules. An occasional hyaline (i.e., proteinaceous) or granular cast can be a normal finding. An increased number of these casts or the presence of any cellular casts is an abnormal finding. Increased hyaline cast formation occurs when there is increased protein loss from the kidney. Cellular casts (erythrocyte, leukocyte, or epithelial) are rarely observed in animal urine, but if found, indicate a severe renal lesion. Cellular casts become granular casts as the cells within the cast degenerate. Granular casts, therefore, are also an indication of a renal lesion. Waxy casts are occasionally seen. They represent the final stage of degeneration of the cellular cast and indicate prolonged local or diffuse intrarenal urine stasis. Broad casts are identified by their width and represent casts formed in collecting ducts or a pathologically dilated portion of the nephron. As with waxy casts, broad casts indicate intrarenal urine stasis. Although cells can originate all along the urogenital tract, cylinduria (increased number of casts) identified renal involvement. As with cells, casts degenerate with prolonged exposure to urine.

Because of the urine collection methods normally used, bacteria are a consistent finding in laboratory animal urine. For true bacteriuria to be confirmed, other collection methods must be used.

Crystals are also common findings in the urine of laboratory animals. The type of crystal is dependent on the urine pH. Triple phosphate, amorphous phosphate, calcium carbonate, and ammonium urate crystals are frequently seen in alkaline urine, whereas urate, oxalate, and hippurate crystals are associated with acid urine. Other types of crystals can be observed. A test material will occasionally form crystals in renal tubules or the development of calculi. Ammonium biurate crystals are associated with liver failure, and hippurate crystals are associated with ethylene glycol toxicity.

SPECIES DIFFERENCES

Rat

Clinical pathology reference ranges for the rat are given in table 12.1.

Hematology

Rats have relatively small erythrocytes; MCV is generally between 50 and 60 femtoliters (fl) in adults. Central pallor is usually observed, and mild to moderate anisocytosis and polychromasia are normal findings that correspond to reticulocyte counts of 1% to 3%. Howell-Jolly bodies and nucleated RBCs are occasionally observed. Crenation, fragmentation, and poikilocytosis of erythrocytes are commonly observed but are usually artifacts of slide preparation. Erythrocyte survival time is approximately 45–68 days (Jain 2000). Hematocrits are generally between 40% and 50% and are slightly higher for males than for females.

WBC counts range from about 3,000 to 12,000/µl; counts for males tend to be slightly higher than those for females. In young rats, lymphocytes comprise as much as 90% of the total cell count. With age, however, the neutrophil:lymphocyte ratio is closer to 40:60. Rate granulocytes sometimes have lobulated, ring-shaped nuclei. Immature granulocytes, such as band and metamyelocyte neutrophils, can appear as distinctive "doughnut" ring forms with a smooth nuclear membrane. Primary granules of the rate neutrophil are small and faint.

Inflammatory lesions in the rat cause a neutrophilic leukocytosis, often accompanied by a lymphocytosis. Older rats with chronic lesions such as cage sores or tumors with ulcerated, infected surfaces can have very high WBC counts (e.g., > 50,000/µl) that must be distinguished from leukemia. A high percentage of Fischer-344 rats develop large granular lymphocyte leukemia after a year of age (Stromberg 1985). In addition to high numbers of neoplastic cells in peripheral blood, the hematologic findings are also characterized by the development of an acute, immune-mediated hemolytic anemia exhibiting spherocytosis and reticulocytosis.

The rat spleen exhibits active hematopoiesis throughout life. In the bone marrow, the M:E ratio is usually between 1:1 and 1.5:1.0. Megakaryocytes are abundant, and lymphocytes are common, comprising up to 20% of the nucleated cell population. Mast cells are more prominent in rat bone marrow than in bone marrow from other laboratory animal species.

Platelet counts in rats are very high, averaging about 1,000,000/µl. Lower counts are frequently due to sampling-induced platelet aggregation, and platelet clumps are often observed on peripheral blood films. PT and partial thromboplastin time in the rat are similar to other species.

Clinical Chemistry

In most respects, clinical chemistry findings and interpretations for the rat are not unusual. Because of the rat's prominence in biomedical research, however, there are numerous references

Table 12.1 Clinical Pathology Reference Ranges, Rat (Young Adult, Sprague-Dawley)

Test	Units	Male	Female
RBC count	$\times 10^6/\mu$	6.7–9.0	5.7–9.0
Hemoglobin	g/dl	13.0–17.0	11.0–17.0
Hematocrit	%	41–58	39–55
Mean corpuscular volume	fl	55–65	55–65
Mean corpuscular hemoglobin	pg	16–22	17–22
Mean corpuscular hemoglobin concentration	%	28–34	28–34
Platelet count	$\times 10^6/\mu$	700–1,500	700–1,500
Prothrombin time	sec	12–17	12–18
Partial thromboplastin time	sec	17–27	17–27
WBC count	$\times 10^3/\mu$	3.0–14.5	2.0–11.5
Segmented neutrophils	$\times 10^3/\mu$	0.3–3.0	.01–2.0
Band neutrophils	$\times 10^3/\mu$	0.0–0.0	0.0–0.0
Lymphocytes	$\times 10^3/\mu$	3.0–12.0	1.0–10.0
Monocytes	$\times 10^3/\mu$l	0.0–0.5	0.0–0.3
Eosinophils	$\times 10^3/\mu$l	0.0–0.3	0.0–0.3
Basophils	$\times 10^3/\mu$	0.0–0.0	0.0–0.0
Nucleated RBC count	/100 WBC	0–2	0–2
Glucose	mg/dl	70–125	70–120
Total protein	g/dl	5.6–7.1	5.5–7.3
Albumin	g/dl	3.9–4.9	4.0–5.2
Globulin	g/dl	1.5–2.3	1.4–2.0
Cholesterol	mg/dl	42–90	45–100
Triglyceride	mg/dl	30–90	15–40
Urea nitrogen	mg/dl	10–16	10–19
Creatinine	mg/dl	0.5–0.8	0.5–0.8
Total bilirubin	mg/dl	0.0–0.2	0.0–0.2
Aspartate aminotransferase	IU/L	60–300	80–250
Alanine aminotransferase	IU/L	25–55	25–50
Alkaline phosphatase	IU/L	85–245	60–110
γ-Glutamyl transferase	IU/L	0–1	0–1
Creatine kinase	IU/L	244–254	241–254
Calcium	mg/dl	8.5–10.5	8.5–10.2
Inorganic phosphorus	mg/dl	6.0–9.5	6.0–9.0
Sodium	mmol/L	139–155	139–155
Potassium	mmol/L	4.4–5.7	4.0–5.5
Chloride	mmol/L	100–115	100–113

in the literature covering a wide range of subjects relating to clinical chemistry. A high percentage of these report on the sources of variation such as the time of day when bled, method of handling, blood collection site, or anesthetic used. Several other references compare the merits of different tests for identifying lesions caused by various models of toxicity. For example, which of several liver enzymes is the best for identifying a specific model of liver toxicity? Rather than list the details of these reports, some generalizations are made.

As with most species, serum glucose concentration is one of the most sensitive parameters to variations in handling and sample collection. Increased concentration due to experimental manipulations other than food consumption is often attributed to the effect of endogenous catecholamines and corticosteroids on glycogenolysis, gluconeogenisis, and insulin antagonism. Although total serum cholesterol in the rat is relatively resistant to atherogenic diets, a variety of disease conditions in older animals are associated with hypercholesterolemia. The reference range for cholesterol in aged rats is quite wide. In contrast to humans, high-density lipoprotein is the predominant lipoprotein in the rat; the same is true of many other laboratory animal species. The γ-globulin fraction of serum protein determined by electrophoresis is remarkably low in the young adult rat, but as in most species, it increases with age of the animal and with exposure to antigenic stimulation.

In young adults, serum urea nitrogen and creatinine concentrations have narrow reference ranges. With age, however, the common occurrence of chronic progressive nephropathy, especially in males, affects the ranges.

Numerous liver enzymes have been studied and variously advocated for the study of hepatic toxicity in the rat. In general, serum ALT, SDH, and GDH activities have been the best indicators of hepatocellular injury. Because ALT activity is the simplest to determine, it has generally been adopted by most laboratories as the standard liver enzyme.

There has been considerable discussion concerning the relative merits of serum AP, GGT, and 5'N activities for the evaluation of hepatobiliary or shocholestatic disease. It has been argued that serum AP activity is not specific or sensitive in the rat and that changes in serum activity are due to changes in the intestinal isoenzyme. The experience of this author is that serum AP activity will increase in the rat in association with some heptobiliary lesions and in the absence of visible histological GI involvement. However, serum AP activity does not appear to be as sensitive to cholestasis in the rat as it is in the dog. Furthermore, depending on the conditions of the experiment, serum AP activity has been shown to either increase or decrease secondary food restriction (Oishi et al. 1979; Schwartz et al. 1973). In either case, the changes are small. Serum GGT activity is essentially nonexistent in normal rats but a few studies have shown increased activity is essentially nonexistent in normal rats, but a few studies have shown increased activity secondary to hepatobiliary toxins (Ghys et al. 1975; Leonard et al. 1984). Serum 5'N has been used infrequently in the rat but has been shown, in certain disease models, to parallel increases in serum AP and GGT activity (Kryszewski et al. 1973).

Electrolyte findings are not notably different in the rat. Like the erythrocytes of mice and humans, however, rat erythrocytes have high potassium concentration and hemolysis will yield spuriously high serum potassium values (Meeks 1989).

The rat is commonly used as a test animal for organophosphate and carbamate products. Plasma cholinesterase activity in females is approximately three to four times higher than in males. Although this difference is absent in very young animals, it becomes noticeable around 6 to 8 weeks of age. RBC cholinesterase activity is similar in males and females.

The primary adrenocorticosteroid in the rat is corticosterone.

Urinalysis

Proteinuria is a common finding in rats, especially males, increasing with age and the development of chronic progressive nephropathy. Males begin to excrete low molecular weight sex-dependent proteins around 8 weeks of age (Alt et al. 1980). Larger proteins, including albumin, are excreted with the development of the nephropathy.

Mouse

Clinical pathology reference ranges for the mouse are given in table 12.2.

Hematology

Mice have the smallest erythrocytes of the common laboratory animal species; MCV is generally between 45 and 55 fl. Central pallor is usually observed, but because of the cell's small size, it is inconsistent. Moderate anisocytosis and polychromasia are normal findings and correspond to reticulocyte counts of 2% to 5%. Mice have the highest reticulocyte counts among adult laboratory animals. Howell-Jolly bodies are a frequent normal finding, and nucleated RBCs are occasionally observed. Fragmentation and crenation of erythrocytes are commonly observed, but these are usually artifacts of slide preparation. Erythrocyte survival time is the shortest among common laboratory animals, approximately 40 to 50 days (Bannerman 1983). Hematocrit is relatively high, especially

Table 12.2 Clinical Pathology Reference Ranges, Mouse (Young Adult, CD-1)

Test	Units	Male	Female
RBC count	$\times 10^6/\mu$	9.0–11.3	9.0–11.5
Hemoglobin	g/dl	13.5–17.0	14.5–17.5
Hematocrit	%	45–55	45–57
Mean corpuscular volume	fl	47–55	45–55
Mean corpuscular hemoglobin	pg	13–16	13–16
Mean corpuscular hemoglobin concentration	%	29–34	29–34
Platelet count	$\times 10^3/\mu$	900–1,900	900–1,800
Prothrombin time	sec	7–20	7–20
Partial thromboplastin time	sec	7–18	7–18
WBC count	$\times 10^3/\mu$	2.0–10.0	1.0–12.0
Segmented neutrophils	$\times 10^3/\mu$	0.3–2.0	0.3–2.5
Band neutrophils	$\times 10^3/\mu$	0.0–0.0	0.0–0.0
Lymphocytes	$\times 10^3/\mu$	1.0–7.0	1.0–9.0
Monocytes	$\times 10^3/\mu$l	0.0–0.3	0.0–0.3
Eosinophils	$\times 10^3/\mu$l	0.0–0.5	0.0–0.5
Basophils	$\times 10^3/\mu$	0.0–0.0	0.0–0.0
Nucleated RBC count	/100 WBC	0–2	0–2
Glucose	mg/dl	80–160	80–150
Total protein	g/dl	5.0–7.4	4.8–7.0
Albumin	g/dl	3.5–5.0	3.4–5.0
Globulin	g/dl	1.5–3.5	1.0–2.5
Cholesterol	mg/dl	90–170	50–130
Triglyceride	mg/dl	60–160	40–130
Urea nitrogen	mg/dl	15–45	15–40
Creatinine	mg/dl	0.3–0.8	0.2–0.6
Total bilirubin	mg/dl	0.1–1.0	0.1–0.9
Aspartate aminotransferase	IU/L	70–400	70–400
Alanine aminotransferase	IU/L	25–200	25–100
Alkaline phosphatase	IU/L	30–80	40–100
γglutamyl transferase	IU/L	0–3	0–3
Creatine kinase	IU/L	142–168	138–163
Calcium	mg/dl	8.5–11.5	8.5–11.5
Inorganic phosphorus	mg/dl	7.0–11.5	7.0–11.5
Sodium	mmol/L	145–167	145–165
Potassium	mmol/L	5.0–8.5	5.0–8.5
Chloride	mmol/L	110–125	110–125

if mice are fasted before sample collection. Although generally between 40% and 50%, hematocrits above 50% are frequently observed. Considering the small erythrocyte size, it is not surprising that mouse RBC counts are the highest encountered, ranging from 8 million to 11 million/ml.

Mice generally have WBC counts ranging from 2,000 to 10,000/µl; counts for males tend to be slightly higher than for females. Lymphocytes normally comprise about 70% to 80% of the total cell count, but the percentage of neutrophils increases with age. Like the rat, mouse granulocytes often have lobulated, ring-shaped nuclei. When the ring is broken, the nuclei appear similar to those of most mammals. As in the rat, immature granulocytes appear as distinctive "doughnut" ring forms with a smooth nuclear membrane. In the mouse, neutrophil primary granules are small and very faint.

As with most species, inflammatory lesions in the mouse result in an increased absolute neutrophil count. Similar to the rat, however, this is often accompanied by an increase in absolute lymphocyte count. Older mice with chronic lesions might get remarkably high WBC counts, easily greater than 50,000/µl. Similar to rabbits, and in contrast to dogs, corticosteroids cause decreased absolute monocyte count in the mouse (Jain 2000).

In addition to bone marrow, the mouse spleen exhibits active hematopoiesis throughout life. Erythropoiesis appears greater than granulopoiesis in the spleen, and the reverse is true in marrow, where the average myeloid-to-erythroid ratio is usually around 1.5–1.0. Megakaryocytes are abundant

in both locations. Lymphocytes are commonly found in bone marrow preparations from mice and can comprise up to 25% of the nucleated cell population.

Mice have the highest platelet counts encountered among laboratory animals. Counts normally range between 1 million and 2 million/ml. Because sample collection is often problematic, it is not unusual to have lower counts due to platelet aggregation and to observe platelet clumps on peripheral blood films. PT in the mouse appears to be similar to other species, but reported partial thromboplastin times (55–110 sec) are longer than for most other laboratory animals (Allen et al. 1962). This could easily be due to methodology differences.

Clinical Chemistry

The limited volume of available serum (or plasma) has a great impact on clinical chemistry procedures in mice. Even with the many advances in biomedical technology, it remains very difficult to complete a moderately full biochemical profile. It is nearly impossible if hematology tests are also desired. Evaporation during handling and testing can have a major effect on areas of concern. The number of tests measuring similar effects, such as urea nitrogen and creatinine for kidney dysfunction or multiple liver enzymes for hepatocellular injury, can be reduced to a single choice. Tests that exhibit large variation among individuals, such as electrolytes, could be of little value. If both hematology and a full biochemical profile are deemed necessary, the number of animals tested should be increased so that individuals can be designated for one or the other test procedure. Pooling of samples for clinical chemistry analysis is not appropriate.

In general, clinical chemistry reference ranges for mice are quite wide. This is probably indicative of some of the difficulties associated with sample collection and analysis. Values for serum glucose and triglyceride concentrations are moderately higher than those normally observed with other species. Serum urea nitrogen values also tend to be higher, and concentrations as high as 40 mg/dl are common in normal mice.

Serum ALT and SDH activities are both useful for the detection of hepatocellular injury. Because serum AST and LDH activities are more easily influenced by muscle injury, such as occurs with handling or blood collection, these enzymes are less desirable if sample volume limitations are a concern. It is important to note, however, that serum ALT activity is also affected by handling. When compared with control mice that were either not handled or tail-handled 1 hr prior to blood collection, mice that were grasped by the body had serum ALT activity nearly four times greater (Swaim et al. 1985). An interesting enzyme activity phenomenon is associated with lactic dehyrogenase virus in mice. This viral infection induced decreased clearance serum enzymes resulting in elevations of LDH and AST activity, among others (Riley et al. 1978). In contrast to most laboratory animal species, salivary amylase is responsible for most of the serum amylase activity in the mouse and can increase with salivary gland injury.

The most notable electrolyte findings in the mouse are the reported reference ranges that are high and wide (e.g., 112–193 mEq/1 for sodium; Everett and Harrison 1983). It is likely that evaporation of the small samples has had some impact on the high end of these ranges. With respect to potassium, mouse erythrocytes have a high intracellular concentration (similar to humans but in contrast to dogs), and hemolysis will cause spuriously high serum potassium concentration.

The primary adrenocorticosteroid in the mouse is corticosterone.

Hamster

Clinical pathology reference ranges for the hamster are given in table 12.3.

Hematology

Many hematological characteristics of hamsters are similar to other rodents, but there are a few differences. Their erythrocytes are morphologically similar to those of rats. The cells are approximately

Table 12.3 Clinical Pathology Reference Ranges, Syrian Hamster (Adult)

Test	Units	Male	Female
RBC count	$\times 10^6/\mu$	6.0–8.5	6.0–8.0
Hemoglobin	g/dl	12.5–16.5	12.0–16.0
Hematocrit	%	35–50	35–45
Mean corpuscular volume	fl	52–65	52–65
Mean corpuscular hemoglobin	pg	19–23	19–23
Mean corpuscular hemoglobin concentration	%	32–36	32–36
Platelet count	$\times 10^3/\mu$	300–900	300–900
Prothrombin time	sec	8–15	8–15
Partial thromboplastin time	g/dl	1.8–3.2	1.8–3.2
WBC count	$\times 10^3/\mu$	4.0–10.0	4.0–10.0
Segmented neutrophils	$\times 10^3/\mu$	0.5–3.5	0.5–3.5
Band neutrophils	$\times 10^3/\mu$	0.0–0.0	0.0–0.0
Lymphocytes	$\times 10^3/\mu$	2.5–8.0	2.5–8.0
Monocytes	$\times 10^3/\mu$	0.0–0.5	0.0–0.5
Eosinophils	$\times 10^3/\mu$	0.0–0.3	0.0–0.3
Basophils	$\times 10^3/\mu$	0.0–0.0	0.0–0.0
Nucleated RBC count	/100 WBC	0–2	0–2
Glucose	mg/dl	50–100	50–100
Total protein	g/dl	5.3–7.0	5.3–7.0
Albumin	g/dl	3.5–4.5	3.5–4.5
Globulin	g/dl	1.8–3.2	1.8–3.2
Cholesterol	mg/dl	50–200	50–200
Triglyceride	mg/dl	30–120	30–120
Urea nitrogen	mg/dl	12–25	12–25
Creatinine	mg/dl	0.3–0.7	0.3–0.7
Total bilirubin	mg/dl	0.1–0.5	0.1–0.5
Aspartate aminotransferase	IU/L	20–100	20–100
Alanine aminotransferase	IU/L	20–50	20–50
Alkaline phosphatase	IU/L	50–200	50–200
γglutamyl transferase	IU/L	0–8	0–8
Creatine kinase	IU/L	200–700	100–600
Calcium	mg/dl	10.0–13.5	10.0–13.5
Inorganic phosphorus	mg/dl	4.5–9.0	4.5–9.0
Sodium	mmol/L	145–155	145–155
Potassium	mmol/L	4.0–7.0	4.0–7.0
Chloride	mmol/L	98–110	98–110

50 to 60 fl in volume, and most exhibit some central pallor. Mild to moderate anisocytosis and polychromasia corresponds to a reticulocyte count of about 1% to 3% in the adult. Howell-Jolly bodies and nucleated RBCs are occasionally observed on blood films from normal adults. Erythrocyte survival time is approximately 60 to 70 days, but it increases greatly during hibernation and can reach as high as 160 days (Brock 1960). Hematocrit is generally in the range of 35% to 45%, and it also increases during hibernation. At the end of hibernation, reticulocyte production increases. Unlike mice and rats, the spleen does not contribute to normal erythropoiesis.

The normal WBC count is approximately 5,000 to 10,000/μl, and lymphocytes are the predominant nucleated cell, making up about 75% of the total. In contrast to RBC count, WBC count decreases during hibernation. Hamster neutrophils are similar to those of mice and rats and frequently appear in the peripheral blood as lobulated ring-forms with fine, faintly eosinophilic primary granules. Band neutrophils and metamyelocytes have a distinctive ring or "doughnut" appearance with a smooth nuclear membrane. They are readily identified in bone marrow smear preparations. Although the normal myeloid-to-erythroid ratio has been reported as high as 10:1 in hamsters (Desai 1968), it is probably closer to 2:1. Lymphocytes, a prominent finding in the bone marrow of other rodents and guinea pigs, do not represent more than a few percent of the marrow cell population.

Although the clotting time for hamster whole blood is fairly rapid, PT and partial thromboplastin time are similar to those of most laboratory species (Desai 1968; Dodds et al. 1977). Platelet counts in hamsters are not quite as high as in mice and rats, and the platelets are more difficult to see on peripheral blood films.

Clinical Chemistry

Serum glucose concentration in the hamster is similar to other species but is said to increase during hibernation (Lyman and Leduc 1953) as do total serum protein and albumin concentration (South and Jeffay 1958). Chinese hamster lines have been developed as models of insulin-dependent diabetes mellitus and, as expected, exhibit hyperglycemia, hypercholesterolemia, and hypoinsulinemia (Gerritsen 1982). Of the small laboratory animals, hamsters appear to have the highest total serum cholesterol concentration (Carroll and Feldman 1989). Amyloidosis is a common disease affecting older hamsters and similar to other species, hamsters with renal ayloidosis develop azotemia, hypoproteinemia, hypoalbuminemia, and hypercholesterolemia (Murphy et al. 1984).

Serum AP and ALT activities appear to be valuable as markers for liver injury, and serum LDH and creatine kinase activities increase in hereditary myopathy in Syrian hamsters (Homburger et al. 1966).

The primary adrenocorticosteroid in hamsters is corticosterone, but cortisol is also present and will increase under the influence of ACTH (Albers et al. 1985; Ottenweller et al. 1985).

Guinea Pig

Clinical pathology reference ranges for the guinea pig are given in table 12.4.

Hematology

Guinea pig erythrocytes are the largest among common laboratory animals. Their MCV is generally between 75 and 90 fl, similar in size to human erythrocytes. The cells have central pallor and exhibit mild anisocytosis and polychromasia that corresponds to a reticulocyte count of 1% to 2.5%. Howell-Jolly bodies and nucleated RBCs are rarely observed. The erythrocytes have a survival time of approximately 80 to 90 days. Hematocrit is generally in the range of 40% to 50%. An interesting phenomenon, unique to the guinea pig, is the development of an acute hemolytic anemia in response to high dietary cholesterol (Yamanaka et al. 1967). The belief is that alterations in membrane lipid content might render the erythrocytes more susceptible to lysis.

The most unique feature of guinea pig leukocytes is the Kurloff body, a cytoplasmic inclusion observed in a small number of mononuclear cells believed to be T-lymphocytes because of their ability to form rosettes of rabbit erythrocytes (Jain 2000). Kurloff bodies are glycoprotein complexes that generally appear as a single, large, round-to-oval, purplish red, homogenous inclusion. On occasion, a cell will have multiple smaller inclusions. Although the function of the Kurloff body is not known, they are observed more frequently in females than males, especially during the first 3 months of life, and appear to increase during pregnancy or with exogenous estrogen administration (Ledingham 1940). In general, Kurloff bodies occur in less than 5% of circulating leukocytes.

Guinea pig neutrophils appear similar to those of humans in that the nuclei are distinctly segmented and the cytoplasm contains conspicuous reddish primary granules. The granules are not as large or prominent as those of the rabbit heterophil, but some authors refer to the guinea pig neutrophil as a heterophil. The cells are easily distinguished from eosinophils. In contrast to other species, guinea pig lymphocytes exhibit alkaline phosphatase activity (Kaplow 1969). This has significance because alkaline phosphatase activity is sometimes used to cytochemically differentiate neutrophils that exhibit activity in most species, from monocytes and lymphocytes, which normally do not.

Table 12.4 Clinical Pathology Reference Ranges, Guinea Pig (Adult)

Test	Units	Male	Female
RBC count	$\times 10^6/\mu$	4.8–6.8	4.5–6.5
Hemoglobin	g/dl	12.0–16.0	12.0–15.5
Hematocrit	%	38–52	36–50
Mean corpuscular volume	fl	70–88	75–90
Mean corpuscular hemoglobin	pg		
Mean corpuscular hemoglobin concentration	%	28–33	28–33
Platelet count	$\times 10^3/\mu$	300–800	300–900
Prothrombin time	sec	20–32	20–32
Partial thromboplastin time	sec	20–35	20–35
WBC count	$\times 10^3/\mu$	2.0–12.0	2.0–12.0
Segmented neutrophils	$\times 10^3/\mu$	0.5–3.5	0.5–4.5
Band neutrophils	$\times 10^3/\mu$	0.0–0.0	0.0–0.0
Lymphocytes	$\times 10^3/\mu$	1.5–10.0	1.5–9.0
Monocytes	$\times 10^3/\mu$l	0.0–0.5	0.0–0.5
Eosinophils	$\times 10^3/\mu$l	0.0–0.5	0.0–0.5
Basophils	$\times 10^3/\mu$	0.0–0.0	0.0–0.0
Nucleated RBC count	/100 WBC	0–1	0–1
Glucose	mg/dl	75–110	75–110
Total protein	g/dl	5.0–6.3	4.5–6.0
Albumin	g/dl	2.5–4.0	2.2–3.5
Globulin	g/dl	2.0–3.2	1.8–3.0
Cholesterol	mg/dl	25–80	30–80
Triglyceride	mg/dl	10–70	10–70
Urea nitrogen	mg/dl	15–30	15–25
Creatinine	mg/dl	0.5–0.8	0.5–0.8
Total bilirubin	mg/dl	0.0–0.3	0.0–0.3
Aspartate aminotransferase	IU/L	30–80	30–75
Alanine aminotransferase	IU/L	30–70	25–65
Alkaline phosphatase	IU/L	60–100	50–100
γglutamyl transferase	IU/L	5–15	5–15
Creatine kinase	IU/L	200–500	15–500
Calcium	mg/dl	9.5–11.5	9.5–11.5
Inorganic phosphorus	mg/dl	4.0–8.5	4.0–8.5
Sodium	mmol/L	130–142	130–142
Potassium	mmol/L	4.0–6.0	4.0–6.0
Chloride	mmol/L	103–112	103–112

The normal WBC count in the guinea pig ranges from approximately 5,000 to 10,000/μl and increases slightly with age. Regardless of age, the neutrophil:lymphocyte ratio remains approximately 1:2. Similar to mice and rats, as many as 25% of the cells in normal bone marrow of guinea pigs are lymphocytes. The typical myeloid:erythroid ratio is about 1.5:1.0.

With respect to coagulation, guinea pigs are unique among laboratory animals because of their relatively low concentration of the factor VII clotting protein. Consequently, one-stage PTs for the evaluation of the extrinsic clotting system are longer in the guinea pig; as long as 50 sec to 100 sec in one study (Kaspareit et al. 1988). Automated coagulation analyzers that automatically stop if an assay has not begun to form fibrin within 50 sec would be inappropriate for use on guinea pig samples. Thrombin time also appears to be relatively prolonged in guinea pigs. On the other hand, activated partial thromboplastin time is similar to other species.

Clinical Chemistry

There is not much information available concerning the clinical chemistry of guinea pigs. One of the most noteworthy items is that serum ALT activity is said to be insensitive and nonspecific as a marker for hepatocellular injury. Part of this stems from the finding that the activity of ALT

in other tissues such as muscle is as high as it is in the liver. In addition, liver ALT activity in the guinea pig is localized primarily within mitochondria, and theoretically would be less likely than cytoplasmic enzymes to enter circulation secondary to hepatocellular damage. Regardless of these considerations, however, liver toxicity should remain the most likely interpretation for elevated serum ALT activity in the guinea pig. The rabbit is another example of a species for which ALT activity is not specific for the liver, but when treated with CCl_4, serum ALT activity is rapidly and markedly elevated. Normal serum ALT activity in the guinea pig is similar to that of other species.

Total serum cholesterol concentration will increase when guinea pigs are fed cholesterol-rich diets, but contrary to most species, the amount of HDL cholesterol also increases. Normally, the guinea pig has a smaller amount of HDL cholesterol than other laboratory animals and humans. Its predominant lipoprotein is LDL.

The primary adrenocorticosteroid in the guinea pig is cortisol. Although there is considerable homology among species with respect to the structure of insulin, guinea pig insulin is substantially different and is associated with differences in activity and receptor concentration (Zimmerman et al. 1974).

Rabbit

Clinical pathology reference ranges for the rabbit are given in table 12.5.

Hematology

Normal rabbit erythrocytes are slightly smaller than those of the dog; MCV is generally between 55 and 70 fl. The cells have central pallor and exhibit moderate anisocytosis and mild polychromasia that corresponds to a reticulocyte count of 1% to 4%. Howell-Jolly bodies and nucleated RBCs are rarely observed on blood films from normal rabbits. The erythrocytes have a survival time of approximately 45 to 70 days. Hematocrit is generally in the range of 35% to 45%.

Rabbit leukocytes have some unique features. The rabbit neutrophil, commonly referred to as a heterophil, is morphologically distinct from neutrophils of other mammals. Although the term *heterophil* has been used to describe the neutrophil of other laboratory animal species, the rabbit's heterophil is clearly unique. The cytoplasm contains many primary granules that are large, irregularly shaped, and darkly eosinophilic. Unless the cells are viewed in the same microscopic field, it might be difficult for the inexperienced observer to distinguish rabbit heterophils from eosinophils. Rabbit eosinophils have larger primary granules that are round and stain dull orange. The granules might partially obscure the eosinophil's bilobed or segmented nucleus. Rabbits are the only laboratory animal species to normally have circulating basophils counted during a standard differential WBC count. These cells have many dark, purplish granules that obscure the nucleus. As many as 10% of the leukocytes in normal animals might be basophils.

The normal WBC count in the rabbit, approximately 5,000 to 12,000/µl, is similar to the dog. The heterophil:lymphocyte ratio is approximately 1:2 in young adult animals and 1:1 in older animals. In response to acute inflammation, the rabbit's heterophil:lymphocyte ratio will increase, but in contrast to the dog, its WBC count might not (Toth and Krueger 1989). Similar to most species, rabbits develop lymphopenia secondary to stress or exogenous corticosteroid administration. In addition, corticosteroids induce monocytopenia in the rabbit.

A rare condition of rabbit leukocytes is Pelger-Huet anomaly. Observed in several species, including humans, this hereditary trait is characterized by failure of granulocyte nuclei to develop normal segmentation. In the heterozygous state, neutrophil morphology give the appearance of a permanent left shift (i.e., the neutrophils always appear to be band forms), but the cells are not dysfunctional. Selective breeding in the rabbit can produce a homozygous state that is invariably lethal.

Table 12.5 Clinical Pathology Reference Ranges, New Zealand White Rabbit (Young Adult)[a]

Test	Units	Male	Female
RBC count	$\times 10^6/\mu$	5.0–7.2	5.0–7.2
Hemoglobin	g/dl	10.5-15.0	10.5–15.0
Hematocrit	%	32–45	32–45
Mean corpuscular volume	fl	55–65	55–70
Mean corpuscular hemoglobin	pg	19–23	19–23
Mean corpuscular hemoglobin concentration	%	30–35	30–35
Platelet count	$\times 10^3/\mu$	300–750	300–750
Prothrombin time	sec	6–9	6–9
Partial thromboplastin time	sec	14–22	14–22
WBC count	$\times 10^3/\mu$	4.0–13.0	4.0–13.0
Segmented neutrophils	$\times 10^3/\mu$	1.0–6.0	1.0–6.0
Band neutrophils	$\times 10^3/\mu$	0.0–0.0	0.0–0.0
Lymphocytes	$\times 10^3/\mu$	2.0–9.0	2.0–9.0
Monocytes	$\times 10^3/\mu$l	0.0–0.5	0.0–0.5
Eosinophils	$\times 10^3/\mu$l	0.0–0.4	0.0–0.4
Basophils	$\times 10^3/\mu$	0.0–1.0	0.0–1.0
Nucleated RBC count	/100 WBC	0–1	0–1
Glucose	mg/dl	105–190	100–190
Total protein	g/dl	5.2–7.5	5.2–7.5
Albumin	g/dl	4.0–5.5	3.5–5.5
Globulin	g/dl	1.0–2.5	1.0–2.5
Cholesterol	mg/dl	25–70	30–100
Triglyceride	mg/dl	50–180	30–180
Urea nitrogen	mg/dl	11–25	11–25
Creatinine	mg/dl	0.9–1.7	0.9–1.7
Total bilirubin	mg/dl	0.1–0.5	0.1–0.5
Aspartate aminotransferase	IU/L	15–45	15–45
Alanine aminotransferase	IU/L	15–50	15–50
Alkaline phosphatase	IU/L	40–140	40–140
γglutamyl transferase	IU/L	0–10	0–10
Creatine kinase	IU/L	100–300	100–300
Calcium	mg/dl	13.0–15.5	12.5–15.5
Inorganic phosphorus	mg/dl	3.0–9.0	2.0–9.0
Sodium	mmol/L	133–152	133–150
Potassium	mmol/L	3.5–6.0	3.5–6.0
Chloride	mmol/L	96–106	96–106

[a] Not fasted.

Because normal serum calcium concentration is much higher in the rabbit than other species, there is a potential for technical difficulties when performing coagulation tests. Calcium chelators such as citrate or oxalate are used for sample collection, and it is possible that overfilling collection tubes that have been prepared using the recommended anticoagulant to blood ratios could allow some fibrin formation to take place. If problems with tests such as PT, APTT, or thrombin time are encountered, consideration should be given to the sample collection method.

Clinical Chemistry

Fasting is recommended prior to blood collection for most species to standardize sampling conditions for the generation of, and comparison to, reference range values. For most experimental purposes, fasting rabbits for sample collection might be unnecessary because it appears to have less effect on serum concentrations of parameters such as glucose and triglycerides than in other species. This could be due in part to the practice of coprophagy. Furthermore, the notable effect of fasting on rabbit body weight data might necessitate bleeding nonfasted animals to maintain proper conditions for other study objectives.

The most conspicuous biochemical differences between rabbits and other laboratory animals involve serum albumin and calcium concentrations. Unmodified, routine chemistry methods (e.g., bromcresol green or bromcresol purple) for determination of serum albumin concentration will yield falsely elevated values. In fact, albumin concentration will often appear to exceed total serum protein concentration. Serum protein electrophoresis results indicate that albumin actually comprises about 60% to 70% of serum protein. The cause of the spuriously high values determined with routine methods is not known. Results can be improved, however, by using a rabbit albumin standard for calibration.

Rabbits have higher total serum calcium than any other laboratory animal. Whereas values as high as 16mg/dl are not uncommon in normal rabbits, they would clearly indicate severe hypercalcemia in other species. The reason for the difference is not known. Despite its relatively high serum calcium, the rabbit does not show mineralization of soft tissue lesions as a prominent histological finding. The VX_2 carcinoma of the rabbit is a model of tumor-associated hypercalcemia (Wolfe et al. 1978).

Serum cholesterol concentration varies more due to strain and gender than other parameters. As with many species, serum cholesterol concentration increases with age and is greater in females. Rabbits are more sensitive to dietary induction of hypercholesterolemia than most species and serve as a model to study atherosclerosis (Clarkson et al. 1974). Diet-induced hypercholesterolemia in rabbits is remarkable in that serum levels can reach as high as 3,000 mg/dl. The serum in these animals is extremely lipemic, and this complicates serum biochemical analysis. Ultracentrifugation might be necessary to clear the serum samples.

The primary adrenocorticosteroid in the rabbit is corticosterone. Rabbits are very sensitive to exogenous corticosteroids and develop typical signs and laboratory results for hyperadrenocorticism including hyperglycemia and increased serum aminotransferase activities. The latter is associated with diffuse vacuolization of hepatocytes.

Urinalysis

Because of its physical characteristics, rabbit urine is not routinely examined. The thick, turbid appearance is due to mucin and abundant calcium and triple phosphate crystals. The crystals can cause falsely elevated urine specific gravity if refractometric readings are made on samples that have not been centrifuged. Furthermore, the crystals make it difficult to visualize other formed elements in the urine sediment. In general, urinalysis is not recommended as part of the routine laboratory database in this species.

FerretClinical pathology reference ranges for the ferret are given in table 12.6.

Hematology

Compared with dogs, ferrets have relatively small erythrocytes; MCV is generally between 50 and 60 fl. Central pallor is usually observed. The cells exhibit only slight anisocytosis and polychromasia, even though reticulocyte counts have been reported as high as 12% to 14% in normal ferrets (Thornton et al. 1979). Others report much lower reticulocyte counts (0%–1%), which correlate better with the erythrocyte morphology (Sherrill and Gorham 1985). Howell-Jolly bodies and nucleated RBCs are occasionally observed as might be expected because the spleen retains some hematopoietic capacity. Hematocrits tend to be slightly higher than those of the dog and generally range from 45% to 55%.

The WBC counts of the ferret range from about 5,000 to 15,000/µl. The neutrophil:lymphocyte ratio ranges from about 40:60 to 60:40. Leukocyte counts, morphology, and response to inflammation are similar to those of rats.

The most unusual feature of ferret hematology is estrus-induced bone marrow hypoplasia (Bernard et al. 1983; Kociba and Caputo 1981; Sherrill and Gorham 1985). Because the ferret is

Table 12.6 Clinical Pathology Reference Ranges, Ferret (Young Adult)

Test	Units	Male	Female
RBC count	$\times 10^6/\mu$	7.0–11.0	6.5–10.0
Hemoglobin	g/dl	14.0–18.0	14.0–17.5
Hematocrit	%	45–55	40–50
Mean corpuscular volume	fl	50–60	50–60
Mean corpuscular hemoglobin	pg	18–20	18–20
Mean corpuscular hemoglobin concentration	%	32–36	32–36
Platelet count	$\times 10^3/\mu$	300–700	300–900
Prothrombin time	sec	14–17	14–17
Partial thromboplastin time	sec	15–22	15–22
WBC count	$\times 10^3/\mu$	4.0–15.0	4.0–15.0
Segmented neutrophils	$\times 10^3/\mu$	2.0–11.0	2.0–11.0
Band neutrophils	$\times 10^3/\mu$	0.0–0.0	0.0–0.0
Lymphocytes	$\times 10^3/\mu$	2.0–7.0	2.0–7.0
Monocytes	$\times 10^3/\mu$	0.0–0.8	0.0–0.8
Eosinophils	$\times 10^3/\mu$	0.0–0.8	0.0–0.8
Basophils	$\times 10^3/\mu$	0.0–0.2	0.0–0.2
Nucleated RBC count	/100 WBC	0–1	0–1
Glucose	mg/dl	90–150	90–150
Total protein	g/dl	5.3–7.5	5.3–7.5
Albumin	g/dl	2.5–4.0	2.5–4.0
Globulin	g/dl	2.0–3.0	2.0–3.0
Cholesterol	mg/dl	100–250	100–250
Triglyceride	mg/dl	10–30	10–30
Urea nitrogen	mg/dl	15–30	15–30
Creatinine	mg/dl	0.3–0.9	0.3–0.9
Total bilirubin	mg/dl	0.1–0.7	0.1–0.7
Aspartate aminotransferase	IU/L	30–120	30–120
Alanine aminotransferase	IU/L	50–200	50–200
Alkaline phosphatase	IU/L	20–100	20–100
γglutamyl transferase			
Creatine kinase	IU/L	60–100	60–100
Calcium	mg/dl	8.5–10.5	8.5–10.5
Inorganic phosphorus	mg/dl	4.0–8.5	4.0–8.5
Sodium	mmol/L	145–160	145–155
Potassium	mmol/L	4.3–6.0	4.3–6.0
Chloride	mmol/L	110–125	110–125

an induced ovulator, estrus can be prolonged if the female is not bred. Under the influence of endogenous estrogen, bone marrow hypoplasia develops in as many as 50% of the females with protracted estrus. The hematological signs are characterized by an initial increase in platelet count and WBC count at the beginning of estrus, followed later by pancytopenia (e.g., platelet count < 50,000/μl; hematocrit < 30%; WBC count < 2,500/μl). The thrombocytopenia might be responsible for hemorrhaging that further lowers the hematocrit and leads to severe, fatal anemia. Systemic bacterial infections are sometimes associated with the leukopenia. Examination of bone marrow from severely affected animals reveals depletion of all hematopoietic cell precursors.

Clinical Chemistry

In general, clinical chemistry test results from normal ferrets more closely resemble those of the dog than any other common laboratory animal. Serum glucose, protein, and lipid concentrations are similar. For example, total serum cholesterol in the ferret and dog is relatively higher than that for most laboratory animals, but comparable to humans. Serum urea nitrogen and creatinine concentrations are similar to the dog. Serum ALT activity tends to be slightly higher than in the dog, but serum AP activity is somewhat lower. As with all other species, serum AP activity decreases

with age. In contrast to mice, rats, hamsters, and monkeys, hemolysis does not spuriously increase serum potassium concentration. Like the dog, it is thought that ferret erythrocytes have a low concentration of intracellular potassium (Lee et al. 1982).

Thyroid and adrenocortical hormone levels and their response to stimulation or suppression tests have been investigated (Garibaldi, Pecquet Goad, Fox, and Murray 1988; Garibaldi, Pecquet Goad, Fox, Sylvina, et al. 1988; Heard et al. 1990). Resting values for serum thyroxine (T_4), triiogothyronine (T_3), and cortisol concentrations are similar to dogs. Stimulation with thyroid-stimulating hormone (TSH) causes increased T_4 but not T_3. Stimulation with ACTH, intravenously or intramuscularly, increases serum cortisol. IV dexamethasone suppression (0.2 mg/animal) appears to decrease cortisol by greater than 50% at 3 hr and 5 hr postdose (Heard et al. 1990), whereas intramuscular dexamethasone (0.1 mg/kg) had little effect at 6 hr postdose (Garibaldi, Pecquet Goad, Fox, Sylvina, et al. 1988). These findings seem to support the belief that the ferret is a relatively steroid-resistant species.

Urinalysis

As in the dog, bilirubinuria is a common finding in the ferret. The threshold for bilirubin excretion is apparently lower in these species than in most others.

Dog

Clinical pathology reference ranges for the dog are given in table 12.7.

Hematology

Dog erythrocytes are smaller than those of humans (MCV is generally between 60–75 fl), but the cells have the classic biconcave disc shape with an obvious area of central pallor. Because the normal reticulocyte count is less than 1%, anisocytosis and polychromasia are minimal. Howell-Jolly bodies and nucleated RBCs are rarely observed in normal adults. Sampling and slide preparation artifacts such as crenation, fragmentation, and poikilocytosis are less frequently observed in dogs than in smaller laboratory animals, especially rodents. Erythrocyte survival time is the longest of the common laboratory animals, approximately 100 to 120 days (Jain 2000). Hematocrits are generally between 40% and 50% and are slightly higher for males than for females. Compared with most species, dog erythrocytes are relatively resistant to osmotic lysis as measured by osmotic fragility tests.

WBC counts range from about 5,000 to 15,000/µl. Laboratory-reared beagles tend to have lower and less variable counts than the general dog population. In contrast to the other species, neutrophils normally outnumber lymphocytes. The neutrophil:lymphocyte ratio is typically about 60:40 to 70:30. Dog neutrophils have segmented nuclei, and their primary granules are much fainter than those in human neutrophils.

Typical inflammatory lesions in the dog generally cause a neutrophilic leukocytosis. The presence of a left shift, as in all species, depends on the severity of the lesion and the inciting agent. Lymphopenia, secondary to stress-induced endogenous corticosteroid, often occurs with serious inflammatory disease. In the dog, leukocyte counts greater than 50,000/µl occur with closed infections such as pyometra or prostatic abscess. Dogs typically respond to exogenous corticosteroid treatment with a mature neutrophilia, lymphopenia, eosinopenia, and monocytosis.

Hematopoiesis is confined to the bone marrow in normal dogs. Extramedullary hematopoiesis, usually in the spleen, can be observed in moderate to severe anemia with marked regeneration. The M:E ration in the bone marrow usually ranges from 1:1 to 2:1. In contrast to rodents, in dogs, lymphocytes generally comprise less than 5% of the nucleated cell population.

Table 12.7 Clinical Pathology Reference Ranges, Beagle Dogs (Young Adults)

Test	Units	Male	Female
RBC count	$\times 10^6/\mu$	5.5–7.7	5.5–7.6
Hemoglobin	g/dl	12.5–17.0	12.3–17.0
Hematocrit	%	38–51	36–50
Mean corpuscular volume	fl	60–71	62–72
Mean corpuscular hemoglobin	pg	20–24	20–24
Mean corpuscular hemoglobin concentration	%	32–35	31–35
Platelet count	$\times 10^3/\mu$	240–550	270–550
Prothrombin time	sec	5.8–8.2	6.0–8.0
Partial thromboplastin time	sec	9.0–13.0	9.5–13.5
WBC count	$\times 10^3/\mu$	6.4–14.6	5.6–15.2
Segmented neutrophils	$\times 10^3/\mu$	3.0–9.5	3.0–10.5
Band neutrophils	$\times 10^3/\mu$	0.0–0.0	0.0–0.0
Lymphocytes	$\times 10^3/\mu$	1.0–6.0	1.5–5.5
Monocytes	$\times 10^3/\mu$	0.0–0.9	0.0–0.8
Eosinophils	$\times 10^3/\mu$	0.0–0.8	0.0–0.7
Basophils	$\times 10^3/\mu$	0.0–0.1	0.0–0.1
Nucleated RBC count	/100 WBC	0–1	0–1
Glucose	mg/dl	82–120	85–125
Total protein	g/dl	5.0–6.5	5.0–6.3
Albumin	g/dl	3.0–3.9	3.1–4.0
Globulin	g/dl	1.7–2.8	1.7–2.8
Cholesterol	mg/dl	120–220	120–230
Triglyceride	mg/dl	20–50	20–50
Urea nitrogen	mg/dl	7–18	7–22
Creatinine	mg/dl	0.5–0.8	0.5–0.8
Total bilirubin	mg/dl	0.0–0.2	0.0–0.2
Aspartate aminotransferase	IU/L	15–45	15–45
Alanine aminotransferase	IU/L	22–48	20–50
Alkaline phosphatase	IU/L	68–190	65–200
γglutamyl transferase	IU/L	0–8	0–8
Creatine kinase	IU/L	35–580	40–540
Calcium	mg/dl	10.2–12.0	9.8–12.1
Inorganic phosphorus	mg/dl	6.6–8.4	6.0–8.2
Sodium	mmol/L	143–158	142–156
Potassium	mmol/L	4.4–6.5	4.3–6.5
Chloride	mmol/L	108–118	109–120

Platelet counts in dogs are generally in the range of 200,000 to 500,000/μl. Inherited factor VII deficiency, an autosomal recessive trait, occasionally affects beagle dogs from commercial breeding colonies. The disease is mild, causing no obvious bleeding problems with the exception of increased tendency to bruise. The dogs might also have increased susceptibility to systemic demodecosis. The disease is usually detected when prothrombin times are measured in a group of dogs and one or more have consistently longer times. The difference is usually about 2 to 3 sec.

Clinical Chemistry

The popularity of the dog as a pet is primarily responsible for the extensive database that has been accumulated concerning its clinical pathology findings in health and disease. The relative ease of sample collection eliminates many variables, such as collection site and anesthesia, that influence the interpretation of data from smaller animals. It is rather natural, therefore, to think of the dog as the norm and to compare other species with it. In this respect, clinical chemistry findings appear fairly straightforward. A few notable features of clinical chemistry in the dog are discussed here.

When compared with the "street" population of dogs, laboratory-reared beagle dogs have very narrow ranges for several parameters, especially total protein, albumin, globulin, urea nitrogen,

creatinine, ALT, and AST. Carbohydrate metabolism in dogs is similar to that in humans, and glucose tolerance tests have been well documented (Thrall 2004). The range for total serum cholesterol concentration is higher and wider than for smaller laboratory species; values over 200 mg/dl are relatively common. Like many laboratory animals, but unlike humans, HDL is the most abundant lipoprotein.

Serum ALT activity is both sensitive and specific for hepatocellular injury, but as in other species, certain compounds might stimulate enzyme production and cause small increases in the absence of pathological change. Serum AP activity in the dog is very sensitive to intrahepatic or extrahepatic cholestasis but will increase with a variety of hepatic lesions. In addition, the dog has a unique steroid-induced AP isoenzyme that can be markedly elevated after administration of exogenous corticosteroids or secondary to hyperadrenocorticism. In these conditions, the hepatic isoenzyme activity will also increase. Serum GGT and 5'N activities parallel serum AP activity during cholestasis and are more specific for biliary diseases. The degree of change for GGT, however, is not as great as for AP. Decreasing activity of the osteoblastic isoenzyme of AP is responsible for the gradual lowering of serum AP activity with age. Serum GGT and 5'N activities are unaffected by changes in bone metabolism.

Total serum bilirubin concentration is slow to increase in cholestatic disease in the dog because the renal threshold for bilirubin is low. Bilirubinuria is a common finding, even for normal dogs.

Findings for electrolytes are similar to those in humans. However, because dog erythrocytes have a low intracellular potassium concentration, hemolysis does not cause spuriously high serum potassium values.

Thyroid and adrenocortical hormone tests are well documented (Kaneko 1997). The size of the dog makes it a very suitable species for stimulation and suppression studies. Cortisol is the primary adrenocorticosteroid in the dog.

Urinalysis

As mentioned previously, bilirubinuria is a normal finding in the dog, especially in concentrated urine.

Nonhuman Primates

Clinical pathology reference ranges for nonhuman primates are given in table 12.8.

Hematology

Although the size of nonhuman primate erythrocytes is somewhat species dependent, they are generally smaller than those of humans. The MCV for cynomolgus monkeys varies according to their origin. Cynomolgus monkeys originating from China and Vietnam have larger erythrocytes (approximately 70–80 fl) than do those originating from Indonesia, Mauritius, or the Philippines (approximately 58–68 fl). The MCV for rhesus monkeys is similar to that for the cynomolgus monkeys from China and Vietnam.

Among laboratory animals, monkey erythrocytes tend to have the largest, most obvious area of central pallor. The cells often appear somewhat hypochromic, and MCHC is generally lower in monkeys than in other species. Values less than 30% for MCHC are frequently observed. Because of these findings, there is a concern that low-grade iron deficiency might be a subclinical problem affecting laboratory primates. The normal reticulocyte count is less than 1%; anisocytosis and polychromasia are minimal. Howell-Jolly bodies and nucleated RBCs are rarely observed in normal adults. Erythrocyte survival time is approximately 85 to 100 days (Jain 2000; Kreier 1970).

Hematocrits are generally between 35% and 45% and tend to be higher for males than for females.

Table 12.8 Clinical Pathology Reference Ranges, Cynomolgus Monkey (Adult, Wild Caught)

Test	Units	Male	Female
RBC count	$\times 10^6/\mu$	5.2–7.8	5.5–7.6
Hemoglobin	g/dl	10.5–14.0	10.0–13.5
Hematocrit	%	36–49	34–48
Mean corpuscular volume	fl	57–75	57–73
Mean corpuscular hemoglobin	pg	16–22	16–22
Mean corpuscular hemoglobin concentration	%	27–32	27–32
Platelet count	$\times 10^3/\mu$	180–650	175–750
Prothrombin time	sec	9.0–12.5	9.0–12.0
Partial thromboplastin time	sec	16.0–29.0	16.0–29.0
WBC count	$\times 10^3/\mu$	5.0–18.0	3.5–18.0
Segmented neutrophils	$\times 10^3/\mu$	0.5–7.5	0.5–9.0
Band neutrophils	$\times 10^3/\mu$	0.0–0.0	0.0–0.0
Lymphocytes	$\times 10^3/\mu$	2.0–12.0	1.0–10.0
Monocytes	$\times 10^3/\mu$	0.0–0.5	0.0–0.5
Eosinophils	$\times 10^3/\mu$	0.0–0.7	0.0–0.7
Basophils	$\times 10^3/\mu$	0.0–0.2	0.0–0.2
Nucleated RBC count	/100 WBC	0–1	0–1
Glucose	mg/dl	45–100	40–100
Total protein	g/dl	7.3–9.8	7.1–9.8
Albumin	g/dl	3.7–5.0	3.5–5.0
Globulin	g/dl	3.2–5.0	3.2–5.4
Cholesterol	mg/dl	110–190	100–200
Triglyceride	mg/dl	20–100	30–80
Urea nitrogen	mg/dl	15–26	12–30
Creatinine	mg/dl	0.8–1.2	0.7–1.1
Total bilirubin	mg/dl	0.1–0.8	0.1–0.8
Aspartate aminotransferase	IU/L	20–100	15–105
Alanine aminotransferase	IU/L	15–200	20–230
Alkaline phosphatase	IU/L	100–1,100	150–600
γglutamyl transferase	IU/L	40–170	30–160
Creatine kinase	IU/L	150–1,100	150–1,100
Calcium	mg/dl	8.9–11.3	8.6–11.0
Inorganic phosphorus	mg/dl	5.1–8.9	4.1–8.1
Sodium	mmol/L	148–169	148–172
Potassium	mmol/L	4.0–6.0	4.0–6.5
Chloride	mmol/L	101–116	102–117

Subclinical malarial infection, recognized by the presence of intracellular *Plasmodium* trophozoies and schizonts is relatively common in imported wild-caught or purpose-bred cynomolgus and rhesus monkeys (Donovan et al. 1983; Schofield et al. 1985; Stokes et al. 1983). Clinical disease is unusual without complicating factors such as disease-induced or drug-induced immunosuppression and splenectomy. Most frequently noted as an incidental finding on peripheral blood films of healthy animals, infections will occasionally cause an acute hemolytic anemia in animals secondary to experimental manipulations.

WBC counts range from about 5,000 to 20,000/μl. The extended reference range is mostly due to physiological leukocytosis observed in "untrained" animals bled without chemical restraint. The release of catecholamies can double the WBC count by mobilizing cells, both neutrophils and lymphocytes, from the marginal pool to the circulating pool. Although this "alarm" or "fright" reaction can probably occur in any laboratory species, it appears to have the greatest effect on nonhuman primates. In nonhuman primates, lymphocytes are increased more than neutrophils during this reaction. The upper limit of reference ranges for anesthetized or trained animals is closer to 12,000/μl. The neutrophil:lymphocyte ratio is generally around 30:70 or 40:60. Neutrophil morphology is similar to that in humans; primary granules are prominent.

As in the dog, hematopoiesis is confined to the bone marrow. The M:E ratio is usually from 1:1 to 1:5:1. Lymphocytes generally comprise less than 5% of the nucleated cell population.

Platelet counts are generally in the range of 300,000 to 600,000/μl. Coagulation times, PT and APTT, are slightly longer than for dogs but similar to man.

Clinical Chemistry

As might be expected, clinical chemistry results for wild-caught monkeys are quite variable; the reference ranges for most parameters are broad. Although fasting serum glucose concentration is lower in the monkey than in other laboratory species, the "alarm" reaction can cause glucose values high enough to be confused with diabetes mellitus. High serum glucose concentrations (e.g., > 150 mg/dl) should be checked with repeated serum glucose tests and urinalysis for glucose and ketones. Monkeys with diabetes mellitus frequently have high serum cholesterol and triglyceride concentrations. Atherogenic diets also cause increased serum cholesterol and triglyceride concentrations.

Wild-caught monkeys often have surprisingly high total serum protein and globulin concentrations; values over 9.0 and 5.0 g/dl, respectively, are not uncommon. The etiology is thought to be subclinical inflammatory or infectious disease with chronic antigenic stimulation causing a polyclonal gammopathy. Serum urea nitrogen and creatinine concentrations are higher and much more variable than in laboratory beagles.

Serum enzyme activities also tend to be highly variable. Increased serum activity of muscle enzymes (e.g., creatine kinase, AST, and LDH) might be observed secondary to iatrogenic muscle injury associated with handling or intramuscular injections of anesthetic. Alanine aminotransferase, although present in muscle tissue in the monkey, is relatively specific and sensitive for hepatocellular injury. A fairly common cause of increased serum ALT activity in cynomolgus and rhesus monkeys is subclinical, enzootic hepatitis A infection (Slighter et al. 1988). Increased activity correlates with seroconversion to the virus and periportal inflammation. Because a percentage of animals entering a facility for use in toxicology studies are not already infected, exposure and infection might take place during the actual study period, causing sporadic, high-serum ALT activities in a few individuals. These results often confound the interpretation of the enzyme data.

Serum AP activity is much higher in cynomolgus monkeys than in any other laboratory animal species, and GGT might be of more value in the diagnosis of hepatobiliary disease in the monkey than in other species.

Values for serum electrolytes are extremely variable in unanesthetized monkeys. Serum sodium and chloride concentrations can range up to 170 and 125 mmol/L, respectively. The reason for these extremely high results is not known, but in the anesthetized animal, values over 155 and 115 mml/L are unusual. In the monkey, serum potassium concentration can be spuriously elevated due to hemolysis because erythrocyte intracellular potassium concentration is high.

Cortisol is the primary adrenocorticosteroid in the monkey. Values in marmosets are higher than in macaques (Loeb and Quimby 1999). The same is true of T3 and T4 (Kaack et al. 1979).

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CHAPTER 13

Model Selection and Scaling

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CONTENTS

This entire volume is directed at the premises that (a) animals can serve as accurate predictive models of toxicity in humans (or other species), (b) the selection of an appropriate species to use is key to accurate prediction in humans, and (c) understanding the strengths and weaknesses of any particular model is essential to understanding the relevance of specific target organ toxicities to what would be expected in humans. Each of these premises requires some examination.

It is a fundamental hypothesis of toxicology that adverse effects caused by chemical entities in animals are generally the same as those induced by those entities in humans. There are many who point to individual exceptions to this and conclude that the general principle is false. Yet, as our understanding of molecular biology advances and we learn more about the similarities of structure and function of higher organisms at the molecular level, the clearer it becomes that the mechanisms of chemical toxicity are largely identical in humans and animals. This increased understanding has caused some of the same people who question the general principle of predictive value to in turn suggest that our state of knowledge is such that mathematical models or simple cell culture systems could be used just as well as intact animals to predict toxicities in man. This last viewpoint also misses the point that the final expressions of toxicity in humans or animals are frequently the summation of extensive and complex interactions on cellular and biochemical levels. Zbinden (1988) has published extensively in this area, including a very advanced defense of the value of animal models. Lijinsky (1988) has reviewed the specific issues about the predictive value and importance of animals in carcinogenicity testing and research. Although it was once widely believed (and still is believed by many animal rights activists) that *in vitro* genotoxicity tests could replace animal bioassays for carcinogenicity, this is clearly not the case on either scientific or regulatory grounds. Although there are differences in the responses of various species (including humans) to carcinogens (Gregory 1988), the overall predictive value of such results (when tempered by judgment) is clear. Additionally, however, many propose that there are few data to support that findings of toxicity in animals have been predictive of adverse effects in humans (because agents found toxic in animals are generally not knowingly given to humans). This is not the case; see Zbinden (1988) or refer to recent events surrounding azidothymidine (AZT) or retionines.

The principal problem in using animal models as predictions of toxicity in humans lies in the second major premise cited at the beginning of this chapter. As addressed in the first major section of this chapter, the theoretical basis for selection of the appropriate animal model is well established and often quoted (Plaa 1976) but rarely adhered to. As the core chapters in this book should have established, each commonly used model species has both strengths and weaknesses. Probably the greatest weakness in the current practice of toxicity testing is a lack of care in selection of the appropriate model. Our third premise is this very point: Each model species has strengths and weakness, and an understanding of these in evaluating findings in the species we actually use is essential to establishing the relevance (or lack of relevance) of findings.

This is, of course, one reason that toxicity is generally evaluated in at least two species. As Plaa (1976) and Litchfield (1962) have pointed out, adverse effects found in two separate and diverse model species have a much higher predictive power for the outcome of exposure in humans. A subset of this consideration (susceptibility factors) is addressed in the next chapter. Susceptibility factors are differences in either the animal model or human population (e.g., age, sex, disease, diet, physiological state, or strain) that can markedly influence the toxicity of a chemical entity and the course of an induced pathogenesis. Many times the predictive value of our animal studies could be improved by altering elements of protocol or experimental design to allow for the existence of susceptibility factors in a target populations. Another corollary of this third premise is that there are times when adverse effects have nothing to do with what will happen in humans. Clayson (1988) has reviewed the case for this in terms of carcinogenicity, but the argument is not limited to that.

MODEL SELECTION

The key points in selecting the most appropriate animal species as a model are to start by clearly identifying the objective of the studies to be performed and then to utilize all available information on both available model species and the mode of action and pharmacokinetics of the compound to be studied.

If the objective of a study is limited to acting for a screen or a single endpoint-type assay (Gad and Chengelis 1998), a much wider range of species will be appropriate than if greater discrimination is required.

Given all that has been presented in the core chapters on test systems, the characteristics of different species, and how one extrapolates from one species to another, the next obvious question is how test species are actually selected.

The obvious theoretical best choice would be the species considered at risk, which would leave us with no difficulty in extrapolating from one species to another.* For some applications (veterinary agents or where the concern is for the effects of potential exposures to domesticated animals or wildlife), it is possible to take this approach. However, for most cases, where the real-life concern is potential toxicity to humans, a laboratory animal species must be selected as, although chemicals are still occasionally administered to people for experimental purposes, there are legal and ethical issues that make this a rare case indeed before at least some acute toxicity data have been gathered in a species other than humans (National Academy of Sciences 1975). Even then, initial toxicity tests carried out in humans are generally at low dose levels compared to the toxic doses predicted from animal experiments. The design of acute toxicity studies in which humans are used is commonly directed at the evaluation of alterations in blood chemistry, measurable physiological variables, and the analysis of the agent and its metabolites in the blood, urine, feces, and tissues (Nosal and Hladka 1968; Rider et al. 1969). With pesticides and a few other environmental agents, useful human acute toxicity data have been obtained by the study of accidentally exposed individuals (Brown 1980).

What then would constitute the best choice of models? There is a set of characteristics that most would agree constitute the ideal animals on scientific grounds. These include (a) similarity of absorption, distribution, metabolism, and excretion to humans; (b) sensitivity of the species to the agent closely resembling that of humans; (c) evolutionary level of the animal; (d) ability of the species to express the full range of responses that humans would (e.g., emesis); (e) ability to make all pertinent measurements in a meaningful way; and (f) stages of the life span should correlate directly to those of humans.

However, there is also a set of desired characteristics for an ideal species to possess from a technical management point of view. These criteria are as follows:

1. Have a low body weight. The weight of the experimental animal is so important because during the early stages of development of new commercial chemicals only small quantities of the test material might be available.
2. Be easy to bleed and large enough to supply a reasonable amount of blood.
3. Be easy to obtain or breed and maintain in the laboratory.
4. Be easy to handle and to administer test agents to by the various desired routes.
5. Have a short life span.
6. Physiology and metabolism should approximate those of humans.
7. Should not pose a disease threat to handlers.

Each of these ideal features is secondary to the desire to have a model that responds exactly as our target species. However, there is no animal species that mimics humans in all respects, so the ultimate choice depends on the balance of conflicting factors. For example, consider the case of selecting a model to predict the effects of agents on the GI tract of humans (Fara et al. 1988). There is not a single best model, with the common species (rat, mouse, and dog) generally being more sensitive than humans to such agents as nonsteroidal anti-inflammatory drugs (NSAIDs). It is all too easy to suggest that the animal of choice should fit the criteria enumerated earlier, but in actuality these are empty words. Actual selections are made generally on practical and "political" criteria rather than these logical points.

* Although, as seen later in this chapter under "Limitations of Models," not all members of the same species (even disregarding sex and strain differences) respond the same.

After a scaled human dose (e.g., the U.S. Food and Drug Administration [FDA] human equivalent dose [HED]) has been determined from the no observable adverse effect levels (NOAELs) from all toxicology studies relevant to human potential risks, the next step is to pick a single HED for subsequent risk assessment. This HED should be chosen from the most appropriate species. In the absence of data on species relevance, a default position is to use the most appropriate species for deriving the potential human exposure.

Human potential risk in adult healthy volunteers is the most sensitive species (i.e., the species in which the lowest HED can be identified).

Factors that could influence the choice of the most appropriate species rather than the default to the most sensitive species include (a) differences in the absorption, distribution, metabolism and elimination (ADME) of the therapeutic between the species; (c) class experience that might indicate a particular model is predictive of human toxicity; or (c) limited biological cross-species pharmacologic reactivity of the therapeutic. This latter point is especially important for biological therapeutics, as many are human proteins that bind to human or nonhuman primate targets (see ICH guidance S6).

When determining the potential human risks in the absence of actual human data, absorption, distribution, and elimination parameters will not be known for humans. Comparative metabolism data, however, might be available based on *in vitro* studies. These data are particularly relevant when there are marked differences in both the *in vivo* metabolite profiles and HEDs in animals. Class experience implies that previous studies have demonstrated that a particular animal model is more appropriate for the assessment of safety for a particular structural class of therapeutics. For example, in the nonclinical safety assessment of the phosphorothioate antisense drugs, the monkey is considered the most appropriate species because monkeys experience the same dose limiting toxicity as humans (i.e., complement activation), whereas rodents do not. For this class of drugs, the potential safe exposure to man would usually be based on the HED for the NOAEL in monkeys regardless of whether it was lower than that in rodents, unless unique dose limiting toxicities were observed with the new antisense compound in the rodent species. Similarities of biochemistry and physiology between the species and humans that are relevant to the limiting toxicities of the therapeutic should also be considered under class experience. If a species is the most sensitive but has differences in physiology compared to humans that sensitize it to the therapeutic, it might not be the most appropriate species for selecting the potential safe exposure to man.

The data necessary to make decisions based on practical considerations have largely been incorporated into the tables in the core chapters of this book. Economic considerations turn out to be among the most important. These include the cost of the animal and its upkeep, availability of test animals, housing requirements, and a host of other factors that tend to push selection toward smaller, established test animal species.

The possibilities for selection are, of course, much wider. The subkingdom of vertebrate animals alone contains a great number of species, which can be classified into distinct categories, as shown in table 13.1.

Table 13.1 Approximate Distribution of the Vertebrate Animal Species

Category	No. of Distinct Species
Fish	23,000
Amphibians	2,000
Reptiles	8,500
Mammals	4,500
All vertebrates	43,000

Source: Rothschild (1961).

However, only a few of this multitude of possible species have actually been employed at any time. In addition, the eight species that are discussed in-depth in this chapter represent virtually all (99.9%) of the animals currently used in toxicology. Why is this?

How Species Are Actually Selected

There are two major sets of factors that actually drive the process of model selection in acute toxicology, with rare exceptions.

First, economic considerations such as ease of commercial production and availability, housing, life span, and so on, have, as already mentioned, favored the use of small laboratory animals. In the resulting enthusiasm for establishing rodents as satisfactory test models, toxicologists have conveniently overlooked the fact that there have been few studies correlating the toxicity of specific compounds in humans and these animal species. The available information suggests a moderate to fair direct correlation (Litchfield 1961, 1962). Difficulties in validating alternative or new test systems have tended to preclude any improvement of the model systems that are employed. There is an urgent need for a nonrodent species with a life span of up to 5 years that does not have the problems inherent in the dog or primate. Ferrets, marmosets, miniature pigs, and a number of other species have been investigated during the last 15 years, but only now have people begun to use any of these (the ferret) to any extent. There is clearly an opportunity for the commercial animal breeder here, but it might be that the essential criteria are impossible to meet and the underlying societal inertia is too great. Stevenson (1979) has discussed these aspects in the wider context of general toxicology.

The second major set of factors can only be classified generally as custom or habit. What the scientists and technicians are used to using, and what the regulators are used to interpreting data from, is generally what we tend to continue doing. The resulting inertia is the greatest impediment not only to proper model selection, but also to adaptation of new or improved study designs and to the development and use of *in vitro* models. The frequently raised issue of "validation" for any proposed change in what the science of toxicology does is all too often more accurately stated as, "Show us that it gives us the same answers—we know how to deal with those, even if they are wrong."

To fulfill these two sets of factors, animals are then actually selected for acute testing based on the following steps:

1. Which species will meet test design needs?
2. What is species availability?
3. How much test compound is available? If the amount is limited, the smallest (body weight) species that will meet other needs is most often selected. However, there are some cases in which less compound might be required to achieve desired levels in larger species than in smaller species (due to pharmacokinetics).
4. What species is the least expensive, both in terms of costs directly associated with the animals and indirect costs (e.g., the easier an animal is to handle, the lower the labor costs associated with the study). There are some special cases of species selection being based on expense. The most common of these is when the compound (usually a drug) being studied is very expensive or in extremely short supply. As a result, using the smallest possible animals reduces compound use. In this sense, small primates are often "less" expensive than dogs.
5. Will the species selected meet regulatory guidelines (usually easy, as these either dictate a species for a particular test or simply specify rodent or nonrodent) and have regulatory "acceptance" (not so easy)?
6. What have we used in the past? This question actually usually comes first, generating a list of candidate species.

Special Cases in Species Selection

There are a number of routes of exposure for which particular species is favored by habit (frequently based on a form of folklore).

In inhalation, there are several special considerations of anatomy and physiology that dictate model selection. The following three factors should be strongly considered in species selection for inhalation studies.

1. Mouth (10μ filtration) versus nose (3μ filtration) breathers. A human versus rodent comparison, with considerable resulting differences in both particular and droplet filtration and regional absorption.
2. Number of "daughter" generations of air passages. These are the number of successive times that air passages in the respiratory tree branches. There are 35 in the human.
3. Distribution of major compartments in the respiratory tract. Humans have the following distribution:
   Nasopharyngeal (NP)
      Nasal cavity
      Mouth
   Tracheobronchial (TB)
      Larynx
      Trachea
      Bronchials
   Alveolar sacs
      Pulmonary (P)

The rat by far the most common species used in inhalation, even though it is an obligatory nose breather and its respiratory morphophysiology is much different than that of humans (it has five lung lobes and a total lung surface area of 7.5 m^2, just 10% that of humans).

Folklore says that primates are the best inhalation model for humans. However, the closest similarity in respiratory structure and function is probably found in the horse or donkey. Besides the rat, commonly used species include none of these, however. Rather, the mouse and dog are the only other commonly used species in inhalation studies.

Likewise, dermal studies are by custom performed on the rabbit because its dermal absorption is "greater" than that of humans, making it the most sensitive model. As presented in a review by Gad and Chengelis (1998), this in fact is not the case.

Neither is it true that in the general case the dermal absorption and skin morphology of the pig most resemble humans (as originally reported by Bartek et al. 1972). Rather, the answers as to which species is either most sensitive or most resembles humans depend very much on the structural characteristics of compounds in question. Recent efforts and suggestions by some that all toxicity tests be performed on a common species, such as the rat, merit wider consideration. If we as scientists are not willing or able to select models on a scientific basis of what will provide us with the best prediction of what would happen in humans, then the argument of at least using a single common model that we understand the weaknesses of becomes compelling.

Caution

Having considered the general process of model selection, one should next be aware of the limitations and peculiarities of the common models, and of some of the variations that occur within a species due to differences between strains of animals.

LIMITATIONS OF MODELS

Despite our best efforts, when human exposures to a chemical entity (e.g., a drug) occur, the results do not always come near what was expected based on animal studies. For the population as a whole, there are a number of possible explanations. Some of these are presented in table 13.2.

Table 13.2 Some Reasons Data Obtained in Animal Studies Does Not Always Match Human Experiences

1. The animal species selected differ in response from humans. The same measurement or experiments in a different animal species might have been more predictive.
2. Differences in absorption, distribution, or metabolism might be present.
3. The anatomy involved in the model might differ from that in people.
4. Different animal strains of the same species might generate different results.
5. The pathological nature of any lesions produced might differ at either a macroscopic or microscopic level.
6. There could be critical differences between the species at subcellular, cellular, receptor, or physiological levels that lead to different responses. This is particularly true in terms of our current use of clinical chemistry findings to identify "target organs" in animals when these enzymes might not have the same relationship to pathogenesis in animals as in humans. Consider, for example, the Animal Clinical Chemistry Association's (1988) review of hepatic function and damage indicators in animals.
7. Experimental conditions in the animal model might yield qualitatively different data over the course of several experiments, and it might be unclear which set is.
8. The "dose" required to produce the observed results in animals is never achieved in humans.
9. The target dose in humans cannot be achieved in test animals.
10. The human population we are concerned about might differ from the population in general, and in so doing might have special characteristics that were not adequately represented in our animal model population.

An example of these types of problems in the extrapolation of toxicity data from one species to another can be found in published studies on fenclozic acid, which was a potential new anti-inflammatory drug (Alcock 1971). No adverse effects were observed to occur in the mouse, rat, dog, rhesus monkey, patas monkey, rabbit, guinea pig, ferret, cat, pig, cow, or horse, but the drug caused acute cholestatic jaundice in humans.

Beyond the difficulties in extrapolating from one or more test animal populations to the overall human population, there are a number of limitations to the standard model populations that are imposed by two forms of "good scientific practice" that are employed in conducting toxicity studies. Both of these practices have as their rationale the maximization of the sensitivity of the test system, with the underlying good intention of therefore providing the greatest possible protection to people. This is not actually the effect, however.

The first of these practices is that toxicity testing has traditionally been performed at high doses. Even in acute toxicity studies that have the objective of predicting potential target organs and mechanisms of toxicity for humans at much lower doses, the study is considered suspect if all (or for some people, any) animals survive at the highest dose level tested. This use of a maximum administrable dose and large fractions thereof is a spillover from carcinogenesis testing and times when our ability to detect effects was crude. It can frequently produce errors or difficulties in prediction of effects in people, such as those listed in table 13.3.

The second practice is that of using test animal populations that are as homogeneous as possible. This current strategy for quantifying toxicity for the most part evaluates toxicity in homogeneous populations, whether in animals or in humans, whether *in vitro* or *in vivo*. Such an approach minimizes the expression of background biological variability and, therefore, generates the most readily quantifiable and "sensitive" estimates of predicted toxicity. In actual target populations that are more heterogeneous than the model population with respect to, among other things, susceptibility and resistance to the compound in question, this might not be the case. The rationale might not be truly applicable to effects on humans, where toxicity in even a relatively small susceptible population would not be acceptable. Adjustments for this wider range of susceptibility in the population of potentially exposed humans is most commonly accounted for as being part of what is involved in

Table 13.3 Reasons High-Dose Toxicity Testing Is Usually Not Predictive of Human Effects

1. Solubility of the compound might be limiting.
2. Kinetics might be nonlinear (e.g., an enzyme might be saturated) and absorption might be decreased.
3. Michaelis-Menten kinetics might be applicable, and the blood levels might be greater than predicted in animals.
4. Metabolites formed in the animal studies might cause toxicity that would not occur with lower doses (high doses of phenacetin are one of many examples).
5. Detoxification mechanisms in the liver or elsewhere could be depleted or saturated (examples are high doses of acetaminophen in the liver or hexavalent chromium in the lungs).
6. Bioavailability of the dose form might be entirely different at lower doses due to local physiological effects (e.g., irritation) in the high-dose animal studies.
7. High dose levels in animals might overwhelm organ systems that would not be affected at lower doses, causing effects that serve to make those seen at lower blood levels.

the use of safety factors in setting allowable limits for human exposure. This might not be either accurate or adequate.

The underlying view is that a potentially toxic exposure occurs with the interaction of the chemical and a model population in a particular space and time. This experimental event of exposure must be characterized by the range of dose; type of exposure; characteristics of the exposed population (weight, sex, strain, etc.); the biological characteristics of the effect at the molecular, cellular, tissue, organ, individual, and population levels; and over a spectrum of effects from physiological through pathological and behavioral. The toxicity of the exposure must be characterized in terms of the severity of the effect; for example, clinical signs, disability, or death. Later, someone must consider the relevance and acceptability of an observed effect to human society. As a corollary of the principles of experimental design, each variable in a protocol is rigidly fixed within a narrow range. We also use a "high-class" test population: the healthiest, most nutritionally adequately fed young adult animal population possible. These laboratory animals have been carefully bred to make them as genetically defined as possible, and are maintained in clean cages under narrowly controlled environmental conditions. Thus, toxicologists traditionally utilized a very robust and (at best) narrowly representative population of animals under the best of environmental conditions.

How then do we predict for the real-life susceptibility of human populations we are most concerned about, or at least allow for them in our predictions? In many cases, perhaps one should utilize special at-risk model populations in such tests. For example, if older individuals or those with compromised cardiovascular function are known to constitute a significant part of the potentially exposed human population, study designs should incorporate groups of animals that can serve to determine if such conditions render the animals (and therefore potentially people) more susceptible to toxic effects or if it changes the nature of the expression of the toxic effect.

Susceptibility to an effect at any particular moment in a biological organism is determined by three sets of variables affecting the biological state of the organism at the time of exposure that are largely "invisible" to the outside world: genetic constitution, previous life experience, and momentary physiological state. Genetic constitution is determined by factors of strain, species, family, congenital abnormalities, and any acquired alterations. Species factors are the result of the selection of major genetic components over the course of evolution, and have already been discussed. Strain factors have largely been determined by selective breeding for concentration of genetic characteristics by the laboratory animal breeder, and considerations of strain are presented later in this chapter.

However, there are a number of factors that are part of the other two preceding variables (life experience and momentary physiological state) that are not generally characteristically represented or considered in our test animal population and yet do exist in humans and do contribute to the biological outcome of a chemical exposure. These can be considered "susceptibility factors." Chapter 14 explores the problem of susceptibility in detail.

CROSS-SPECIES EXTRAPOLATION

For all the other words in this volume about the relevance of the test systems that are described, one should never forget that none of the animal species we use is other than a model for the human being. Ultimately the continued use of animals in predictive testing must depend on how well we can use the data from these animal models to predict the outcome in people. The activity of transforming results in members of one animal species (e.g., rats) to one or more populations of another species (e.g., people) is called cross-species extrapolation, or scaling (although technically scaling is actually limited to the act of making adjustments for differences in sizes or rates).

Each step in the scaling process adds an additional degree of uncertainty to the final product. Wise and prudent scientific practice calls for at least three courses of action in seeking to give the best quality (i.e., least uncertain) final product in the form of what this means to humans. These courses of action are: (a) have as few steps in the prediction process as possible; (b) have as little uncertainty as possible associated with each step; and (c) understand the places and ways in which the selected model fails as a predictor. Each of these courses of action is not only an integral part of the extrapolation process, but also contributes heavily to proper model (test species) selection.

All the efforts so far in this book have focused primarily on the last two steps: performing various tests in a manner that gives us the least imprecise and most relevant data possible, and understanding the associated weaknesses of the model systems we employ. As such, our efforts and resulting extrapolations to this point have generally been for the animal species in which data were being developed. Ultimately it is necessary to predict what these data would mean in humans. With the wide range of effects we are concerned about here, what conversion factor (or factors) can we derive that would allow us to equate X dose or exposure in rats or Y effect in dogs with what would be seen in humans at the same or different doses? The tools at hand for the effort consist of a collection of mathematical methods (generally based on either body weight or body surface area as a means of quantitatively bridging the gap) and a set of logical and empirical rules that have been developed over the years.

The mathematical aspects are addressed primarily in this section of the chapter, whereas the "rules" appear in the sections that follow. Although there is some scientific basis for these mathematical conversions, it is not on a point-for-point basis and from one to two orders of magnitude of uncertainty are generally involved. Such extrapolations clearly have both quantitative and qualitative aspects, and the rules seek to limit the uncertainty about the qualitative aspects. Some form of pharmacokinetic and metabolic study would provide us with active agent concentrations at target organ sites as well as other information of value in safety assessments (Hawkins and Chasseaud 1985). Although this would be the best approach to such qualitative modeling, even these methods have both difficulties and limitations of their own accompanying their clear advantages (Gillette 1979), and are expensive and generally not available for support of most cases of data interpretation. An extension of this approach is that of physiological modeling (Gibson and Starr 1988), which seeks to develop a quantitative compartmental model for each identified organ or organ system.

The qualitative aspects of species-to-species extrapolations are best addressed by a form of classification analysis tailored to the exact problem at hand. This approach identifies the known physiological, metabolic, and other factors that might be involved in the risk-producing process in the model species (e.g., the skin sensitization process in the guinea pig); establishes the similarities and differences between these factors and those in humans; and comes up with means to bridge the gaps between these two (or at least identifies the fact that there is no possible bridge).

Table 13.4 presents an overview of the classes of factors that should be considered in the initial step of a cross-species extrapolation. Examples of such actual differences that can be classified as one of these factors are almost endless.

The absorption of compound from the GI tract and from the lungs is generally comparable among vertebrate and mammalian species. There are, however, differences between herbivorous

Table 13.4 Classes of Factors to Be Considered in Species-to-Species Extrapolations of Toxicity

I.	Relative sensitivity of model (compared to humans)
	A. Pharmacological
	B. Receptor
	C. Life span
	D. Size
	E. Metabolic function
	F. Physiological
	G. Anatomical
	H. Nutritional requirements
	I. Reproductive and developmental processes
	J. Diet
	K. Critical reflex and behavioral responses (e.g., emetic reflex)
	L. Behavioral
	M. Rate of cell division
	N. Other systemic defense mechanisms
	O. Can endpoint of interest be expressed in both species?
II.	Relative population differences
	A. Size
	B. Heterogeneity
	C. Selected nature of test population (model populations are "high class" compared to the human population)
III.	Differences between test and real-world environment
	A. Physical (temperature, humidity, etc.)
	B. Chemical
	C. Nutritional

animals and omnivorous animals due to the differences in stomach structure. The problem of distribution within the body probably relates less to species than to size, and is discussed later. Primarily endogenous metabolism, xenobiotic metabolism of foreign compounds, metabolic activation, or toxicantion or detoxification mechanisms (by whatever name) is perhaps the critical factor, and this can differ widely from species to species. The increasing realization that the original administered compound is not necessarily the ultimate toxicant makes the further study of these metabolic patterns critical.

In terms of excretory rates, the differences between the species are not great: Small animals tend to excrete compounds more rapidly than large animals in a rather systematic manner. Boxenbaum (1982) has generalized this to the concept of pharmacokinetic time, which is related to relative body size.

The various cellular and intracellular barriers seem to be surprisingly constant throughout the vertebrate phylum. In addition, it is becoming increasingly clear that the various receptors, such as DNA and the neurotransmitters, are comparable throughout the mammalian species.

There are life-span (or temporal) differences that are not considered adequately, nor have they been in the past. It takes time to develop, for example, a cellular immune response, and at least some of this time can be taken up by actual cell division processes. Cell division rates appear to be significantly higher in smaller animals. Mouse and rat cells turn over appreciably faster than do human cells, perhaps at twice the rate. On the other hand, the latent period for expression of many effects is also much shorter in small animals than in large ones.

Another difficulty is that the life span of humans is from 4.4 to 66.0 times (Gad and Chengelis 1998) that of common test species. Thus, there is generally a much longer time available for many toxicities to be expressed or developed in people than in test animals. These sorts of temporal considerations are of considerable importance, and this area of chronotoxicology has not yet begun to really be explored.

Body size, irrespective of species, seems to be important in the rate of distribution of foreign compounds throughout the body. A simple example of this is that the cardiac output of the mouse

is on the order of 1 ml/min, and the mouse has a blood volume of about 1 ml. The mouse is, therefore, turning its blood volume over every minute. In humans, the cardiac output per minute is only 1/20th of the blood volume. Therefore, the mouse turns its blood over 20 times faster than the human, which has clear implications for the comparative rates at which xenobiotics are systemically distributed in these two species.

Another aspect of the size difference that should be considered is that the large animal has a much greater number of susceptible cells that can interact with potential toxic agents, although there is also a proportionately increased number of "dummy" (hyporesponding) cells.

Rall (1979), Oser (1981), and Borzelleca (1984) have published articles reviewing such factors, and Calabrese (1983) had published an excellent book on the subject.

Having delineated and quantified species differences (even if only having factored in comparative body weights or relative body surface areas), one can now proceed to some form of quantitative extrapolation (or scaling).

Historically, there have been two major approaches to scaling for use with general toxicities. These are by body weight and body surface area (Calabrese 1983; Schmidt-Nielsen 1984). Both of these are single-variable or two-dimensional models, and represent alternate simple forms of what are called allometric equations. Davidson et al. (1986) have presented the generalized form of such equations as

$$Y = aW^n$$

where W is body weight, n is the slope of the derived line, and a is a scaling factor. Certain authors (e.g., Yates and Kugler 1986) proposed that a multidimensional model would be more accurate. Such a form of allometric equation is probably too complicated for use in most cases in toxicology, however, and its use would be inappropriate considering the relatively imprecise nature of the data generated.

The body weight approach has historically been the most common general approach to scaling in toxicology, particularly in regulatory toxicology testing. There are several ways to perform a scaling operation on a body weight basis, the most often employed being to simply calculate a conversion factor (K) as

$$K = \frac{\text{Weight of human (70 kg "standard")}}{\text{Weight of average test animal}}$$

More exotic methods for doing this, such as that based on a form of linear regression, are reviewed by Calabrese (1983), who believes that the body weight method is preferable.

A difficulty with this approach is that the body weights of any population of animals or people change throughout life, and in fact even at a common age will present considerable variation. Custom is, therefore, to use an "ideal person" (70 kg for men and 50 kg for women now set as a "standard" of 60 kg) or "ideal" animal weight (for which there is considerably less consensus).

The alternative is the body surface area methods, which attempt to factor in differences in metabolic rates based on the principle that these changes are in proportion to body surface area (because as the ratio of body surface area to body weight increases, relatively more energy is required to maintain a constant body temperature). As long ago as 1938, Benedict published a comparison of body weight and basal metabolic rates for species from mice to elephants that showed a linear relationship between the two variables. Pinkel (1958) and Freireich et al. (1966) later found a similar relationship for effective and tolerated doses of cancer chemotherapeutics, and most recently Otterness and Gans (1988) have reported that the effective dose of NSAIDs could be scaled between species and models by surface area. There are several methods for making such conversions, each having a ratio of dose to the animal's body weight (in mg/kg) as a starting point, resulting in a conversion factor with mg/m^2 as the units for the product of the calculations.

The U.S. Environmental Protection Agency (EPA) version of the surface area scaling equation has generally been calculated as

$$(M_{human}/M_{animal})^{1/3} = \text{Surface area}$$

where M = mass in kilograms. Another form is calculated based on constants that have been developed for a multitude of species of animals by actual surface area measurements (Spector 1956). The resulting formula for this approach is

$$A = KW^{2/3}$$

where A = surface area in square centimeters, K = a constant, specific for each species, and W = body weight in grams. A scaling factor is then simply calculated as a ratio of the surface area of a human over that of the model species.

Direct measurements of indicators of damage (much as tissue DNA damage; Slaga 1988; Visek 1988) have also been proposed as the ultimate means of a cross-species scaling of exposures.

The FDA (2002) released a draft guideline for cross-species extrapolation in using animal safety data to set initial levels for clinical trials in man, called the HED approach. This was also heavily based on the work of Boxenbaum and DiLea (1995) and Mahmood (2004). After the NOAELs in the relevant animal studies have been determined, they are converted to HEDs. A decision should be made regarding the most appropriate method for extrapolating the animal dose to the equivalent human dose. Toxic endpoints for therapeutics administered systemically to animals, such as the maximum tolerated dose (MTD) or NOAEL, are usually assumed to scale well between species when doses are normalized to body surface area (i.e., mg/m^2). The basis for this assumption lies primarily with the work of Freireich et al. (1966) and Schein et al. (1970). These investigators reported that, for antineoplastic drugs, doses lethal to 10% of rodents (LD_{10}) and MTDs in nonrodents both correlated with the human MTD when the doses were normalized to the same administration schedule and expressed as mg/m^2. Despite the subsequent analyses showing that the MTDs for this set of drugs scale best between species when doses are normalized to $W^{0.75}$ rather than $W^{0.67}$ (inherent in body surface area normalization), normalization to body surface area has remained a widespread practice for estimating an HED based on an animal dose.

An analysis of the impact of the allometric exponent on the conversion of an animal dose to the HED was conducted. Based on this analysis and on the fact that correcting for body surface area increases clinical trial safety by resulting in a more conservative starting dose estimate, it was concluded that the approach of converting NOAEL doses to an HED based on body surface area correction factors (i.e., $W^{0.67}$) should be maintained for selecting starting doses for initial studies in adult healthy volunteers. Nonetheless, use of a different dose normalization approach, such as directly equating the human dose to the NOAEL in mg/kg, might be appropriate in some circumstances. Deviations from the surface area approach should be justified. The basis for justifying direct mg/kg conversion and examples in which other normalization methods are appropriate are described in the following subsection.

Although normalization to body surface area is an appropriate method for extrapolating doses between species, consistent factors for converting doses from mg/kg to mg/m^2 have not always been used. Given that body surface area normalization provides a reasonable approach for estimating HED, the factors used for converting doses from each species should be standardized. Because surface area varies with $W^{0.67}$, the conversion factors are therefore dependent on the weight of the animals in the studies. However, analyses conducted to address the effect of body weight on the actual body surface area–conversion factor (BSA–CF) demonstrated that a standard factor provides a reasonable estimate of the HED over a broad range of human and animal weights. The conversion factors and divisors shown in table 13.5 are therefore recommended as the standard values to be used for interspecies dose conversions for NOAELs in the Center for Drug Evaluation and Research

(CDER) and the Center for Biologics Evaluation and Research (CBER). These factors can also be applied when comparing safety margins for other toxicity endpoints (e.g., reproductive toxicity and carcinogenicity) when other data for comparison (i.e., areas under the curve [AUCs]) are unavailable or are otherwise inappropriate for comparison.

Table 13.5 Conversion of Animal Doses to HEDs Based on Body Surface Area

Species	To Convert Animal Dose in mg/kg to Dose in mg/m², Multiply by km Below	To Convert Animal Dose in mg/kg to HED[a] in mg/kg, Either	
		Divide Animal Dose By:	Multiply Animal Dose By:
Human	37	—	—
Child (20 kg)[b]	25	—	—
Mouse	3	12.3	0.08
Hamster	5	7.4	0.13
Rat	6	6.2	0.16
Ferret	7	5.3	0.19
Guinea pig	8	4.6	0.22
Rabbit	12	3.1	0.32
Dog	20	1.8	0.54
Primates:			
Monkeys[c]	12	3.1	0.32
Marmoset	6	6.2	0.16
Squirrel monkey	7	5.3	0.19
Baboon	20	1.8	0.54
Micropig	27	1.4	0.73
Minipig	35	1.1	0.95

[a] Assumes 60-kg human. For species not listed or for weights outside of standard ranges, HED can be calculated from the formula:

$$\text{HED} = \text{animal dose in mg/kg} \times (\text{animal weight in kg/human weight in kg})^{0.33}.$$

[b] This km is provided for reference only as healthy children will rarely be volunteers for phase 1 trials.

[c] For example, cynomolgus, rhesus, stumptail.

The factors in table 13.5 for scaling animal NOAEL to HEDs are based on the assumption that doses scale 1:1 between species when normalized to body surface area. However, there are occasions for which scaling based on body weight (i.e., setting the HED (mg/kg) = NOAEL (mg/kg)) might be more appropriate. To consider mg/kg scaling for a therapeutic, the available data should show that the NOAEL occurs at a similar mg/kg dose across species. The following factors should be satisfied before extrapolating to the HED on a mg/kg basis rather than using the mg/m^2 approach. Note that mg/kg scaling will give a 12-, 6-, and 2- fold higher HED than the default mg/m^2 approach for mice, rats, and dogs, respectively.

NOAELs occur at a similar mg/kg dose across test species (for the studies with a given dosing regimen relevant to the proposed initial clinical trial).

If only two NOAELs from toxicology studies in separate species are available, one of the following criteria should also be true:

- The therapeutic is administered orally and the dose is limited by local toxicities. GI compartment weight scales by $W^{0.94}$. GI volume determines the concentration of the therapeutic in the GI tract. It is thus reasonable that the toxicity of the therapeutic would scale by mg/kg ($W^{1.0}$).
- The toxicity in humans (for a particular class) is dependent on an exposure parameter that is highly correlated across species with dose on a mg/kg basis. For example, complement activation by systemically administered antisense oligonucleotides in human is believed to be dependent on C_{max} (Geary et al. 1997). For some antisense drugs, the C_{max} correlates across nonclinical species with mg/kg dose and in such instances mg/kg scaling would be justified.

- Other pharmacologic and toxicologic endpoints also scale between species by mg/kg for the therapeutic. Examples of such endpoints include the MTD, lowest lethal dose, and the pharmacologically active dose.

Other Exceptions to mg/m² Scaling Between Species

Therapeutics can be administered by alternative routes (e.g., topical, intranasal, subcutaneous, intramuscular) for which the dose is limited by local toxicities. Such therapeutics should be normalized to concentration (e.g., mg/area of application) or amount of drug (mg) at the application site. Therapeutics administered into anatomical compartments have little subsequent distribution outside of the compartment. Examples are intrathecal, intravesical, intraocular, intrapleural, and intraperitoneal administration. Such therapeutics should be normalized between species according to the compartmental volumes and concentrations of the therapeutic. Biological products administered intravascularly with $M_r > 100{,}000$ daltons should be normalized to mg/kg.

Body Weight

Accurate conversion of mg/kg dose to a mg/m² dose depends on the actual weight (and surface area) of the test species. A popular formula for converting doses is:

$$\text{mg/m}^2 = km \times \text{mg/kg} \tag{i}$$

where $km = 100/\text{K} \times \text{W}0.33$ where K is a value unique to each species or $km = 9.09 \times \text{W}0.365$ where a K value unique to each species is not needed.

The *km* is not truly constant for any species, but increases within a species as body weight increases. The increase is not linear, but increases approximately proportional to $W^{2/3}$. For example, the *km* in rats varies from 5.2 for a 100-g rat to 7.0 for a 250-g rat. Strictly speaking, the *km* value of 6 applies only to rats at the reference weight of 150 g. For standardization and practical purposes, a fixed *km* factor for each species is preferred. An analysis was undertaken to determine the effect of different body weights within a species on the conversion of an animal dose to the HED using *km* factors. The *km* factor was calculated for a range of body weights using $km = 100/\text{K} \times \text{W}^{0.33}$. In table 13.6, a working weight range is shown next to the reference body weight. This is the range within which the HED calculated by using the standard *km* value will not vary more than ±20% from that which would be calculated using a *km* based on exact animal weight. This is a relativity small variance considering dose separation generally used in deriving the NOAEL, in toxicology studies, which are often twofold separations. For example, suppose a NOAEL in rats is 75 mg/kg and the average rat weight is 250 g. The *km* for a 250g rat is 7.0:

$$\text{HED} = 75 \times (7/37) = 14 \text{ mg/kg in humans.}$$

Using the standard *km* of 6 for rats,

$$\text{HED} = 75 \times (6/37) = 12 \text{ mg/kg in humans.}$$

The HED calculated with the standard *km* of 6 is within 15% of the value calculated using the actual *km* of 7. The body weights producing *km* factors for which the nominal, integer conversion factor was within 20% of the calculated factor covered a broad range (table 13.6). This working weight range encompassed the animal weights expected for the majority of studies used to support starting doses in humans.

For the typical species used in nonclinical safety studies, table 13.6 also shows the body surface area in m² for an animal at a particular reference weight. For example, a 400-g guinea pig has a

Table 13.6 Conversion of Animal Doses to HEDs Based on Body Surface Area

Species	Reference Body Weight (kg)	Working Weight Range[a] (kg)	Body Surface Area (m^2)	To Convert Dose in mg/kg to Dose in mg/m^2 Multiply by *km* Below	To Convert Animal Dose in mg/kg to HED[b] in mg/kg, Either	
					Divide Animal Dose by:	Multiply Animal Dose by:
Human	60	—	1.62	37	—	—
Child[c]	20	—	0.80	25	—	—
Mouse	0.020	0.011–0.034	0.007	3	12.3	0.081
Hamster	0.080	0.047–0.157	0.016	5	7.4	0.135
Rat	0.150	0.080–0.270	0.025	6	6.2	0.162
Ferret	0.300	0.160–0.540	0.043	7	5.3	0.189
Guinea pig	0.400	0.208–0.700	0.05	8	4.6	0.216
Rabbit	1.8	0.9–3.0	0.15	12	3.1	0.324
Dog	10	5–17	0.50	20	1.8	0.541
Primates						
Monkeys[c]	3	1.4–4.9	0.25	12	3.1	0.324
Marmoset	350	0.140–0.720	0.06	6	6.2	0.162
Squirrel monkey	600	0.290–0.970	0.09	7	5.3	0.189
Baboon	12	7–23	0.60	20	1.8	0.541
Micropig	20	10–33	0.74	27	1.4	0.730
Minipig	40	25–64	1.14	35	1.1	0.946

[a] For animal weights within the specified ranges, the HED for a 60-kg human calculated using the standard *km* value will not vary more than ±20% from the HED calculated using a km based on the exact animal weight.

[b] Assumes 60-kg human. For species not listed or for weights outside the standard ranges, human equivalent dose can be calculated from the formula: HED = animal dose in mg/kg × (animal weight in kg/human weight in kg)$^{0.33}$.

[c] The km is provided for reference only as healthy children will rarely be volunteers for phase 1 trials.

[d] For example, cynomolgus, rhesus, stumptail, and so on.

body surface area of approximately $0.05 m^2$. These values come from published sources with surface area determined experimentally by various methods. Compilations of this type of data can be found in published references.

For animal weights outside the working weight range in table 13.6, or for species not included in the table, an alternative method is available for calculating the HED. In these cases the following formula can be used:

$$\text{HED} = \text{Animal dose (mg/kg)} \times [\text{animal weight (kg)} / \text{human weight (kg)}]^{0.33}$$

For example, assume that NOAEL of 25 mg/kg was determined in a study using rabbits weighing 4.0 kg. The 4.0-kg animals are outside the working range for rabbits of 0.9 kg to 3.0 kg indicated in table 13.6.

$$\text{HED} = 25 \text{ mg/kg} \times (4.0 / 60)^{0.33} = 25 \times (0.41) = 10 \text{ mg/kg}$$

Alternatively, if the standard conversion factor was used to calculate the HED

$$\text{HED} = 25 \text{ mg/kg} / 3.1 = 8.1 \text{ mg/kg}$$

The value of 10 mg/kg for the HED is 25% greater than the value of 8.1 mg/kg that would be calculated using the standard conversion factor.

The *km* analysis addresses only half of the HED conversion process. The range of human sizes must also be considered to convert the mg/m^2 dose back to an HED dose in mg/kg. To examine the effect of both animal and human weights on the conversion factor, the principle of allometry was used. Interspecies biologic parameters are often related by the power function $Y = aW^b$ where W is body weight and b (allometric exponent) is the slope of the log-log plot, logy = bxlogW + C. HED in mg/kg, this equation is:

$$\text{HED} = \text{animal NOAEL} \times (W_{\text{animal}}/W_{\text{human}})^{(1-b)} \qquad \text{(ii)}$$

Because body surface area is believed to scale with an allometric exponent (b) of 0.67, one can explore how the animal and human body weights affect the conversion factor $(W_{\text{animal}}/W_{\text{human}})^{0.33}$.

The conversion factor was calculated over a range of animal weights and a range of human weights from 50 kg to 80 kg. The results are summarized in table 13.7. Column B is the weight range of the animals used to calculate, in conjunction with the 50 kg to 80 kg range in humans, the conversion factor. The extremes of the conversion factors for the permutations chosen are shown in columns C and D. The proposed standard conversion factors are shown in column E. The percentage difference of these extremes from the standard is shown in column F. Finally, the range of animal weights that produced a conversion factor for a 60-kg human within 20% of the standard factor are shown in column G.

The conclusions from these analyses are as follows:

- The ±20% interval around the standard conversion factor includes a broad range of animal and human weights.
- Given that the human weights will vary broadly, it is not usually necessary to be concerned about the impact of the variation of animal weights within a species on the HED calculation.
- If an extreme animal weight is encountered in a toxicology study, one can calculate an accurate conversion factor using $(W_{\text{animal}}/W_{\text{human}})^{0.33}$.

The best scaling factor is not generally agreed on. Although the majority opinion is that surface area is preferable where a metabolic activation or deactivation is known to be both critical to the

Table 13.7 Effect of Body Weight on HED Conversions[a]

Species	Animal Weight Range[b] (kg)	Conversion Factor[c] Small Animal Large Human	Large Animal Small Human	Standard[d]	% Difference of Extreme from Standard	±20% Range[f] for 60-kg Human (kg)
Mouse	0.018–0.033	0.060	0.089	0.081	–22%	0.015–0.051
Rat	0.090–0.400	0.106	0.213	0.162	–35%	0.123–0.420
Rabbit	1.5–3.0	0.269	0.395	0.324	+22%	1.0–3.4
Monkey	1.5–4.0	0.319	0.435	0.324	+34%	1.0–3.4
Dog	6.5–13.0	0.437	0.641	0.541	–19%	4.7–16.2

[a] Conversion factor = $(W_{animal}/W_{human})^{0.33}$.

[b] Human weight range used was 50 kg to 80 kg (110–176 lb).

[c] HED in mg/kg equals animal dose in mg/kg multiplied by this value.

[d] See Standard Table

[e] Extreme from column C or D.

[f] Range of animal weights that produced a calculated conversion factor within 20% of the standard factor (column E) when human weight was set at 60 kg.

adverse effect-causing process and present in both humans and the model species, these assumptions might not always be valid. For the conditions under which most toxicity testing is performed, these facts are generally unknown. Table 13.8 presents a comparison of the weight and surface area extrapolation methods for the eight common laboratory animal species and humans. Brown et al. (1988), on reviewing the currently available data, likewise have concluded that though "correlations exist among risk levels in various species, many factors appear to influence toxicity that are not captured in a simple scaling rule." These factors are commonly those pointed out in table 13.4.

Table 13.8 Extrapolation of a Dose of 100 mg/kg in the Mouse to Other Species

Species	Weight (g)	Surface[a] Area (cm^2)	Extrapolated Dose Based on (mg) Body Weight (A)	Body Surface Area (B)	Ratio A/B
Mouse	20	46.4	2	2	1.0
Rat	400	516.7	40	22.3	1.80
Hamster	50	126.5	5	5.4	1.08
Guinea pig	400	564.5	40	24.3	1.65
Ferret	500	753.9	50	32.5	1.54
Rabbit	1,500	1,272.0	150	54.8	2.74
Dog	12,000	5,766.0	1,200	248.5	4.82
Monkey	4,000	2,975.0	400	128.2	3.12
Man	70,000	18,000.0	7,000	775.8	9.8

[a] Surface area (except in the case of man) values calculated from the formula

$$\text{Surface area } (cm^2) = KW^{2/3}$$

where K is a constant for each species and W is the body weight (values of K and the surface area for man are taken from Spector 1956).

Schneiderman et al. (1975) and Dixon (1976) have published comparisons of these methods, but Schmidt-Nielsen (1984) should still be considered the primary source on scaling in interspecies comparisons.

When one is concerned about specific target organ effects, frequently the earliest indicator of such an effect is an alteration in organ weight out of proportion to what is expected due to changes in overall body weight (Gad et al. 1984) or to changes in a standard such as brain weight. It should be pointed out that a form of scaling is involved in detecting such effects, as adjustments to organ weight to account for alterations in overall body weight can take several forms. Either simple ratios

(Angervall and Carlstrom 1963; Gad and Weil 1980; Weil and Gad 1980), analysis of covariance, or species- or organ-specific allometric methods (Lutzen et al. 1976; Trieb et al. 1976) can be employed.

An alternative approach to achieving society's objective for the entire risk assessment process (i.e., protecting the human population from unacceptable levels of voluntary risk) is the classic approach of using safety factors. This is still the methodology used in determining what are acceptable risks, given the uncertainties involved, in phase I human clinical trials of a new drug based on animal safety data. The presumed degree of uncertainty in these cases is instructive. Weil (1972) summarized this approach as:

> In summary, for the evaluation of safety for man, it is necessary to: (1) design and conduct appropriate toxicologically tests, (2) statistically compare the data from treated and control animals, (3) delineate the minimum effect and maximum no ill-effect levels (NIEL) for these animals, and (4) if the material is to be used, apply an appropriate safety factor, e.g., (a) 1/100 (NIEL) or 1/50 (NIEL) for some effects or (b) 1/500 (NIEL), if the effect was a significant increase in cancer in an appropriate test.

This approach has served society reasonably well over the years, once the experimental work has identified the potential hazards and quantified the observable dose–response relationships. The safety factor approach has not generally been accepted or seriously entertained by regulatory agencies for carcinogens, mutagens, or teratogen, but is well established for other toxic effects of drugs and chemicals (Weil 1972), and Johnson (1988), after reviewing a broad range of developmental and maternal toxicity data proposed that "where the effect in the embryo is only seen at maternally toxic doses and exposure is below the adult toxic doses, relatively modest safety factors are sufficient for safe cross species extrapolation." Until such time as more elegant risk assessment procedures can instill greater public and scientific confidence, the use of the safety factor approach to bridge our collective uncertainty about the difference between species responses should perhaps not be abandoned so readily for more "mathematically precise and elegant" procedures, but rather should be reviewed and perhaps revised for some areas of greater uncertainty (Dawson and Stara 1983). Indeed the FDA has returned to this approach as part of the guidance on safe starting doses for clinical trials (FDA 2002).

Once the HED of the NOAEL in the most appropriate species has been determined, a safety factor is then applied to provide a margin of safety for protection of human subjects receiving the initial clinical dose. This safety factor allows for variability in extrapolating from animal toxicity studies to studies in humans resulting from (a) uncertainties due to enhanced sensitivity to therapeutic activity in humans versus animals, (b) difficulties in detecting certain toxicities in animals (e.g., headache, myalgias, mental disturbances), (c) differences in receptor densities or affinities, (d) unexpected toxicities, and (e) interspecies differences in ADME of the therapeutic. These differences can be accommodated by lowering the human starting dose from the HED of the selected species NOAEL.

In practice, the maximum safe starting dose for the clinical trial is determined by dividing the HED derived from the animal NOAEL by the safety factor. The default safety factor used is 10. This is a historically accepted value, but it should be evaluated based on available information.

Although a safety factor of 10 can generally be considered adequate for protection of human subjects participating in initial clinical trials, this safety factor might not be appropriate for all cases. The safety factor should be raised when there is reason for increased concern, and lowered when concern is reduced due to available data that provide added assurance of safety. This can be visualized as a sliding scale, balancing findings that mitigate the concern for harm to healthy volunteers with those that suggest greater concern is warranted. The extent of the increase or decrease is largely a matter of judgment, using the available information. It is incumbent on the evaluator to clearly explain the reasoning behind the applied safety factor when it differs from the default value of 10, particularly if it is less than 10.

Increasing the Safety Factor

The following considerations indicate a safety concern that might warrant increasing the safety factor. In these circumstances, the maximum recommended starting dose (MRSD) would be calculated by dividing the HED by a safety factor that is greater than 10. If any of the following concerns are defined in review of the nonclinical safety database, an increase in the safety factor might be called for. If multiple concerns are identified, the safety factor should be increased accordingly.

- *Steep dose response curve*. A steep dose response curve for significant toxicities in the most appropriate species or in multiple species might indicate a greater risk to humans.
- *Severe toxicities*. Qualitatively severe toxicities or damage to an organ system (e.g., central nervous system) indicate increased risk to humans.
- *Nonmonitorable toxicity.* Nonmonitorable toxicities can include histopathologic changes in animals that are not readily monitored by clinical pathology markers.
- *Toxicities without prodromal indicators.* If the onset of significant toxicities is not reliably associated with premonitory signs in animals, it might be difficult to know when toxic doses are approached in human trials.
- *Variable bioavailability.* Widely divergent bioavailability in several species, with poor bioavailability in the test species used to derive the HED, suggest a greater possibility for underestimating the toxicity in humans.
- *Irreversible toxicity.* Irreversible toxicities in animals suggest the possibility of permanent injury in human trial participants.
- *Unexplained mortality.* Mortality that is not predicted by other parameters raises the level of concern.
- *Large variability in doses or AUC levels eliciting effect*. When doses or exposure levels that produce a toxic effect differ greatly across species, the ability to predict a toxic level in humans is reduced and a greater safety factor might be called for.
- *Questionable study design or conduct*. Poor study design or conduct casts doubt on the accuracy of the conclusions drawn from the data. For instance, few dose levels, wide dosing intervals, or large differences in responses between animals within dosing groups might make it difficult to characterize the dose–response curve.
- *Novel therapeutic targets*. Therapeutic targets that have not been previously clinically evaluated might increase the uncertainty of relying on the nonclinical data to support a safe starting dose in humans.
- *Animal models with limited utility.* Some classes of therapeutic biologics might have very limited interspecies crossreactivity or pronounced immunogenicity, or might work by mechanisms that are not known to be conserved between (nonhuman) animals and humans; in these cases, safety data from any animal studies could be very limited in scope and interpretability.

Decreasing the Safety Factor

Safety factors of less than 10 might be appropriate under some conditions. The toxicologic testing in these cases should be of the highest caliber in both conduct and design. Most of the time, candidate therapeutics for this approach would be members of a well-characterized class. Within the class, the therapeutics should be administered by the same route, schedule, and duration of administration; should have a similar metabolic profile and bioavailability; and should have similar toxicity profiles across all the species tested including humans. A smaller safety factor might also be used when toxicities produced by the therapeutics are easily monitored, reversible, predictable, and exhibit a moderate to shallow dose–response relationship with toxicities that are consistent across the tested species (both qualitatively and with respect to appropriately scaled dose and exposure).

An additional factor that could suggest a safety factor smaller than 10 would be a case in which the NOAEL was determined based on toxicity studies of longer duration compared to the proposed clinical schedule in healthy volunteers. In this case, a greater margin of safety is often built into

the NOAEL, as it was associated with a longer duration of exposure than that proposed in the clinical setting. This assumes that toxicities are cumulative, are not associated with acute peaks in therapeutic concentration (e.g., hypotension), and did not occur early in the repeat dose study.

As a final sanity check to any multistep process of hazard assessment, the data points generated by any other studies (particularly any human exposures) of the endpoint of interest should be evaluated to determine if they fall within the range expected based on the assessment. If we find that the available real-world data do not fit our extrapolation model at this point, then as scientists we have no choice but to reject such a model or assessment and start anew.

Embodied in the safety factor approach are two of the "rules" for cross-species extrapolation, which are actually general comparative statements of relationships between species.

In general, as animal species become larger, they also become more sensitive to short-term toxicities. This effect can be credited to a number of mechanisms (e.g., increases of available target tissues and decreases of metabolic rate as size increases), but it is true even for nonmammalian species such as fish (Anderson and Weber 1975) and birds (Hudson et al. 1979). The rule even applies somewhat to differences in body size within the same age class of the same sex of the same species.

What this means is that as a rule of thumb, the sensitivity of a larger species (e.g., a dog or human) to a short-term toxicity will be greater than that of a smaller species (e.g., a mouse or rat). There are, of course, exceptions and wide variations from linearity in this relationship (such as hamsters being much less sensitive to the neurotoxicity of DDT than are mice, as reported by Gingell and Wallcave 1974). Those toxicities that are mediated or modulated by structurally different features (such as those toxicities associated with the skin, where general rules fall completely apart across broad ranges of structural classes; Campbell and Bruce 1981; Nixon et al. 1975) are subject to even less certainty under this rule.

There are also those who believe that humans are more sensitive than any test species, even if that species is larger than humans (e.g., a cow or horse). Lehman (1959) published, for example, the relationships shown in table 13.9.

Table 13.9 Some Relations of Drug Toxicity in Experimental Animals Compared to Humans

Species	Weight (kg)	Weight Ratio Animal/Human	Drug Dose Ratio	Sensitivity: Drug Dose Ratio/Weight Ratio
Man	60	1	1	1
Cow	500	8	24	Human 3× as sensitive
Horse	500	8	16	Human 2× as sensitive
Sheep	60	1	3	Human 3× as sensitive
Goat	60	1	3	Human 3× as sensitive
Swine	60	1	2	Human 2× as sensitive
Dog	10	1/6	1	Human 6× as sensitive
Cat	3	1/20	1/2	Human 10× as sensitive
Rat	0.4	1/150	1/15	Human 10× as sensitive

Note: The values in this table are averages and their validity cannot be checked against original data, as Lehman (1959) only reported them as being from numerous sources.

It should be noted that many data on effects in humans are biased by humans being better (or at least more sensitive and louder) indicators of adverse effects. Differences in sensitivity between the sexes are, in the majority, such that females are more sensitive than males. Data to support this are reviewed later in this chapter.

SPECIAL CASES: MATCHING CHARACTERISTICS FOR SPECIAL POPULATIONS

There are many factors that can alter the physiological state of an individual (or the fraction of available chemical moiety in an individual; see table 13.10), and in so doing make them more (or,

in some few cases, less) susceptible to the adverse effects of a chemical. These include (but are not limited to) immunological experience; physiological factors such as stress, age, and illness; conditioning factors (e.g., obesity and malnutrition); and sex. Proper model selection and experimental design require that these factors be identified and considered. Chapter 14 (on susceptibility factors) considers these and related factors on a theoretical basis in more detail.

Table 13.10 Factors That Can Increase the Fraction of Available Chemical Moiety in the Systemic Circulation

1. Renal impairment
2. Liver impairment
3. Hypoalbuminea
4. Presence of other moieties that displace test agent from proteins in circulation
5. Pregnancy

Sex

Sex hormones might be the target or sex hormones might modify a particular toxic response, which then can account for differential responses between the sexes to toxic materials. The current consensus is that (as pointed out earlier) females are more susceptible than males to the acute toxic effects of many chemicals, although males and females of the same strain, age, and general condition will react in a qualitatively similar manner.

As a result of reviewing the acute oral and dermal toxicities of 98 pesticides to rats, Gaines (1969) concluded that by the oral route the majority were more toxic to females than to males. He found the reverse true for only 9 out of the 98 pesticides tested: aldrin, chlordane, heptachlor, abate, imidan, methyl parathion, fenchlorphos, schradan, and metepa. Pallotta et al. (1962) found the same pattern for the antibiotic acetoxycycloheximide in rats but not in dogs (where there was no sex difference). Indeed, a review of the published literature on pesticides by Kato and Gillette (1965) found that such sex differences are common in rodents but less so in other mammals, although the information on these other species is not as definitive. Even with rodents and pesticides, however, it should be remembered that this is a general rule and not a universal truth. Steen et al. (1976) found mevinphos to be more toxic to male Mongolian gerbils than to females, whereas Gaines (1960) found the reverse to be the case for this compound in rats. These observations were in accord with published data on hexabarbitone sleeping times, as shown in table 13.11. In general, most barbiturates cause longer sleeping times in females than in males. Likewise, as a class, organophosphates are lethal in lower doses in female rats than in males.

Table 13.11 Relative Hexobarbitone Sleeping Times for Each Sex in Two Different Rodent Species

	Mean Sleeping Time (min)		
Sex	Mongolian Gerbil[a]	Rat[b]	Mouse[c]
Male	105 + 9.6	22 + 4	34 + 5
Female	70 + 6.9	67 + 15	31 + 5

Note: The reported sex difference did not occur in rats less than 4 weeks old.

[a] Maines and Westfall (1971).

[b] Quinn et al. (1958).

[c] Vessell (1968).

Shanor et al. (1961) found that in humans there is a statistically significant difference between the plasma cholinesterase levels of healthy young males and females (activity levels in females are

from 64%–74% of males), but that this difference disappeared in older people. There was no significant variation between the sexes in erythrocyte cholinesterase, a finding confirmed by Eben and Pilz (1967). Naik et al. (1970) likewise found there to be no significant difference between males and females in either total brain cholinesterase or in brain acetylcholine. These findings suggest that the distribution characteristics of toxicants working by cholinesterase inhibition mechanisms might be critical to their acute toxicity and that these distribution characteristics can be altered by the sex of the animal.

Krasovskij (1975) reviewed data on the acute toxicities of 149 chemicals and compared the results for males versus those for females. For both rats and mice, he found that the females tended to be more sensitive than the males, although not by large amounts (generally the differences were on the order of 8%–12%, being a little greater in rats than in mice).

Depass et al. (1984) looked at oral and dermal lethality of a number of previously studied compounds on which the results had largely been published. To assess the effect of sex, they calculated the correlation coefficient (r) between the LDP results for the two sexes. For 91 oral studies, r was found to be .93, whereas for 17 dermal studies with skin abrasion and 28 without, the r values were .73 and .96, respectively. The LD_{50} values between the two sexes were, in other words, strongly associated. However, when the values were compared using paired t tests, there was a statistically significant trend toward higher LD_{50}s in the males.

Similarly, Bruce (1985) reviewed studies from files on 48 chemicals and found that for only 3 of these was there evidence of lower LD_{50} values among the males than the females, and that none of these differences approached statistical significance. In 13 cases, however, the males had significantly higher LD_{50} values than the females. For these 13 studies, male LDP values averaged 29% higher than those for females.

Imbalances of hormones other than those related to sexual function have also been shown to alter the susceptibility of animals to the toxic effects of chemicals. Hyperthyroidism, hyperinsulinism, adrenalectomy, and stimulation of the pituitary have all been demonstrated to be capable of modifying the effects of selected toxicants (Dauterman 1980; Doull 1980).

Stress

Stress and the variability of animals underlying biological rhythms (the complex interactions of physiological responses to chronologically ordered external factors) are among the least accounted for variables in toxicology. Although they have both been studied and identified as important determinants of sensitivity to toxicity, standard practice does not evaluate these effects or seek to factor them in predicting human effects.

Stress is a broad term for specific morphological, biochemical, physiological, or behavioral changes experienced by an organism in response to a stressful event or stressor (Vogel 1987). Such changes can be quite drastic. Plasma levels of epinephrine in resting rats are approximately 100 pg/ml, but in a stressed rat can approach 2,000 pg/ml. Cessation of the stressful event usually terminates the stress response and the organism returns to its baseline homeostasis. However, if the stress response is very intense or long lasting, a return to the original homeostasis might not occur and a new biological equilibrium could be established. The consequences of this new condition can be either beneficial (e.g., exercise-induced stress strengthening the heart) or detrimental (e.g., job-induced stress causing ulcers or hypertension) to the organism.

The typical behavioral stress responses are fear, tension, apprehension, and anxiety. Physiological stress responses can include changes in gastric secretion and motility and increases in blood pressure and heart rate. Biochemical changes are widespread during stress and include significant increases in the levels of plasma catecholamine and corticosteroids or marked changes in brain neurotransmitter concentrations. Although these are only typical response examples, most biochemical and physiological systems are probably affected during stress. Thus, potential toxicants interact

with quite different physiological and biochemical systems during stress and the resulting outcomes of such interactions are bound to be quite different under these altered conditions.

In experimental toxicology, it is customary to use nonstressed animals to evaluate the extent and modes of action of chemicals. However, animals and humans are seldom nonstressed, but rather are frequently challenged by stressful events in the real world, responding with stress manifested as some of the previously mentioned responses. Thus, agents acting at specific biochemical sites or on physiological processes will encounter different conditions during rest and stress, resulting in differences in their effects. In addition, the true action of some agents might only be revealed during stress. Thus, the variable of stress should probably be included during experimentation to better approximate (or model) various real-life situations and to predict more accurately the actions of chemicals under all types of environmental conditions.

The literature does very clearly reflect that the actions of biologically active substances can be altered during stress. Toxic effects can be increased or decreased, and the results must be interpreted in this context before they can be generalized or extrapolated to the human population. Guinea pigs show an increased susceptibility to the lethal effects of ouabain during stress. Natelson et al. (1979) report that only 9% of nonstressed animals die, whereas 50% succumb to the same dose of ouabain if the animals are stressed.

Similarly, the delayed neuropathology to triorthotolyl phosphate in hens is almost tripled by stress (Ehrich and Gross 1983), and Stokinger (1953) indicated the considerable influence that stress can have on the dose-dependent distribution of some of the elements in the body.

It has also been demonstrated that injections of adrenocorticotropic hormone (Vaccarezza and Willson 1964a, 1964b) caused increases both in plasma and cell cholinesterase in rats, but that adrenalectomy caused a progressive decline in the circulating RBC cholinesterase but had no effect on plasma cholinesterase in rats. In people, injections of adrenocorticotropic hormone also gave rise to increases in plasma and circulating cell cholinesterases (Vaccarezza and Peltz 1960).

In reporting investigations of parathion in rats, Kling and Long (1969) demonstrated the influence that dietary stress could have on the time course of the response of cellular cholinesterase, while not altering the overall quantitative outcome. In fact, much of life (for at least the laboratory animals species) consists of a habitat that exhibits a recurrence of a sequence of events in an ordered manner relative to time. The effect of many biologically active agents, particularly toxicants, must interfere with these normal patterns and resulting biological cybernetics. Although Scheving et al. (1974) have held that there was little evidence that acute toxicity displayed significant differences relative to circadian rhythms, this conclusion seems suspect. There are clear relationships among biorhythms, stress, and the endocrine functions.

Circadian differences in response to a range of chemicals such as nikethamide, ethanol, chlordiazepoxide (Librium), methopyrapone, and ouabain have been observed in the mouse. Halberg et al. (1960) demonstrated that there was a potency ratio alteration of from 3.2 to 1.0 for the bioassay of *Escherichia coli* endotoxin carried out at 12-hr time intervals. Working with rats, Lenox and Frazier (1972) demonstrated that the mortality due to methadone was influenced by a circadian cycle.

Stress due to fasting has been shown to alter the permeability of the blood–brain barrier to some chemicals (Angel 1969). Indeed, selective starvation can also influence sensitivity. Boyd et al. (1970) demonstrated that feeding protein-deficient or protein-rich diets to rats could markedly alter the LD_{50} values for many pesticides.

Likewise, for some toxicants the influence of single or multiple housing can also significantly alter the results of a range of outcomes in acute toxicity tests, with marked variations in sensitivity (and the direction even of the influence) to this housing variable between different species.

Age

Age is an endogenous factor that alters an organism's response to exposure to a test chemical. Very young or old animals might be either more or less sensitive to toxic effects than fully developed

young or mature animals, and indeed might even have qualitatively different responses. Older rats, for example, are almost immune to the carcinogenic actions of most chemicals. Neonates are more susceptible to the actions of opiates.

Traditionally, young adult animals are used to perform tests in our studies. However, much of our human population is either very young or old, and clearly not physiologically comparable to young adults.

Age variation might give rise to differences in susceptibility to acute intoxication by different chemicals and there is not a simple rule for relating age to the sensitivity or nature of the toxic response. Goldenthal (1971) published an extensive review of the comparative acute toxicity of agents to newborn and adult animals. During the early stages of life, anatomical, physiological, metabolic, and immunological capabilities are not fully developed.

Substantial differences in susceptibility can sometimes even be related to small age differences. With rodents, a difference of a few months in age can markedly alter the response to chemicals that influence either the central nervous or immunological systems.

Biological aging is both time and species dependent (Mann 1965). For the purposes of acute toxicity, it is generally convenient to consider the stages of biological age as being neonatal, infant, young adult, and old. There is no clear dividing line between these stages in any species, although their length was loosely defined at the beginning of this chapter. Rather, development and aging are a continuum on which there is both species and individual variation. For some laboratory-bred species, however, such as the rat, there is a fairly linear relationship between the logarithm of body weight and the reciprocal of the animal's age (Gray and Addis 1948), which can be expressed as:

$$\log_{10}[W] = \frac{-K}{d}\ a + \log_{10}A$$

where W = weight in grams, K = slope, d = age in days, and A = the estimated asymptote or limit for W.

There is, in fact, an entire family of statistical methods for adjusting different structural and functional characteristics for age. Mattfeldt and Mall (1987) give an excellent overview of these allometric methods.

The toxicological response to both exogenous and endogenous physiological chemicals (e.g., epinephrine and acetylcholine) can vary with age. Brus and Herman (1971) demonstrated that newborn mice were significantly less sensitive to epinephrine and norepinephrine than were adults, but that the reverse held true for acetylcholine. Naik et al. (1970) found that brain acetylcholine concentrations increased with body weight and age until maturity, whereas brain cholinesterase activities were variable at lower weight and age and became less variable as weight and age increased. Shanor et al. (1961), using a large population sample of young adults (ages 18–35 years) and older people (ages 70–80 years) found that the plasma cholinesterase activity was approximately 24% higher in the young males than in the old males, but that no such difference existed for females or for RBC cholinesterase.

Older animals also show a large number of alterations in their response to potential toxicants when compared to young adults.

Disease

Disease states can modify a variety of kinetic and physiological parameters, altering the baseline homeostatic condition. Earlier, it was pointed out that a number of conditions (e.g., liver or renal disease) could increase the amount of available drug moiety in the systemic circulation. The ability to understand how pathological conditions can modify the kinetics and effect of exogenous chemicals requires an understanding of the interrelationships among these various parameters.

Stress due to infection can alter the responses of animals to biologically active chemicals. Safarov and Aleskerov (1972) found that the dipping of sheep in an ectoparasiticide depressed antibodv production and reduced the ability of the sheep to survive infections. It has been shown that some chemicals adversely affect the natural immunological defense systems, although this appears to be associated more with persistent agents retained in the organism than with those agents that are rapidly cleared. Liver (by decreasing biotransformation) and renal (by disrupting both excretory and metabolic functions) diseases associated with a preexisting condition or old age might contribute to a greater sensitivity to a toxicant. Hyperthyroid states also have been shown to increase sensitivity to the toxic effects of several classes of drug, particularly selected psychoactive agents (Zbinden 1963).

Physiological State

The influence of diet on the response of animals and humans to toxins is well established. The toxicity of specific agents can be increased or decreased by alterations of dietary protein or the various micronutrients (e.g., the decreased sensitivity of protein-deficient animals to CCl_4). Two conditions that occur in humans and are not generally recognized as diseases, obesity and subclinical malnutrition or marginal nutrition, can also alter the biological affects of chemicals and serve to increase the susceptibility of individuals to toxic actions.

Obesity could well alter the distribution and storage of a xenobiotic, especially when it is markedly lipophilic. Obesity is also generally accompanied in humans and test animals by reduced or impaired respiratory, cardiovascular, and renal function, all of which will also alter the manner and degree to which an agent might be toxic.

Subclinical malnutrition or marginal nutrition is the other end of the scale from obesity usually, but not always or absolutely. An individual person's or animal's diet might be calorically adequate (or even oversupplied), but nutritionally marginal in terms of vitamins, proteins, minerals, and other nutrients. Any such marginal nutrient state clearly presents the possibility of an increased susceptibility to a toxic or adverse outcome of exposure to a xenobiotic, particularly if said nutritional state means limited or deficient metabolic or enzymatic defense mechanisms, or if the potential toxicant acts to reduce the available limiting nutrient. The principal biotransformation of toxicants is performed by the microsomal mixed function oxidase (MMFO) system, which is depressed by a deficiency of essential fatty acids, vitamin A, or proteins.

Boyd et al. (1970) and Boyd (1972) reviewed the effects of nutritional status on acute toxicity, showing alteration in the responses of rodents. Mehrle et al. (1973) demonstrated that the LC_{50}s of chlordane in rainbow trout were altered by the brand of commercial diet the fish were maintained on beforehand. Furthermore, the nutritional status of animals used to prepare or provide tissues for *in vitro* studies can change the microsomal metabolism and other aspects of responsiveness of the tissue (Kato and Gillette 1965).

Toxins also have the potential to induce nutritional deficiencies, but these are generally of concern only in cases of longer term exposure.

MODELS

This entire volume has focused on animal models in toxicology and is based on the premise that these models are not only a valid approach to predicting the adverse effects of chemicals in humans, but are the primary model for such predictions.

In vivo models are not the only means, however, and a brief review of the status of alternatives to them is called for. Classes of *in vitro* alternatives are *in vitro* models (which do not use intact higher organisms but do use some form of test system) and mathematical or structure-activity

relationship (SAR) approaches (which construct theoretical analogies but require no actual generation or interpretation of new data).

In Vitro Models

In vitro models, at least as screening tests, have been with us in toxicology for some 20 years now. The last 5 to 10 years have seen a great upsurge in interest in such models. The increased interest is due to economic and animal welfare pressures and technological improvements.

In vitro systems per se have a number of limitations that can contribute to their unacceptability as models. Some of these reasons are detailed in table 13.12.

Table 13.12 Possible Interpretations When *in Vitro* Data Do Not Predict Results of *in Vivo* Studies

1. Chemical is not absorbed at all or is poorly absorbed in *in vivo* studies.
2. Chemical is well absorbed but is subject to first-pass effect in liver.
3. Chemical is distributed so that less (or more) reaches the receptors than would be predicted on the basis of its absorption.
4. Chemical is rapidly metabolized to an active or inactive metabolite that has a different profile of activity or different duration of action than the parent drug.
5. Chemical is rapidly eliminated (e.g., through secretory mechanisms).
6. Species of the two test systems used are different.
7. Experimental conditions of the *in vitro* and *in vivo* experiments differed and might have led to different effects than expected. These conditions include factors such as temperature or age, sex, and strain of animal.
8. Effects elicited *in vitro* and *in vivo* differ in characteristics.
9. Tests used to measure responses will probably differ greatly for *in vitro* and *in vivo* studies, and the types of data obtained might not be comparable.
10. The *in vitro* study did not use adequate controls (e.g., pH, vehicle used, volume of test agent given, samples taken from sham-operated animals).
11. *In vitro* data cannot predict the volume of distribution in central or in peripheral compartments.
12. *In vitro* data cannot predict the rate constants for chemical movement between compartments.
13. *In vitro* data cannot predict whether linear or nonlinear kinetics will occur with specific chemical *in vivo*.
14. *In vitro* data cannot predict whether linear or nonlinear kinetics will occur with specific dose of a chemical *in vivo*.
15. Pharmacokinetic parameters (e.g., bioavailability, peak plasma concentration, half-life) cannot be predicted based solely on *in vitro* studies.
16. *In vivo* effects of chemical are due to an alteration in the higher order integration of an intact animal system, which cannot be reflected in a less complex system.

At the same time there are substantial potential advantages in using *in vitro* systems. The scientific advantages of using cell or tissue culture in toxicological testing are isolation of test cells or organ fragments from homeostatic and hormonal control, accurate dosing, and quantification of results. It is important to devise a suitable model system that is related to the mode of toxicity of the compound. Tissue and cell culture have been used in two very different ways in screening studies. First, they have been used to examine a particular aspect of the toxicity of a compound in relation to its toxicity *in vivo*. Second, they have been used as a form of rapid screening to compare the toxicity of a group of compounds. Additionally, target-organ-specific *in vitro* models offer a powerful means of explaining the mechanisms behind toxicities observed in intact organisms.

SUMMARY

If the human population we are concerned about is such that one or more of these susceptibility factors is present in a substantial portion of the members, steps should be taken to design studies so that such individuals are adequately represented (i.e., "matched") by an appropriate model in the test animal population. Barring that, or in the face of having existing data on studies performed in a standard manner, consideration should be given to these factors when attempting to predict the outcome of exposures in people.

Individual Biological Variation

There are also individual animal-to-animal variations in temperature, health, and sensitivity to toxicities that are recognized and expected by experienced animal researchers, but are only broadly understood. The resulting differences in response are generally accredited to individual biological variation. This same phenomenon has been widely studied and observed among humans, and is expected by any experienced clinician. Examples of such individual variations in humans include isoniazid, succinylcholine, and glucose-6-phosphate levels or activities. In the first of these, slow inactivators who are deficient in acetyltransferase, and therefore process acetylate agents such as isoniazid only slowly, are thus more liable to suffer from the peripheral neuropathy caused by an accumulation of isoniazid. At the same time, people with more effective acetyltransferase require larger doses of isoniazid to benefit from its therapeutic effects, but in so doing are more likely to suffer liver damage.

Likewise, individuals with atypical or low levels of serum cholinesterase can exhibit prolonged muscle relaxation and apnea following an injection of a standard dose of the muscle relaxant succinylcholine. And glucose-6-phosphate dehydrogenase deficiency is responsible for the increased probability of some individuals given primaquine or antipyrine to suffer from a hemolytic anemia.

Species Variation

Although anyone who has had to work in biological research with intact animals should be aware of the existence of wide variability between species, examples that are specific to toxicology should be presented here along with some degree of comparison of relative species sensitivity for a number of specific agents. Table 13.13 presents an enumeration of target organ toxicities that are specific to several model-specific species commonly used in toxicology.

Table 13.13 Species-Specific Toxic Effects

Type of Toxicity	Structure	Sensitive Species	Mechanism of Toxicity
Ocular	Retina	Dog	Zinc chelation
Ocular	Retina	Any with pigmented retinas	Melanin binding
Stimulated basal metabolism	Thyroid	Dog	Competition for plasma binding
Ocular	Retina and optic nerve	Primates	Formic acid formation
Porphyria	Liver	Human, rat, guinea pig, mouse, and rabbit	Estrogen-enhanced sensitivity
Tubular necrosis	Kidney	Rats (males)	Androgen-enhanced sensitivity[a]
Urolithiasis	Kidney and bladder	Rats and mice	Uricase inhibition
Teratogenisis; mortality	Fetus	Rats and mice	Uricase inhibition
Cardiovascular	Heart	Rabbits	Sensitivity to microvascular constriction

[a] More sensitive than humans for many agents (e.g., caprolactam and halogenated solvents).

Source: Adapted from Gralla (1986) with modification.

The rodenticide zinc phosphide is dependent on the release of phosphine by hydrochloric acid in the stomach for its activation and efficacy (Johnson and Voss 1952). As a result, dogs and cats are considerably less sensitive than rats and rabbits because the former species secrete gastric hydrochloric acid intermittently whereas the latter secrete it almost continuously.

That this case is not a rare one can be quickly established by examining some data sets in which we have comparative oral lethality data on several species (including humans), such as those presented in table 13.14.

Hottendorf (1987) has published a review of the predictive value of seven animal model species across a range of compounds with different target organs, finding the rat, mouse, and monkey to

Table 13.14 Comparative Human Acute Lethal Doses and Animal LD_{50}s (mg/kg via Oral Route)

Chemical	Human LD_{LO}[a]	LD_{50} Values			
		Mouse	Rat	Rabbit	Dog
Aminopyrine	220	358	685	160	150
Aniline	350	300	440		195
Amytal	43	345	560	575	
Boric acid	640	3,450	2,660		
Caffeine	192	620	192	224	140
Carbofuran	11	2	5		19
Carbon tetrachloride	43	12,800	2,800	6,380	
Cycloheximide		133	3		
Lindane	840		125	130	120
Fenoflurazole		1,600	283	28	50

[a] LD_{LO} = lowest observed lethal dose.

be generally the best individual predictors, whereas the guinea pig and hamster were the least accurate predictors.

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CHAPTER 14

Susceptibility Factors

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CONTENTS

Susceptibility factors are conditions or characteristics that make individual animals or discrete groups of animals (or people) differentially more sensitive to the toxicity of an agent or to expression of manifestations of the toxicity. Such factors should be considered from two different perspectives. First, if undesired they might serve to either confound the results of experiments, or by increasing the variability of response in text populations, decrease the sensitivity of test systems. Second, if our concern is the health and safety of more sensitive individuals in the population (as opposed to the young, healthy adult population our usual test animal pool represents), then incorporation of such factors in selecting model populations is essential. Unfortunately, this is something we still do not do well.

There are many factors that can alter the physiological state of an individual (or the fraction of available chemical moiety; see table 14.1), and in so doing make them more (or, in a few cases, less) susceptible to the adverse effects of a chemical. These include (but are not limited to) immunological experience; psychological factors such as stress, age, and illness; conditioning factors (e.g., obesity and malnutrition); and sex. There are also environmental factors such as temperature, humidity, and time of day that can serve as susceptibility factors.

Table 14.1 Factors That Might Increase the Fraction of Available Chemical Moiety in the Systemic Circulation (and Therefore Potential Toxicity)

1. Renal impairment
2. Hepatic impairment
3. Impaired GI absorption
4. Hypoalbumineia
5. Presence of other moieties that displace test agent from proteins in circulation
6. Pregnancy
7. Gender

As a starting place for examining the influence (and possible uses) of susceptibility factors, we should first consider the base case; that is, what is the nature of the commonly used animal model?

THE DEFINED ANIMAL MODEL

Since at least the middle 1970s, it has become accepted practice in toxicology to use bred-to-purpose, genetically defined (usually inbred) and specific pathogen-free (SPF) experimental animals. Such animals are most commonly obtained from commercial vendors and put on study while they are young adults in the log phase of growth.

Festing (1979b), in his review of the development of these animal stocks, has called them "defined" animals in terms of their microbiological flora, genetic background, and environmental and nutritional care. It must be noted that the influence of these are further confounded (or possibly) accentuated by age and gender.

Renal Impairement and Genobiotic Metabolism and Toxicity

It should not be surprising that there is special interest in patient populations in which there is compromised function of eliminating or clearing organs (Brater et. al. 1992; Matzke and Milikin 1992). The organs of particular interest are the kidney and liver, the clearing organs primarily responsible for drug elimination. Impairment of either of those organs has implications in terms of drug accumulation and potential toxicity. There is need, therefore, to adjust dosing, especially for those drugs having a narrow therapeutic concentration range. The U.S. Food and Drug Administration (FDA) issued a draft guidance titled "Pharmacokinetics and Pharmacodynamics in Patients with Impaired Renal Function: Study Design, Data Analysis, and Impact on Dosing and Labeling."

It makes sense to examine the pharmacokinetics and pharmacodynamics of those xenobiotics primarily or substantially excreted unchanged via the kidney (e.g., lithium). It would also make sense to extend such studies to those compounds that are primarily metabolized by the liver but whose metabolites are predominantly excreted into the urine; the latter is a common pathway for many if not most metabolites. The concern here is with metabolites that are pharmacologically active or toxic, as such metabolites will accumulate in the presence of renal insufficiency (e.g., N-acetyl procainamide, the active metabolite of procainamide).

A further complication is that renal impairment is associated with other conditions that might have an impact on the pharmacokinetics and pharmacodynamics of the drug. These changes in, for example, pharmacokinetics might be the result of indirect effects. For example, in some renal diseases, plasma proteins could be excreted into the urine, resulting in a lowering of plasma protein concentration. The latter could influence (i.e., decrease) plasma protein binding (f_U increases), which might, in turn, influence (i.e., increase, for low-clearance drugs) the elimination of the drug by other clearing mechanisms (e.g., hepatic). It is apparent that one needs to have a reasonably good understanding of the pharmacokinetics of the drug, especially route of elimination, plasma protein binding, and clearance, to decide whether or not to conduct studies in renal-impaired subjects. The latter are complex, expensive, and time-consuming studies.

It is quite fortunate that we have a direct physiological measure of renal function that can be used in assessing the degree of renal insufficiency and can be used quantitatively to alter drug dosing regimens. That measure is creatinine clearance (CLCr). Creatinine is an end product of muscle metabolism that is exclusively excreted via the kidney. Although passive glomerular filtration appears to be the predominant excretory pathway, there have been some suggestions of active secretion. In either event, the compound serves as a good marker of renal function and it has an additional advantage; it is produced endogenously. As a consequence, it is not necessary to administer a test compound (e.g., inulin; a large molecular weight polysaccharide that is cleared exclusively by glomerular filtration). CLCr can be measured experimentally by obtaining a complete (and it must be complete) urine collection, generally for 24 hr, and a single blood sample to determine serum creatinine concentration. The latter is often determined at the beginning or middle of the urine collection period and one assumes that the serum concentration remains essentially constant during that time, which is generally a good assumption. One caution here: Some cooked meats (especially boiled beef) contain creatinine which, following absorption, increases urine creatinine excretion and results in an overestimate of CLCr (assuming that the serum creatinine concentration does not reflect this absorption).

It is unusual to actually measure CLCr in the subject for a variety of reasons, including inconvenience, errors in urine collection, and so on. It is more usual that we estimate renal function, CLCr, from a measure of serum creatinine concentration (Scr). The latter is quite convenient because it represents one of many biochemical measures routinely determined in a battery of blood chemistries. However, CLCr is not directly related to Scr and we need to rely on a relationship between the two that has been determined in studies of relatively large numbers of subjects. Such a relationship exists in several very similar forms often referred to as the Cockcroft -Gault or Siersback-Nielson relationships or equations. The Cockcroft-Gault equation is based on a study of about 200 subjects with varying degrees of renal function:

$$\mathrm{CLCr(ml/min)_{MALE}} = \frac{140\text{-age(yr)} \bullet \mathrm{Weight(kg)}}{72 \bullet \mathrm{S_{cr}(mg\%)}}$$

It is important that the correct units be used: age in years, weight (as lean or ideal weight) in kg, and Scr in mg%. The resulting clearance will have units of ml/min. The preceding equation is appropriate for males. For females the equation needs to be adjusted downward by multiplying by 0.85. The latter is on the basis of a smaller muscle mass in females.

$$\text{CLCr(ml/min)}_{\text{MALE}} = \frac{\text{140-age(yr)} \bullet \text{Weight(kg)}}{72 \bullet \text{S}_{\text{cr}}\text{(mg\%)}}$$

There remains some controversy concerning which weight to use: lean or ideal weights. The latter is a special problem in obese subjects. If the actual weight is less than ideal, use the actual weight. Some investigators use the following equations for estimating ideal body weight (IBW) in males and females:

$$\text{IBW}_{\text{MALE}}\text{(kg)} = 50 + [2.3 \times (\text{height in inches} - 60]$$

$$\text{IBW}_{\text{MALE}}\text{(kg)} = 45.5 + [2.3 \times (\text{height in inches} - 60]$$

Serum creatinine concentration and CLCr are related in a nonlinear fashion. A similar nonlinear relationship is also seen between the elimination half-life of a drug and CLCr.

A linear relationship will exist between K or CL of the drug and CLCr and it is those relationships that are most often used in adjusting a dosing regimen. The following relationship would be expected:

$$K = m \bullet \text{CLCr} + k_{NR} \qquad K = m \bullet \text{CLCr} + \text{CL}_{NR}$$

The relationships just noted provide a basis for the estimation of K or CL in a given subject. A value of Scr is obtained and from that an estimate of CLCr is calculated using the equations (or nomogram) noted previously. The value for CLCr is then entered into one of the preceding relationships to estimate K or CL and from which a value of half-life is obtained. The information is then used to calculate an individualized dosing regimen for the subject with renal impairment. Alternatively, one can locate tables that contain values for K or CL for normal and anephric subjects (and corresponding values for CLCr). From those two values one can estimate a slope and intercept and use that information to estimate K or CL in the subject of interest whose value for CLCr has been estimated.

The more substantial the renal excretory process relative to all pathways of elimination (i.e., the larger the ratio, CLR/CLs), the greater the impact of renal insufficiency on the pharmacokinetics of the drug.

There are several complications that need to be considered here with regard to the influence of renal impairment on the pharmacokinetics of drugs. These considerations are in addition to the obvious direct effect of renal impairment on the elimination of the parent drug, as discussed earlier.

One consideration is the accumulation of active or toxic metabolites of the parent drug that would normally be excreted via the kidney. Such metabolites will accumulate in renal insufficiency because they will be renally cleared less efficiently. Examples include the parent and metabolite pairs shown in table 14.2.

Table 14.2 Parent and Metabolite Pairs

Parent Drug	Metabolite
Allopurinol	Oxipurinol
Cilazapril	Cilazaprilat
Meperidine	Normeperidine
Primidone	Phenobarbital
Procainamide	N-acetylprocainamide
Proxyphene	Norpropoxyphene
Ofloxacin	Desmethylofloxacin

The dosing regimen of the parent drug might need to be reduced as a consequence of the accumulation of the active metabolite.

An additional complication is the alteration in the plasma protein binding of drugs as a result of two mechanisms. During renal insufficiency, endogenous waste materials will accumulate and such compounds have the potential to compete with drugs for protein binding sites (e.g., fatty acids). Plasma protein concentrations can be reduced in association with renal diseases such as uremia and nephrotic syndrome. A reduction in protein concentration will result in a decrease in the number of binding sites and this could result in reduced drug binding. The implications of this effect will depend on the drug, especially its value of clearance. A good example is phenytoin, which is highly bound to albumin and is completely metabolized by the liver. If the drug has a low (restrictive) clearance, such as phenytoin, f_U will increase and there will be a corresponding increase in clearance because $CL \approx f_U \bullet CL_{u,i}$. Most important, however, there will be no change in the unbound concentration of phenytoin, even though the total concentration declines:

$$C_T \downarrow = \frac{\text{dose rate}}{\uparrow f_U \cdot CL_{U,i}}$$

$$C_T \leftrightarrow = \frac{\text{dose rate}}{CL_{U,i}}$$

Alteration in plasma protein binding would suggest that the apparent volume of distribution might increase or be minimally changed. There is at least one drug example of a decrease in apparent volume of distribution in renal insufficiency, digoxin. Digoxin has a very large apparent volume of distribution (ca. 10 Ukg), indicating that most of the drug is outside of the vascular region. The drug binds extensively to muscle tissue. It appears that the latter binding process is altered during renal insufficiency, resulting in digoxin movement from the tissue into the blood. The latter results in a smaller apparent volume of distribution. The only clinical significance that this has is in the calculation of a loading dose for the drug (it must be reduced).

A somewhat more complex and far more limited situation has been referred to as *futile metabolism*. In this instance the metabolite formed is unable to be excreted into the urine because of renal insufficiency. However, if the metabolite can undergo reversible metabolism to the parent drug, the drug will have a prolonged residence time in the body; the metabolic step is futile in terms of getting rid of the substance. An example appears to be acyl-glucuronide metabolites (e.g., those of clofibrate, diflunisal, and some arylpropionic nonsteroidal anti-inflammatory agents). The paradox is the observation that the parent drug, which undergoes metabolism, accumulates in the presence of renal insufficiency, even though metabolic function has not been altered.

The FDA Guidance offers several possible study designs depending on the characteristics of the drug under study. A full study is recommended if the drug is excreted primarily by the kidney. There are four groups to be studied. The control group is the typical patient population or equivalent. A normal healthy group can be included but not to replace the control group. The study design can be single dose (if linear kinetics) or multiple dose (if nonlinear kinetics). A concentration-controlled study is also acceptable. Unbound and total plasma concentrations should be determined (if highly bound) and CLCr should be measured. A sufficient number of subjects should be selected to determine if there is a pharmacokinetic difference necessitating dose adjustment. The groups suggested are shown in table 14.3.

Alternatively, it might be acceptable to perform a reduced study in only two groups (groups 1 and 4 in table 14.3). If no pharmacokinetic differences are found, no further study is necessary. A population-based pharmacokinetic study might also suffice, if there is a sufficiently wide range of renal function within the population.

Table 14.3 Suggested Groups for Renal Study

Group	Renal Impairment	% Normal Renal Function
1	Controls	> 80%
2	Mild	40%–80%
3	Moderate	10%–40%
4	Severe	< 10%

A study in hemodialysis or peritoneal dialysis patients would not be necessary if the drug has an unbound apparent volume of distribution greater than about 350 L. Drugs of the latter ilk undergo very poor dialysis (i.e., less than about 10% removal during typical dialysis).

Renal excretion changes dramatically with aging. Glomerular filtration as measured by inulin clearance (A) decreases with age as does creatinine clearance (not shown). Tubular excretory capacity (B), renal blood flow (C), and renal plasma flow (D) all decline with age. Interestingly, if all of those values are plotted as a percentage of the value at age 20 to 29, the lines almost superimpose. This suggests that all aspects of renal function decline in parallel with age in the absence of disease states.

Some care needs to be taken in interpreting serum creatinine clearance in the elderly and its relationship with CLCr, the latter being the better estimate of renal function. Serum creatinine concentration remains relatively constant with age (in the absence of renal disease). The following equation relates steady-state creatinine serum concentration to CLCr and, as with all similar steady-state equations, it is a function of rate in or production rate (numerator) and rate out or clearance (denominator),

$$\text{Serum Creatinine Concentration} = S_{CR} = \frac{\text{creatinine production rate}}{\text{creatinine clearance}} = \frac{\text{production rate}}{CL_{CR}}$$

The only way that S_{CR} could remain constant in the face of a decreasing CL_{CR} is if the production rate declines in parallel with clearance. In fact, this is quite likely on the basis that creatinine is an end product of muscle metabolism. Because muscle mass declines with age it is reasonable to expect that the production rate of creatinine would decline.

The general conclusion is that renal function declines with age.

Hepatic Impairment: Xenobiotic Metabolism and Toxicity

Unfortunately, and unlike the situation with renal impairment, there is no reliable quantitative endogenous measure of liver function that one can relate to drug metabolism, such as CL_H or ER_H. CL_{CR}, which is an excellent index of renal function, quantitatively correlates with measures of drug excretion, such as CL_R, CL_S, or K. The search for an endogenous biochemical marker that would quantitatively relate to hepatic metabolic efficiency has been ongoing but, for the most part, unsuccessful. Currently we are able to use the metabolism of certain probe markers or model compounds to assess the relative efficiency of certain enzyme systems. For example, the results of metabolic tests using select probe compounds that undergo metabolism via specific isozymes of the cytochrome P-450 system will permit placing subjects into phenotype groups of rapid or slow metabolizers. The former is more an "all-or-none" assessment; there is no continuous measure of enzyme activity on a relative scale. The rapid developments in molecular biology and the area called pharmacogenomics hold out the promise that such an assessment could be achieved through DNA testing.

It is difficult to make generalizations concerning the influence of hepatic impairment on drug disposition for several reasons, including the fact that there is no reliable quantitative index for measuring hepatic function. With regard to hepatic disease states, there are acute versus chronic hepatic disease conditions and these conditions vary according to the effect that they have on hepatic function. The term *hepatic impairment* might imply one cause or one effect, but in fact there are

numerous factors involved. In terms of metabolic processes, there are a wide variety of isozymes with different intrinsic enzymatic activities and different cofactors and variables that influence activity. Each drug–isozyme pair will have a different set of enzymatic parameter values for metabolism (i.e., k_m and V_{MAX} or $CL_{U,i}$). It is not surprising, therefore, to find conflicting data, making it difficult to reach unequivocal conclusions about liver disease and its effects on drug disposition.

Hepatic diseases will exert an effect on drug metabolism only when impairment results in measurable changes in endogenous biochemical factors (e.g., serum albumin levels). Table 14.4 lists a variety of biochemical parameters that change in response to liver disease and the resulting potential change in xenobiotic disposition.

Table 14.4 Biochemical Changes and Potential Effects on Drug Disposition (Brouwer et al. 1992)

Biochemical Measurement	Physiologic/Pathologic Alteration	Potential Pharmacokinetic Alteration	Problems in Interpretation
Prothrombin time	Acute ↓protein synthesis	↓ metabolism	Low vitamin K
Serum albumin	Chronic ↓protein synthesis	↓ metabolism, ↓ protein binding; ↑ Vd	Poor nutrition
Serum bilirubin Conjugated (direct)	Cholestasis	↓ biliary elimination of drugs	Prolonged elevations despite return of normal function
Unconjugated (indirect)	Hepatocyte dysfunction or ↓ extraction from blood	↓ metabolism	Hemolysis
Serum alkaline phosphatase	Cholestasis	↓ biliary elimination of drugs	↑ production
Serum ammotransferase [Alanine (ALT)] [Aspartate (AST)]	Hepatocyte damage	↓ metabolism	Normal in chronic disease; high elevations in acute disease might not reflect hepatic malfunction.

Table 14.5 presents the most frequently used classification system for assessing the degree of liver impairment. Severity increases from Grade A to C from 1 point to 3 points per test (i.e., 5–6 points = mild impairment; 7–9 = moderate impairment; more than 9 = severe impairment). These scales are useful clinically for following the progression of the disease but they offer little in terms of quantifying alterations in pharmacokinetic parameters.

Table 14.5 Classification Systems Used to Characterize Liver Impairment (Brouwer et al. 1992)

Child: Turcotte Classification			
	Grade A	**Grade B**	**Grade C**
Bilirubin (mg/dL)	< 2.0	2.0–3.0	> 3.0
Albumin (gm/dL)	> 3.5	3.0-3.5	< 3.0
Ascites	None	Easily controlled	Poorly controlled
Neurological disorder	None	Minimal	Advanced
Nutrition	Excellent	Good	Poor
Pugh's Modification of Child's Classification[a]			
	1 Point	**2 Points**	**3 Points**
Encephalopathy (grade)	None	1 or 2	3 or 4
Ascites	Absent	Slight	Moderate
Bilirubin (mg/dL)	1–2	2–3	> 3
Albumin (gm/dL)	> 3.5	2.8–3.5	< 2.8
Prothrombin time (sec > control)	1–4	4–10	> 10

[a] 5–6 total points = mild dysfunction: 7–9 = moderate dysfunction: > 9 = severe dysfunction.

Liver disease can influence absorption and drug disposition by altering the primary parameters: unbound intrinsic clearance, $CL_{u,i}$; unbound plasma fraction, f_U; and liver blood flow, Q_H. Alteration in those parameters can then produce measurable changes in several absorption and disposition parameters, as shown in table 14.6.

Table 14.6 Primary Parameter Alterations and Effect on Pharmacokinetic Parameters

Altered Primary Parameter	Pharmacokinetic Parameter Effected
CLu,I	CLs $\rightarrow T_{1/2}$ (restrictive clearance)
	$ER_H \rightarrow F$ (nonrestrictive clearance)
f_U	$V \rightarrow T_{1/2}$
	CLs $\rightarrow T_{1/2}$
Q_H	CLs $\rightarrow T_{1/2}$ (nonrestrictive clearance)

Table 14.7 summarizes the pharmacokinetic changes for a number of (low) restrictively cleared compounds during liver disease (mostly cirrhosis). The healthy control values are listed in parentheses. In most cases the value for CLs decreases and $T_{1/2}$ increases (due to a decrease in $CL_{U,i}$). The latter might in part be due to an increase in apparent volume of distribution. In some cases, notably compounds undergoing phase II conjugation metabolism (e.g., lorazepam, oxazepam), there is no change in CL_S. In at least one case, tolbutamide, there is an increase in CL_S, which is probably a result of an increase in f_U.

Antipyrine is a frequently used marker for CYP 450 oxidative metabolism because it is completely metabolized, not plasma protein bound, and has a low clearance. For the influence of various liver diseases on antipyrine $t_{1/2}$, the normal value of about 10 hr increases markedly to more than 25 hr for all liver disease states. Note, however, there is an extremely wide variation in the values (from about 10 hr to more than 50 hr). One reason for this is the lumping together of all liver disease states into one category. The average values for $t_{1/2}$ depend on the specific disease state, but variation remains wide in all cases. Clearance would be a better parameter to compare. Variability remains very high in both the control and disease groups and the raw data indicate considerable overlap of the clearance values. If the clearance values are normalized for estimates of liver volume, both variability and overlap decrease considerably.

Reduced enzyme activity, as measured by $CL_{u,i}$, would be expected to decrease the hepatic clearance of low or restrictively cleared drugs, as noted previously. In contrast, such changes should have no influence on the clearance of high or nonrestrictively cleared drugs. The clearance of such drugs will be influenced by changes in liver blood flow, Q_H, which is associated with many liver disease conditions. Table 14.8 summarizes the data for a number of high-clearance drugs. Clearance is reduced in most all cases and this is a reflection of altered blood flow.

Note also that bioavailability increases substantially in all cases. This is a reflection of a reduction in $CL_{u,i}$. A reduction in $CL_{u,i}$ results in a reduction in the hepatic extraction ratio, which in turn results in a decrease in the hepatic first-pass effect. In other words, the absolute oral bioavailability (F) increases. This will be most dramatic for drugs having a very high hepatic extraction ratio. Thus, if ER_H is 0.95 in normals and the value changes to 0.90 (less than a 5% decrease) in liver disease, the value for bioavailability (F) increases twofold, from 0.05 to 0.10. (Recall that, $F = 1 - ER_H$). An additional part of this increase in F is due to the existence of portal shunts that permit some of the absorbed dose to go directly from the GI tract into systemic circulation. The propranolol and nicardipine concentrations following oral dosing in cirrhosis patients are much higher than those in normals.

Table 14.7 The Influence of Liver Disease on the Pharmacokinetics of (Low) Restrictively Cleared Drugs (Williams 1984)

Drug	Disease	Volume	$T_{1/2}$	Clearance
Ampicillin	C	59.1 ± 43.1 liters	1.90 ± 0.56 hr[a]	280 ± 136 ml/min
	C	(19.5 ± 4.6) (V_{ss})	(1.31 ± 0.15)	(342 ± 80)
Chloramphenicol		49.9 ± 4 liters[a]	10.45 ± 1.14 hr[a]	59.2 ± 8.4 ml/min[a]
		(65.9 ± 4) (V_{ss})	(4.6 ± 0.3)	(168.6 ± 9)
Chlordiazopoxide	C	428 ± 108 ml/kg	40.1 ± 5.1 hr[a]	7.6 ± 1.08 ml/kg/hr
		(321 ± 77) (V)	(16.5 ± 3.6)	(13.8 ± 1.2)
	C	0.48 ± 0.14 liter/kg	62.7 ± 27.3 hr[a]	7.7 ± 2.1 ml/min[a]
		(0.33 ± 0.06) (V_{ss})	(23.8 ± 11.6)	(15.4 ± 4.4)
Cimetidine	L	1.4 ± 0.6 liters/kg	2.9 ± 1.1 hr	463 ± 145 ml/min
		(1.1 ± 0.4) (V)	(2.3 ± 0.7)	(511 ± 93)
Diazepam	C	1.74 ± 0.21 liters/kg[a]	105.6 ± 15.2 hr[a]	13.8 ± 2.4 ml/min
		(1.13 ± 0.28) (V)	(46.6 ± 14.2)	(26.6 ± 4.1)
Flurosemide	C	533 ml/kg[a]	2.2 hr	192 ml/min
		(210) (V)	(0.79)	(194)
	C	12 ± 3.5 liters	129 ± 75 min	120 ± 36 ml/min
		(9.3 ± 3.7) (V_{ss})	(74 ± 18)	(142 ± 42)
Hexobarbital	C	1.14 ± 0.26 liter/kg	509 ± 174 min[a]	1.88 ± 0.70 ml/min/kg[a]
	(compensated)	(1.25 ± 0.24)(V)	(340 ± 140)	(3.32 ± 0.99)
	C	1.57 ± 0.64 liters/kg	1.017 ± 450 min[a]	1.26 ± 0.49 ml/min/kg[a]
	(uncompensated)	(1.25 ± 0.24) (V.)	(340 ± 110)	(3.32 ± 0.99)
Lorazepam	C	2.01 ± 0.82 liters/kg	31.9 ± 9.6 hr[a]	0.81 ± 0.48 ml/min/kg
		(1.28 ± 0.34) (V)	(22.1 ± 5.4)	(0.75 ± 0.23)
	AVH	1.52 ± 0.61 liters/kg	25.0 ± 6.4 hr	0.74 ± 0.34 ml/min/kg
		(1.2 ± 0.34) (V)	(22.1 ± 5.4)	(0.75 ± 0.23)
Oxazepam	C	60.9 ± 9.5 liters	5.8 ± 1.1 hr	155.5 ± 70.4 ml/min
		(61.2 ± 12.2) (V)	(5.6 ± 0.8)	(136.0 ± 46.3)
	AVH	51.7 ± 17.2 liters	5.3 ± 0.7 hr	137.4 ± 51.4 ml/min
		(47.7 ± 16.7)(V)	(5.1 ± 1.3)	(113.5 ± 30.7)
Prednisolone	CAH	69 ± 13 liters	3.0 ± 1.0 hr	278 ± 79 ml/min
		(70 ± 8) (V)	(3.3 ± 1.0)	(256 ± 56)
Rantidine	C	115 ± 32 liters	166 ± 41 min	476 ± 139 ml/min
		(106 ± 35)	(124 ± 16)	(543 ± 126)
Theophylline	C	0.563 ± 0.08 liter/kg	28.8 ± 14.3 hr[a]	18.8 ± 11.3 ml/hr/kg[a]
		(0.482 ± 0.08) (V)	(6.0 ± 2.1)	(63.0 ± 28.5)
Tolbutamide	C	0.15 ± 0.03 liter/kg	4.0 ± 0.9 hr[a]	26 ± 5.4 ml/hr/kg[a]
		(0.15 ± 0.03) (V)	(5.9 ± 1.4)	(18 ± 2.8)
Warfarin	AVH	0.19 ± 0.04 liter/kg	23 ± 5 hr	6.1 ± 0.9 liters/hr
		(0.21 ± 0.02)(V)	(25 ± 3)	(6.1 ± 0.7)

Note: C = cirrhosis; L = type of liver disease not cited; AVH = acute viral hepatitis; CAH = chronic active hepatitis.

Table 14.8 The Influence of Liver Disease on the Pharmacokinetics of (High) Nonrestrictively Cleared Drugs (Williams 1984)

Drug	Extraction Ratio	Disease	Bioavailability (% Change)	Volume[a]	$T_{1/2}$	Clearance
Chlormethiazole	0.9	C	+1,000		8.7 ± 4.0 hr (6.6 ± 2.4)[b]	12.8 ± 4.8 ml/min/kg[c] (18.1 ± 29)
Labetalol	0.7	C	+91	526 ± 31 liters[c] (805 ± 91) (V_{area})	170 ± 24 min (187 ± 26)	
Lidocaine	0.7	C		2.22 ± 0.94 liters/kg (1.70 ± 0.21) (V_{area})	343 ± 234 min[c] (108 ± 70)	5.2 ± 2.1 ml/min/kg[c] (9.2 ± 0.8)
		AVH		310 ± 180 liters/kg (2.00 ± 0.5) (V_{ss})	160 min (90)	13.0 ± 3.9 ml/min[c] (20.0 ± 3.9)
Lorcainide		C			12.5 ± 4.5 hr (7.7 ± 2.0)	814 ± 144 ml/min[c] (1,002 ± 304)
Meperidine	0.5	C	+81	263 ± 28 liters (232 ± 53) (V_{ss})	359 ± 77 mind (213 ± 25)	523 ± 158 ml/min[c] (900 ± 316)
		AVH		5.56 ± 1.8 !iters/kg (5.94 ± 2.65) (V)	6.99 ± 2.74 hr[c] (3.37 ± 0.82)	649 ± 228 ml/min[c] (1,261 ± 527)
Metoprolol	0.15	C	+65	4.0 ± 0.3 liters/kg (3.2 ± 0.2) (V)	7.2 ± 1.2 hr (4.2 ± 1.1)	0.61 ± 0 13 liter/ min (0.01 ± 0.11)
Morphine	0.6–0.8	C		23 ± 1 3 liters/kg (2.9 ± 2 4) (V_{ss})	2.2 ± 1 3 hr (25 ± 1.5)	1,153 ± 345 ml/min[c] (1,233 ± 427)
Pentazocine	0.8	C	+278	356 ± 94 liters (415 ± 107) (V_{area})	396 ± 115 min[c] (230 ± 28)	675 ± 296 ml/min (1.246 ± 236)
Propranolol	0.6	C	+42	380 ± 41 liters[c] (290 ± 17) (V_{area})	11.2 ± 12 hr (4.0 ± 0.3)	580 ± 140 ml/min (860 ± 90)
Verapamil	0.87	C		481 ± 141 liters[c] (296 ± 67) (V_{ss})	815 ± 516 min[c] (170 ± 72)	0.545 ± 0.181 liter/min[c] (1.571 ± 0.405)
		C	+140[d]	9.17 liters/kg (6.15) (V_{ss})	840 min (220)	1.22 liter/min (1.26)

Note: C = cirrhosis; AVH = acute viral hepatitis

[a] V_{ss}, volume of distribution at steady state; V = volume of distribution (one compartment); V_{area} clearance $\times$ 0.693/$T_{1/2.}$

[b] Numbers in parentheses indicate values observed in healthy controls.

[c] Statistically significant differences between patients and healthy controls.

[d] Because of a sixfold variability, caution is suggested in interpretation of this change in bioavailability.

The increased apparent volume noted for a number of compounds in table 14.9 is a reflection of the altered (reduced) plasma protein binding of those compounds. The increase in the unbound fraction, f_U, is the result of decreased plasma protein concentrations (especially albumin) or the accumulation of endogenous compounds that would normally be eliminated by hepatic metabolism. The latter compounds might compete with drug for protein binding sites. Table 14.9 lists the percentage increase in the unbound fraction to plasma proteins for a variety of drugs. Binding either decreases or does not change, as noted in the table.

Table 14.9 Increase in the Unbound Fraction to Plasma Proteins in Liver Disease (Williams 1984)

Drug	Diseases	Percentage Increase in Fraction Unbound
Highly extracted drugs		
Udocaine	AVH	No change
Meperidine	AVH	No change
Morphine	AVH/C	15
Propranolol	AVH/C	38
Poorly extracted drugs		
Amobarbital	AVH/C	38
Azapropazone	CAH/C	477
Diazepam	C	210
Diazepam	C	65
Phenylbutazone	C	400
Phenylbutazone	AVH/C	500
Phenytoin	AVH	33
Phenytoin	C	40
Quinidine	C	300
Tolbutamide	AVH	28

Note: AVH = acute viral hepatitis; C = cirrhosis; CAH = chronic active hepatitis.

Hepatic metabolism and aging is very difficult to discuss as there do not appear to be any general rules. The most likely reason for this, unlike renal function, is that there are a host of factors that influence the efficiency of drug metabolism (e.g., genetics, nutrition, drugs, disease, live size, etc.). There is huge variation among the population for hepatic clearance at any given age. It is not unusual to find a tenfold range of clearances among otherwise normal, healthy subjects at a given age. On that basis it is difficult, if not impossible, to tease out the effect of age per se on hepatic clearance (Rowland and Tozer 1995)

The general impression is that hepatic drug clearance either decreases or remains unchanged with age at least for phase I metabolic processes. Although not well proven, it appears that age has less of an effect on those compounds that undergo phase II metabolism (i.e., conjugation).

Impaired GI Absorption

GI absorption has been claimed for many years to be impaired in the elderly. There is simply no good evidence for this and, in fact, the statement has been made because of poor experimental design and incorrect interpretation of data. There are definitely many changes in the gut that could effect absorption efficiency, including the following:

- GI fluid pH
- GI fluid contents
- Gastric emptying rate
- Intestinal transit rate

- GI blood flow
- GI surface area and "membrane" characteristics
- Nutritional intake and eating habits
- Age-related drug ingestion altering physiology or affecting absorption of other drugs
- Age-related GI disease

There is not very good information about a number of these factors. There is a greater incidence of achlorhdria (lack of acid secretion in the stomach) that could have some implications in terms of drug dissolution and drug stability, but at present this condition does not appear to be an important clinical issue. Gastric emptying rate and intestinal transit rate are expected to decrease with age; there is the general thought that gut activity slows with aging. This is difficult to conclude, however, and the data shown in table 14.10, which are the only data available, are directly conflicting. In one case there is no change and in the other the elderly empty more slowly than younger subjects. Even less is known about intestinal transit rates but, once again, the prevailing thought is that they decrease with age.

Table 14.10 Two Studies That Have Examined the Influence of Age on Gastric Emptying Half-Time (in Minutes, Inversely Related to Emptying Rate)

	Age, Years		Liquid Meal		
n	**M**	**Range**	**M**	**Range**	
	26	23–31	50	21–132	
	77	72–86	123	67–4,541	$p < .001$

	Age, Years		Liquid Phase		Solid Phase	
n	**M**	**Range**	***M***	***SEM***	**M**	**SEM**
10	31	24–51	68	7 ns	104	10 ns
10	76	71–88	94	13 ns	105	17 ns

Note: n = number of subjects; SEM = standard error of the mean; ns = not significantly different

One of the most interesting stories concerns the use of d-xylose for the estimation of GI absorption. That carbohydrate is often used to assess the presence of malabsorption syndromes, especially in the case of sprue. It has also been applied to the elderly and pediatric populations. Unfortunately, the data have been totally misinterpreted and this has led to the general statement about impaired absorption with aging. The basis of the method is quite simple: Ingest a 5- or 25-g dose of d-xylose and collect urine for 5 hr. The amount excreted is then compared to a normal range. In reviewing the literature one can plot, from a number of different studies, the percentage dose recovered in 5 hr as a function of age. Note the lines decrease, suggesting impaired absorption with age. However, it is very difficult to explain line A, which is obtained following an IV dose of d-xylose; it also declines with age! The only explanation for this is not a decrease in absorption (after all, absorption is complete, 100%, after IV dosing) but a reduction in elimination. The latter makes sense, when we realize that, as discussed later, renal function declines with age and that we are therefore obtaining an incomplete urine collection.

The general conclusion with regard to GI absorption is that the rate of absorption might decline with age but there are no differences in the extent of absorption as a function of age. The only exceptions to the latter rule are drugs that have a high hepatic extraction ratio and undergo substantial first-pass metabolism. For such drugs, absorption might increase with age as a result of reduced hepatic clearance.

There are virtually no data about the influence of age on drug absorption by other routes of administration such as intramuscular, transdermal, pulmonary, and so on.

Distribution might be altered as a result of changes in plasma protein concentration, changes in anatomy, and blood flow differences. Changes in plasma protein binding can result from this

observation, but it might also occur due to the use of many drugs by the elderly and the consequent interaction in displacing one compound by one or more other drugs.

Another major change that occurs with aging is the relative body content of water and adipose tissue. In both males and females the relative percentage of weight that is adipose tissue increases with age, whereas water content (or lean body mass) declines with age.

Another important issue in drug distribution onvolves blood flow. Age per se does not appear to alter cardiac output. However, among the population in which there is substantial coronary artery disease, there appears to be an inverse relationship with age. One needs to first define the question to provide a correct answer. The conclusions with regard to drug distribution and aging are as follows:

- Plasma protein binding either decreases or does not change with age.
- Clearance could change (increase) as a result of altered binding (low-clearance drugs).
- Apparent volume of distribution will increase for lipid-soluble drugs and decreases for water-soluble drugs.
- Half-life could change as a result of a change in apparent volume.
- Blood flow decreases in most elderly subjects and this can alter the clearance of nonrestrictively cleared drugs.

Pregnancy

There are numerous and substantial changes that occur during pregnancy and, although there is not a great deal of quantitative information with regard to drug disposition and response, several of these factors can be discussed. There is little quantitative information with regard to the effect of pregnancy on the efficiency of the GI absorption process or, for that matter, absorption by other routes of administration. Several changes in the GI tract do occur, however, including a reduction in gastric acid secretion, an increase in mucous secretion, and a slowing in gastric emptying and intestinal motility. Any of these changes can affect the drug dissolution and absorption processes, at least in theory, but as yet we do not have the data to indicate the existence of such alterations in absorption. Increased peripheral blood flow might also suggest more rapid absorption following intramuscular or subcutaneous and, perhaps, transdermal dosing.

There are considerable anatomical changes that occur during pregnancy and might affect drug distribution. This increase in weight in terms of fluid volumes, blood, and fat is illustrated in table 14.11.

Table 14.11 Change in Blood Distribution During Pregnancy

Parameter	Late Pregnancy	Nonpregnant State	Increase (%)
Blood volume (mL)	4,820	3,250	48
RBC volume (cells/mm^3)	1,790	1,355	32
Hematocrit (%)	37.0	41.7	

Note: RBC = red blood cell.

Source: Pritchard (1965).

The increased volumes of both water and fat will likely increase the apparent distribution space for water-soluble and lipid-soluble drugs. An additional consideration, however, is the distribution space afforded by the fetus (assuming the drug can traverse the placenta) and breast milk (assuming the drug can undergo mammillary transfer).

The other major factor that can affect distribution volume as well as clearance (and, therefore, half-life) is plasma protein binding. As with other factors, one must consider the time course of any possible change over the normal duration of pregnancy. Serum albumin concentration declines and, for many of the drugs studies to date, plasma protein binding also decreases, resulting in a greater fraction of unbound drug.

There are substantial changes in hemodynamic functions such as cardiac output and blood flow to different body regions, as shown in table 14.12.

Table 14.12 Hemodynamilc Parameters Throughout Pregnancy

	Position	1st Trimester	2nd Trimester	3rd Trimester	Postpartum
Heart rate (beats/min)	L	77 ± 2	85 ± 2	88 ± 2	69 ± 2
	5	76 ± 2	84 ± 2	92 ± 2	70 ± 2
Stroke volume ml/min)	L	75 ± 3	86 ± 4	97 ± 5	79 ± 3
	S	82 ± 5	85 ± 4	87 ± 5	79 ± 3
Cardiac output 1/mm/m^2	L	3.53 ± 0.21	4.32 ± 0.22	4.85 ± 0.27	3.30 ± 0.17
	S	3.76 ± 0.24	4.19 ± 0.21	4.54 ± 0.28	3.33 ± 0.21
Left ventricular election time (msec)	L	302 ± 2	290 ± 5	281 ± 4	310 ± 5
	S	301 ± 3	286 ± 4	260 ± 4	307 ± 5
Systolic blood pressure (mmHg)	L	98 ± 2	91 ± 2	95 ± 2	97 ± 2
	S	106 ± 2	102 ± 2	106 ± 2	110 ± 2
Diastolic blood pressure (mmHg)	L	53 ± 2	49 ± 2	50 ± 2	57 ± 2
	S	57 ± 2	60 ± 1	65 ± 2	65 ± 1

Note: L = lateral: S = supine.

The implications of these changes in terms of drug distribution have not been clearly delineated but the findings suggest that there would be an increase in distribution volume during pregnancy that then returns to normal sometime following birth of the child. There is a very dramatic increase in renal function as judged by estimates of glomerular filtration (e.g., creatinine and inulin clearances). This is illustrated in table 14.13.

Table 14.13 Changes in Kidney Function in Pregnancy

Time	Renal Plasma Flow (ml/min)	Glomerular Filtration Rate (ml/min)
13.0 weeks pregnancy	804.67	161.33
20.8 weeks pregnancy	749.13	157.11
38.0 weeks pregnancy	589.00	146.00
20.0 weeks postpartum	491.00	100.00
80.0 wk postpartum	549.00	97.00

This increase in renal function has its direct counterpart in the renal clearance of drugs that undergo excretion by the kidney. Ampicillin, for example, is primarily excreted unchanged by the kidney and has a greater clearance in the same women during pregnancy (613 ml/min) compared to the value after giving birth (394 ml/min). Elimination half-life is also shorter and the apparent volume of distribution is larger.

Findings similar to these have been made for other water-soluble antibiotics (e.g., cephalosporins) and digoxin, among other renally excreted drugs. Therefore, it is very likely that larger than usual doses of such drugs will need to be given to pregnant women to achieve the desired steady-state plasma concentration and response.

As noted earlier in the discussion of age, it is far more difficult to address the issue of drug metabolism because of the large number of variables that affect hepatic metabolism and, once again, there are only a limited number of studies available. Two studies examined the difference in disposition of the drug metoprolol in the same women during and after pregnancy. These results are summarized in table 14.14.

These data suggest that there is an increase in hepatic metabolic activity during pregnancy, at least for the enzyme system responsible for metoprolol metabolism. General conclusions concerning the metabolism of other drugs during pregnancy are far from being unequivocal. Metabolic clearance

Table 14.14 Metoprolol Absorption and Disposition Parameters in Women During and Following Pregnancy

Parameter	Pregnant	Nonpregnant
Oral dose (100 mg)		
CL_o (ml/min/kg)	362	82
$T_{1/2}$ (hr)	1.27	1.70
IV dose (10 mg)		
CL_s (L/min)	1.38	0.65
V (L/Kg)	6.87	3.85
$T_{1/2}$ (hr)	5.38	5.36
Oral dose (100 mg)		
CL_o (L/min)	9.56	1.71
F	0.21	0.42

Note: Studies done in 5 women during third trimester of pregnancy and 3 to 6 months after giving birth.

is affected by several factors, some of which have already been noted. First, intrinsic hepatic or metabolic clearance might change in response to, for example, hormonal alterations during pregnancy. In fact, it appears that many if not all of the changes in metabolic efficiency are related to hormonal activity, as will be noted later when we consider the influence of oral contraceptives on drug metabolism. Furthermore, the effects of hormones on drug metabolism are not always clear-cut but often appear contradictory. The latter point probably reflects the fact that there are other factors, currently not well recognized or understood, that have an impact on the overall metabolic disposition of drugs. For example, an increase in testosterone concentrations increases hepatic microsomal enzyme activity, thereby enhancing the rate of hepatic metabolism of some drugs. In contrast, progesterone and estradiol can act as inhibitors of certain enzymatic processes and thus reduce the rate of metabolism.

The other factors that play a role in hepatic clearance are plasma protein binding and hepatic blood flow. The former has already been discussed and, assuming a general decrease in binding during pregnancy, one would expect an increase in hepatic clearance. Liver blood flow, on the other hand, does not appear to be dramatically affected during pregnancy and, therefore, should have a minimal effect on drug metabolism.

The preceding issues have important ramifications in terms of appropriate drug dosing during pregnancy to maintain the desired therapeutic response and minimize adverse, toxic effects. One can make a good argument for therapeutic drug monitoring for those drugs with a narrow therapeutic range along with careful, continued monitoring of the response to the drug. Making the foregoing issues more complicated are concerns for the fetus, which is exposed to the drugs that the mother is taking. To minimize adverse effects on the fetus, it might be necessary to select a drug that undergoes minimal placental transfer or exerts little effect on the fetus. An additional complication arising after birth is the mammillary transfer of drug and subsequent exposure of the breastfeeding infant.

We have long known that there are certain cycles or rhythms that biological systems undergo that can have a dramatic affect on various aspects of the system. The term given to this area of study is *chronobiology* and it has received considerable interest in recent years. The menstrual cycle is a good example of chronobiology, yet its implications are poorly understood with regard to drug disposition and pharmacologic response. It has been only in very recent years that we have begun to learn of the impact that menstruation might have on the outcome of therapy.

It is not clear whether or not GI absorption is altered during the menstrual cycle as this process has not been thoroughly studied. One well-designed study that examined the oral absorption of d-xylose indicates no significant differences during the follicular (days 4 and 5), ovulatory (days 16 and 17) or luteal (days 23 and 24) phases.

A 20% increase in creatinine clearance has been reported between the beginning and end of the menstrual cycle, which is consistent with the approximate 24% increase in d-xylose renal clearance. In contrast to these findings, however, no changes were noted in creatinine clearance and tobramycin pharmacokinetics during the corresponding phases of the menstrual cycle in another study. As a result, and because there is a lack of other basic studies, one cannot reach a general conclusion concerning the changes in renal function during the menstrual cycle.

Unfortunately, but not surprisingly, similar disparities exist with regard to consideration of drug metabolism during the menstrual cycle. To date there have been at least two different studies that have examined the pharmacokinetics of the model compound antipyrine during different times of the menstrual cycle. The results of both of these studies suggest that there is no time-dependent alteration in antipyrine metabolism during the menstrual cycle.

One positive finding is the results of a study that examined methaqualone pharmacokinetics during days 1 and 15 of the cycle and these results are shown in table 14.15. There is a very dramatic difference in the values for clearance and half-life and, although the mechanism(s) for this alteration is not currently known, the investigators suggest a hormonal action.

Table 14.15 Pharmacokinetic Parameters of Methaqualone During the Menstrual Cycle

	Day of Cycle	
Parameter	**Day 1**	**Day 15**
Half-life (hr)	16.3	11.6
Clearance (mL/min.kg)	1.72	3.20
Volume (L/kg)	2.12	2.84

Influence of Gender on Xenobiotic Absorption, Disposition, and Toxicity

It has become quite clear in recent years that women's health and health issues have not been adequately studied. As a result, we understand far less about the diagnosis of illness and its treatment in women than in men. For the same diagnosis, women are less likely than men to receive important diagnostic or therapeutic modalities (e.g., renal transplantation, cardiac catheterization). The issue, however, is not so straightforward and is complicated by several factors such as age associated with heart disease (earlier age in men than in women) and the dangers of certain therapeutic maneuvers (e.g., catheterization) at those ages. It is important to recognize that the medical treatment for women is based on a male model: The results of medical research on men are generalized to women without sufficient evidence of applicability to women. This statement is best supported by consideration of the large-scale clinical research studies that have been conducted in this country over the past several decades:

- The Physicians Health Study examined the use of aspirin for coronary artery disease (22,071 male, 0 female).
- The Multiple Risk Factor Intervention Trial examined the modification of risk factors to prevent heart disease (15,000 male, 0 female).
- The Veterans Administration Cooperative Study showed the benefits of coronary surgery in angina patients.
- The Baltimore Longitudinal Study of Aging, which has been ongoing since 1958, began to include women only since 1978.

Consider the following facts:

- Women will constitute the larger population and will be the most susceptible to disease in the future (see table 14.16 and table 14.17).
- Overall, women have worse health than men.
- Certain health problems are more prevalent in women than in men.
- Certain health problems are unique to women or affect women differently than they do men.

Table 14.16 Life Expectancy: 1989

	Men	Women
Total population	71.8	78.6
White	72.7	79.2
Black	64.8	73.5
Hispanic	69.6	77.1
Native American[a]		
Asian Pacific Islanders[a]		

[a] No information.

Table 14.17 Percentage of Women Within the Aging Population

Year	65+	85+
1900	49.5%	55.6%
1980	59.7	69.6
1990	59.7	72.0
2020	60.0	73.0

The Assistant Secretary of Health established a Public Health Task Force on Women's Health Issues in 1983. One result was the publication of *Women's Health: Report of the Public Health Service Task Force on Women's Health Issues* (Vol. 1 1985; Vol. 2 1987). One of the most important recommendations of this Task Force was the recommendation that "biomedical and behavioral research should be expanded to ensure emphasis on conditions and diseases unique to, or more prevalent in, women in all age groups."

A consequence of these activities was the creation of the Office of Research on Women's Health (ORWH) within the Office of the Director of the National Institutes of Health (NIH). Currently, consideration is being given to establishing a permanent office for the ORWH within NIH. The objectives of the ORWH are the following:

- Any research supported by NIH must adequately address issues related to women's health.
- Women must be appropriately represented in any clinical research, especially clinical trials.
- An increase should be fostered in the enrollment of women in biomedical research, especially in decision-making roles within clinical medicine and the research environment.

A public hearing was held in June 1991 for the purpose of determining the major needs for research into women's health and the testimony given indicated that the following issues should receive attention (among other issues):

- Cancer prevention (especially breast cancer)
- Cardiovascular disease
- Osteoporosis
- Autoimmune diseases affecting women
- Hormonal cycles and how these might affect absorption, disposition, action, and elimination of drugs
- Sexually transmitted diseases
- Work site safety
- Domestic violence
- AIDS
- Pre- and postnatal care

The ORWH has begun the largest clinical project of its kind ever undertaken in the United States, the Women's Health Initiative. The purposes of this project are to do the following:

- Decrease prevalence of cardiovascular disease, cancer (especially breast cancer), and osteoporosis.
- Develop recommendations on diet, hormone replacement therapy, diet supplements, and exercise.
- Evaluate effectiveness of various strategies for motivating older women to adopt health-enhancing behaviors.

The project is expected to involve 150,000 women at 45 centers for up to 14 years and cost about $625 million. The health issues to be addressed will be divided according to age and processes:

- Consideration of age
  - Birth to young adulthood (birth–15 years)
  - Young adulthood to perimenopausal years (15–44 years)
  - Perimenopausal to mature years (45–64 years)
  - Mature years (65+ years)
- Consideration of process
  - Reproductive biology
  - Early developmental biology
  - Aging processes
  - Cardiovascular function and diseases–
  - Malignancy
  - Immune function and infectious diseases

Certain conditions are more prevalent among women (see table 14.18) than in men and these include the following.

Table 14.18 Leading Causes of Death in Women

Condition	Number
Heart disease	379,754
Cancer	226,960
Cerebrovascular	90,758
Pneumonia/influenza	40,828
Chronic obstructive pulmonary disease	33,191
Accidents	31,279
Diabetes	23,393
Atherosclerosis	13,759
Septicemia	11,793
Nephritis	11,512

Source: National Center for Health Statistics (1998).

- Cardiovascular disease
  - Stroke accounts for a greater percentage of deaths among women than in men at all stages of life.
  - Half of all women, but only 31 % of men, who have heart attacks die within 1 year.
  - About 90% of all heart disease deaths among women occur after menopause.
  - One in 9 women 45 to 64 years old have some clinical cardiovascular disease, increasing to 1 in 3 at age 65 and older.
- Mental disorders
  - Rate of affective disorders (depression, etc.) is 7%, about twice the rate in men.
  - In elderly women depression affects about 3.6% versus 1% in men.
- Alzheimer's disease
  - Higher incidence among women
- Osteoporosis
  - Affects more than 24 million Americans, primarily women (see table 14.19).
  - Hip fractures are the most serious consequence of osteoporosis (about 250,000/year).
  - Osteoporosis causes 1.3 million bone fractures per year.
  - 500,000 vertebrae fractures occur per year, and about one-third of women over age 65 will suffer at least one vertebral fracture.

Table 14.19 Incidence of Osteoporosis in Women

Age Group	% Incidence
45–49	17.9
50–54	39.2
55–59	57.0
60–64	65.6
65–69	73.5
75+	89.0

- Sexually transmitted diseases
  - 6 million women per year are affected (one-half are teenagers).
  - Fifteen to 20 million women have chronic genital herpes or human papillomavirus.
  - Women are the fastest growing population with AIDS.
- Immunologic diseases
  - Autoimmune thyroid diseases have a 15:1 ratio of women to men.
  - Rheumatoid arthritis has a 3:1 ratio of women to men.
  - Systemic lupus erythematosus occurs nine times more often in women than men.
  - Systemic sclerosis affects women four times as often as men.
  - Diabetes mellitus and multiple sclerosis occur more often in women.

Certain health problems are unique to women or affect women differently than they do men.

- Cancer is the second leading cause of death and the leading cause of premature death in women.
- Lung cancer has exceeded breast cancer as the leading cause of death due to cancer (51,000 vs. 45,000 in 1991).
- One in 9 women will develop breast cancer versus 1 in 20 in the 1960s.
- Two and one-half million women acquire chlamydial genital infections annually.
- One million women are treated for pelvic inflammatory disease annually.
- Incidence of involuntary infertility and ectopic pregnancies have quadrupled in past decade due to sexually transmitted disease.
- Due to perinatal transmission, AIDS is the leading cause of death among Hispanic children and the second leading cause of death among Black children.

The FDA restricts the inclusion of women of childbearing age in early clinical trials except for studies involved with life-threatening diseases. These restrictions are currently being reconsidered.

Gender-related differences in drug disposition in nonhuman mammals, especially in metabolism, have been known for some time. There are also several impressive differences in the magnitude of response to selected drugs in male and female rats (e.g., anesthetics and barbiturates). The prevailing thought up to just a few years ago was that these differences did not apply to humans.

The gender-related differences in drug metabolism in animals have been shown to depend on the specific drug, metabolizing enzyme system, age, and animal species among other variables. The differences in drug metabolism between the genders appear to be a result of hormonal differences between the sexes.

The lack of unequivocal information concerning differences in drug disposition between males and females has been the result of limited studies, small numbers of participants in each study, and the influence of confounding variables. With regard to confounding variables there is often no control for or consideration given to factors such as differences in age, smoking status, use of other drugs (especially oral contraceptives, caffeine, and ethanol), time during the menstrual cycle, and diet. The latter factors might exert a profound influence on the results of any study attempting to examine and compare the disposition of a drug as a function of gender. Several reviews on this topic have appeared in the literature.

Few if any studies have indicated substantial gender-related differences in the rate or extent of drug absorption, which might prove to be clinically important. The detection of such differences

requires intensive sampling that is seldom applied in the typical clinical study. Furthermore, the incorrect analysis of data could lead to an erroneous conclusion (e.g., comparisons of blood concentrations only, which do not take into account differences in drug clearance).

There is one important and interesting exception to this general statement that might have a counterpart for certain other drugs. Women have greater blood ethanol concentrations compared to men following oral doses of ethanol and this has been ascribed to differences in body build (i.e., smaller percentage of body weight that is fat-free in women and into which ethanol distributes). Blood concentrations are similar, however, following IV dosing. Ethanol is metabolized in the stomach by the enzyme alcohol deydrogenase before it is absorbed into the systemic blood circulation. The latter is an example of GI or gastric first-pass metabolism. It appears that women have a much lower gastric alcohol dehydrogenase enzyme activity than men, which results in less metabolism in the stomach and, consequently, a greater part of the ethanol dose being absorbed into the blood stream. Women, therefore, will have a greater blood ethanol concentration than men at comparable oral doses. The approximate absolute oral bioavailability of ethanol in women was 91% compared to 61% for men.

There are examples of other drugs that undergo some form of GI metabolism prior to absorption that might be prone to the same effect noted for ethanol. For example, L-DOPA is inactivated in the stomach by a decarboxylase enzyme. Does the activity of this enzyme vary between the genders?

Another potential gender-related difference in oral bioavailability could involve hepatic first-pass metabolism. The concept is identical to that noted earlier for gastric metabolism, with the only difference being the site of metabolism. What determines the significance of the hepatic first-pass effect is the hepatic clearance value of the drug; the greater the clearance, the greater the first-pass effect and the lower the systemic oral bioavailability. Therefore, if there are gender-related differences in the hepatic clearance of high-clearance drugs, one would expect there to be a corresponding difference in GI absorption or bioavailability. Although there is currently little information on which to make a conclusion, there are suggestions that hepatic clearance of certain drugs differs between the genders (discussed later).

Drug distribution throughout the body depends on several factors, including plasma protein and tissue binding and body build with regard to relative amounts of adipose and fat-free tissues. These factors, in turn, determine the apparent volume of distribution of a drug that affects the elimination half-life (i.e., half-life increases as the apparent volume of distribution increases, assuming that elimination clearance of the drug remains the same). Plasma protein binding might also affect the clearance of a drug, depending on the nature of the clearing process.

The current literature with regard to plasma protein binding differences between the genders is conflicting and inconclusive. Some studies suggest a lower binding in women compared to men for certain highly bound drugs and other studies suggest no differences for the same drugs. The reasons for this disparity include the inclusion of few subjects and the lack of control for a number of variables that might alter binding (e.g., plasma protein concentration, age, health and nutritional status, other drugs, smoking, etc.). As a result no definitive conclusion can be reached, but the general impression is that differences, if they exist, are not dramatic.

There are substantial differences between the genders, however, with regard to body build that are further magnified with age (as discussed later). The major difference here is the fact that women have a greater percentage of body weight that is adipose tissue compared to men; conversely, women have a smaller percentage of body weight that is fat-free or that contains water. Therefore, a lipid-soluble drug (e.g., thiopental) will have a larger apparent volume of distribution (i.e., it will occupy a larger space) in women compared to men of the same age on a body weight basis (e.g., volume/kg). In contrast, a water-soluble drug (e.g., ethanol) will have a smaller volume of distribution in women compared to men of the same age. These differences can become important when administering a loading dose of a drug and in terms of the elimination half-life.

This is the most difficult topic to discuss rigorously because there is virtually no consensus about how gender affects drug metabolism in general, although specific drug examples can be

discussed. The reason for this dilemma is the fact that there have been far too few adequate research studies that have employed a sufficient number of participants and the fact that there are a host of variables, in addition to gender, that can affect drug metabolism. A brief listing of those factors would include consideration of: genetics, age, health status, nutritional status, smoking status, and the use of other drugs (including ethanol, caffeine, and drugs of abuse). Furthermore, when considering gender per se, one must also control for the time of the menstrual cycle and the possibility of pregnancy in addition to the use of oral contraceptives. Clearly, it is very difficult to factor out gender differences as being responsible for any observed differences in metabolism when there are so many other variables. Exquisite care must be given to the control of all aspects of such a comparative study to obtain statistically valid conclusions.

Furthermore, it would be far more instructive to obtain estimates of metabolic (or hepatic) clearance of a drug compared to elimination half-life, because the former is an adequate measure of the inherent ability of the liver to metabolize drug, whereas the latter reflects clearance as well as volume. Unfortunately, not all investigations recognize this difference and they report half-life more often than clearance.

The relationship among these three parameters is presented in this equation.

$$T_{1/2} = \frac{0.693 \times \text{Volume}}{\text{Clearance}}$$

Differences in clearance as a function of gender must also consider the possibility of differences in plasma protein binding that could affect the clearance of certain drugs (those with low clearance values). A further complication in comparisons of clearance values is whether or not the values are adjusted for body weight, as this normalized value is usually greater in women by virtue of their weighing less than men.

At this time it is almost counterproductive to list the results of the several studies that have attempted to compare metabolic drug efficiency in men and women, because the results of one study are often diametrically opposite the findings from another.

This is the case, for example, for many benzodiazepine derivatives that undergo oxidative (phase I) biotransformation (e.g., diazepam, chlordiazepoxide, etc.) and the frequently used model or test compound, antipyrine.

One recent study examined the role of gender in propranolol kinetics subsequent to an earlier report that suggested that females have higher concentrations of the drug compared to males after long-term oral dosing following a myocardial infarct. The investigators report that the oral clearance of propranolol is greater in males (leading to a greater first-pass effect, as discussed earlier). Females, therefore, have greater plasma concentrations of the drug compared to males, supporting the preliminary observation already noted. Complicating this finding, however, is the fact that whereas metabolic clearance was greater for two pathways (a side-chain oxidation and glucuronidation), it was not different for at least one other pathway (ring hydroxylation).

Further complicating this issue are the recently discovered gender- and age-specific enantiomer-selective metabolic differences. The considerable recent interest in drug enantiomers (on the basis of different pharmacologic and pharmacokinetic behaviors) has lead to a greater ability to assay these different stereoisomers in biological fluid with the resulting greater understanding of what factors influence the disposition of those forms. One recent study, for example, indicates that there is an age-dependent gender effect as well as a gender-dependent age effect in the metabolism of the R-enantiomer of mephobarbital. In contrast, little difference exists in the metabolism of the S-enantiomer.. Although we have been aware of the age dependence in the metabolism of enantiomers (e.g., hexobarbital), this is the first example of a gender dependence. Undoubtedly, other examples will be forthcoming.

In contrast to the difficulty in reaching any unequivocal conclusions about gender-related differences in phase I metabolic processes, one investigator concludes that there is a more consistent

gender-related trend in the metabolic clearance of those benzodiazepine derivatives that undergo conjugation reactions (phase II). A comparison among several of those drugs is shown in table 14.20.

Table 14.20 Clearance of Selected Benzodiazepine Derivatives Undergoing Phase II Conjugation Reactions in Normal, Young Males and Females

	Clearance, ml/min	
Drug	**Male**	**Female**
Lorazepam	77	55
Oxazepam	88	50[a]
Temazepam	97	68[a]

[a] Significantly different from male value.

These data, however, remain far too limited to allow us to reach any valid general conclusions concerning consistent differences between the sexes for the metabolism of those drugs that undergo phase II metabolism. For that reason recommendations cannot be made with regard to the need to alter dosing regimens as a function of gender. It is quite clear that there is a need for additional studies to clarify the influence of gender on drug metabolism. As alluded to earlier and noted later, the effect of gender is further complicated by consideration of age.

In addition to metabolism, urinary excretion is another major route of drug elimination from the body. Unlike metabolic processes, there are fewer variables that affect renal excretion of drugs (e.g., urine flow, urine pH). As a result, the renal clearance of a drug is reasonably consistent among people with similar kidney function. Renal clearance is often estimated by measurement of creatinine clearance or, more often, by serum creatinine concentration. The latter, however, can be quite misleading because factors such as age and body build affect the relationship between concentration and clearance. The following useful relationship between CLCr and Scr has been determined from many male subjects:

$$\mathrm{CL_{CR}(ml/min)_{MALE}} = \frac{(\text{ideal body weight, kg})(144\text{-age, yr})}{72 \times \mathrm{S_{CR}(mg\%)}}$$

The corresponding equation for females requires multiplication by a factor of 0.86. The latter correction is a result of the fact that creatinine is an end product of muscle metabolism and females have smaller muscle mass compared to males. This relationship is rewritten here. Therefore, at equal body weight, age, and serum creatinine concentration the male will have a greater creatinine clearance and, presumably, more efficient kidney function and a greater ability to excrete drugs. To the best of this writer's knowledge, surprisingly, there has not been a systematic investigation of gender-related differences in renal function and renal excretion of drugs.

$$\mathrm{CL_{CR}(ml/min)_{FEMALE}} = \frac{0.86(\text{ideal body weight, kg})(144\text{-age, yr})}{72 \times \mathrm{S_{CR}(mg\%)}}$$

Figure 14.1 is a plot of the percentage function remaining for several body functions versus age. Note that this is a linear scale indicating that the decline in function occurs at a constant rate (also note that there is no precipitous drop at age 65 years).

The reasons for considering the elderly as a special segment of the population and some of the complications in conducting gerontologic research are outlined in table 14.21.

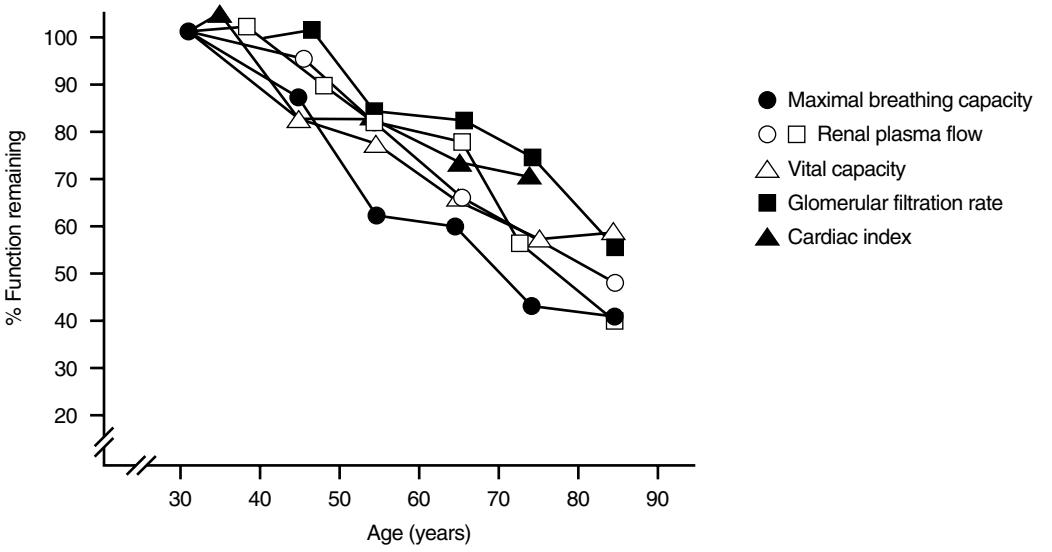

Figure 14.1 Plot of the percentage function remaining for a variety of body functions versus age. (Mayersohn 1992).

Table 14.21 Characteristics of the Elderly That Warrant Their Being Considered as a Special Segment of the General Population

Consideration	Characteristics
Population	The elderly (i.e., those older than age 65) currently represent about 12% of the U.S. population; this percentage increased to about 16% by year 2005.
Health	The elderly experience a greater incidence of disease, physical impairments, and physiological disorders than do younger adults.
Institutionalization	The elderly occupy a greater share of hospital beds (≈ 33%) and long-term care facilities than do younger adults.
Drug use	The elderly consume more drugs (≈ 25% of total use) per capita than do younger adults.
Drug effects	The elderly experience a greater incidence of adverse drug effects and drug interactions than do younger adults.
Factor	Complication
Age: Definition	Chronological versus biological age
Age: Comparisons	Continuum over years versus arbitrary definition of elderly
Age: Changes	Longitudinal design (age changes) versus cross-sectional design (age differences)
Health status	Chronic or acute illness versus good health; institutionalization versus living at home
Drug therapy	Acute or chronic drug therapy versus no drug use
Nutritional status	Good versus poor nutrition
Environment	Smoking versus not smoking, prior environmental exposure of elderly when young versus current exposure in young

Source: Mayersohn (1992).

Microbiological Definition

Animals, like humans, suffer from infectious diseases ranging from those causing only mild symptoms to acute disease outbreaks with high mortality. Although some of these diseases can be partially controlled by medication, both the disease and the medication could seriously interfere with experimental work. Fortunately, most of the infectious diseases of laboratory animals can be controlled simultaneously by the use of SPF techniques (Bleby 1976).

Stocks of SPF animals were initially developed by hysterectomy of the pregnant females just prior to parturition using aseptic surgical techniques. The young were resuscitated in a building designed to prevent the entry of disease-causing organisms, or in closed isolators, and then hand-reared using a sterile milk substitute. This procedure immediately eliminates a wide range of pathogenic organisms that are normally transmitted from mother to offspring because young within the uterus are usually microbially sterile. Such SPF animals are normally free of all parasites, many viruses, and most pathogenic bacteria. Once established, such colonies breed well and can be used to supply high-quality breeding stock that does not carry the risk of introducing disease into an existing colony. Commercial breeders supply SPF mice, rats, guinea pigs, rabbits, and cats. Although some other species have been derived into SPF conditions, they are not freely available at present. Such SPF animals have (or offer) the several advantages.

A colony of animals carrying infectious pathogenic organisms is likely to be more variable than a group of SPF animals, and therefore more animals will be needed to achieve the same degree of statistical precision. For example, it has been shown that underweight mice carried five times as many parasitic nematodes as their normal-weight littermates (Eaton 1972). Although in this case cause and effect cannot be separated, it seems reasonable to assume that such an uneven parasite burden will lead to increased variability among the experimental animals. This means that for the same statistical precision, more "parasitized" animals might be needed than if the animal were free of such parasites.

In conventional animals, there is the danger that the effects of disease might be mistaken for the actions of the experimental treatment. Vitamin A deficiency, for example, has been recorded as causing pneumonia and lung abscesses in rats, when in fact all that the deficiency is doing is increasing the severity of the infectious chronic respiratory disease found in all non-SPF rats (Lindsey et al. 1971). Pneumonia does not occur in SPF rats that are vitamin A deficient. The activation of latent disease through the experimental treatment can be extremely misleading as the control animals not subjected to the same degree of stress could be unaffected (Baker et al. 1971).

In some cases, mild infections might mask the results of an experimental treatment. For example, rats are widely used in inhalation toxicology, yet the lesions of chronic respiratory disease could completely obscure the effects of the experimental treatment. In one case, chlorine gas caused lung lesions, but as the animals grew older the differences between the treated and control groups were completely obscured by the chronic respiratory disease, so that the two groups became histologically indistinguishable (Elmes and Bell 1963).

Many long-term studies require a substantial number of animals to reach old age. As fewer SPF animals die as a result of infectious disease, fewer animals need to be started in each experimental group (Lindsey et al. 1971). Thus, for long-term studies SPF animals might be substantially more economical than conventional ones. A sidelight of this is that the use of SPF animals has advanced survivability to the point that consideration has been given to lengthening some study types (e.g., carcinogenicity).

Genetic Definition

Laboratory animals should also be genetically defined. Behavior; response to drugs; size, weight, and shape of many organs; numbers and types of spontaneous tumors; and response to antigens depends not only on the species, but also on the strain of animal (Festing 1979b). Inbred strains of mice, rats, hamsters, and guinea pigs produced as a result of at least 20 generations of brother–sister mating are readily available. They are much better experimental subjects than the more widely used outbred "white" mice and rats for most studies. Strong (1942) wrote that:

> It is the conviction of many geneticists that the use of the inbred mouse in cancer research has made possible many contributions of a fundamental nature that would not have been otherwise. Perhaps it would not be out of place to make the suggestion that within the near future all research on mice should

be carried out on inbred animals or on hybrid mice of known (genetically controlled) origin where the degree of biological variability has been carefully controlled.

Gruneberg (1952) even went so far as to state that, "The introduction of inbred strains into biology is probably comparable with that of the analytical balance into chemistry."

The main characteristics of genetically defined inbred strains are as follows:

1. All individuals of a strain are genetically identical (isogenic). The genetic uniformity of inbred strains means that each strain can be genetically typed for characteristics such as their blood group in the knowledge that all animals within that strain will be the same. Such data are essential in many immunological and cancer research studies, and cannot be gathered in outbred stocks, where each individual is genetically unique. Isogenicity also leads to phenotypic uniformity for all highly inherited characters, and this means that the statistical precision of an experiment using these animals is increased.
2. Inbred strains are genetically stable. Once a strain has been developed it stays genetically constant for many years. Noninbred strains might change as a result of selective forces, but such forces cannot act on inbred strains, which can only change as a result of the accumulation of mutations, a slow process. This stability means that background data on strain characteristics remains constant for long periods—allowing for the use of such information in planning experiments.
3. Inbred strains are internationally distributed. This means that experiments conducted on some of the more common inbred strains, which are maintained in laboratories throughout the world, can easily be confirmed at laboratories in entirely different parts of the world. Moreover, if many laboratories are working with the same strain, background data on the strain are accumulated much faster.
4. Each strain has a unique set of characteristics that could be of value in research. In some cases, a strain might have a disease that in some way models a similar condition in humans. The best known of such models are the strains with a high incidence of a particular type of cancer. The inbred mouse strain C3H develops a very high incidence of breast tumor, strains AKR and C58 develop leukemia, SJL develops reticulum-cell sarcoma (Hodgkin's disease), and some sublines of strain 129 develop teratomas. Other strains develop autoimmune anemia (NZB), amyloidosis (YBR and SJL), congenital cleft palate (A and CL), hypertension and heart defects (BALB/c and DBA/2 mice, and SHR and GH rats), obesity and diabetes (NZO, PBB, and KK mice), and even a preference for alcohol when given a free choice of 10% alcohol or plain water (strain C57BL mice).

Each of these strains can be studied to obtain a better understanding of the disease in the mouse or rat. Once it is understood in the animal, it will be easier to understand in the human, even though it is unlikely that the conditions are exactly comparable in animals and humans. In fact, it is clear from study of a disease such as hypertension in the rat that the cause of the hypertension in SHR and GH is entirely different (Simpson et al. 1973), emphasizing that diseases of this sort in humans can have several different causes. Obviously, in such cases, some animal models might mimic a human disease relatively closely, whereas in other cases there is little resemblance. Table 14.22 lists some examples of the models of disease that can be found among inbred strains of mice.

Inbred stains do not need to model any human disease to be of value in search. Strains can usually be found to differ for almost every characteristic studied, including many aspects of behavior, response to a wide range of drugs and chemicals, response to antigens, response to infectious agents, incidence of spontaneous diseases, and even anatomical features. These differences can be of great value in research in a number of different ways. At the most trivial level, if a scientist is studying a response to some treatment effect, it is often possible for him or her to find, by surveying a number of inbred strains, a strain that is highly sensitive to his or her experimental treatment. In some cases this will mean that fewer animals are needed to achieve the same degree of statistical precision in future experiments. In other cases, the more sensitive strain might show the effect sooner than resistant strains, and this could reduce the time and facilities needed to complete the experiment.

Table 14.22 Examples of Disease Models and Characteristics of Interest in Inbred Strains of Mice

Character	Strain(s)
Alcohol (10%) preference	C57BL, C57BL, C57BR/cd
Aggression/fighting	SJL, NZW
Audiogenic seizures	DBA/2
Autoimmune anaekia	NZB
Amyloidosis	YBR, SJL
Cleft palate	CL, A
Chediak-Higashi syndrome	SB
Hypertension and/or heart defects	BALB/c, DBA/1, DBA/2
Hyperprolinaemia and prolinuria	PRO
Obesity and/or diabetes	NZO, PBB, KK, AY
Osteoarthropathy of knee joints	STR/1
Polydipsia	SWR, SWV
Resistance to myxovirus infection	A2G
Tumors	
Leukemia	AKR, C58, PL, RF
Reticulum-cell sarcoma (Hodgkin's disease)	SJL
Lung tumors	A
Hepatomas	C3Hf
Mammary tumors	C2H, C2HA-A^{vy}, GRS/A, RIII
Ovarian teratomas	LT
Induced plasmacytomas	BALB/c, NZB
Testicular texatomas	B129/terSV
Complete absence of spontaneous tumors	X/Gf
Whisker eating	A2G

Source: From Festing (1978).

At a slightly more sophisticated level, a comparison of sensitive and resistant strains could give extremely valuable information about the mechanism of some treatment effect. For example, if two strains differ in sensitivity to a drug, it would be of great interest to know whether this is because of differences in absorption, metabolism, excretion, or target organ sensitivity. Such a study could give information that would be extremely useful in evaluating the likely effect of the drug in humans. Preferably, such studies should be carried out on two or more sensitive and two or more resistant strains to show whether the results are uniquely strain dependent, or can be generalized.

Any two inbred strains will normally differ from each other at several thousand different genetic loci. However, sets of inbred strains that differ from one another at only one or a few loci have been developed to study in greater detail those loci that are of particular importance in biomedical research. These are known as sets of congenic strains, and most of them have been developed to study the major histocompatibility complex (MHC). This complex locus is responsible for a range of immunological reactions, including immune responses and graft rejection. Obviously, if two strains can be developed that are genetically identical apart from the MHC, it becomes possible to study the MHC in detail simply by comparing the two strains. Such strains can be developed either as a result of a fortuitous mutation within an inbred strain, or by deliberate breeding using conventional genetic backcrossing techniques. Several hundred strains of this type have been developed, and they are not widely used in immunology and cancer research. They have undoubtedly given much insight into the biology of the mouse MHC, which in many respects is very similar to the MHC in humans. There are more than 500 known mutants and variants in the mouse, and a further 100 in the rat, although in the rat many of these have now been lost. Some of these mutants appear to mimic similar mutants in several species, including humans, and can therefore be regarded as "models" of human disease. Other mutants lack an organ such as the thymus, spleen, tail, or eyes, or they suffer from some hormone deficiency or a developmental defect. Such mutants can be extremely valuable for certain types of research even though they might not resemble any human condition. A list of some of these mutants, classified into

models of disease, a genetic alterations and deficiencies, and biochemical and immunological polymorphisms is given in table 14.23. There are, for example, a number of types of genetically determined obesity and diabetes that have been extremely useful as models of similar conditions in humans

Table 14.23 Examples of Mouse Mutants of Medical Interest and of Biochemical and Immunological Polymorphisms

	Mouse Mutants of Biomedical Interest	
Models of Disease	**Gene**	**Name**
Anemia	sla	Sex-linked anemia
	Sl	Steel
	W	Dominant spotting
Chediak-Higashi syndrome	bg	Beige
Diabetes and obesity	A^y	Yellow
	A^{vy}	Viable yellow
	db	Diabetes
	db^{ab}	Adipose
	ob	Obese
Inborn errors of metabolism	his	Histidinaemia
	pro	Prolinemia
Kidney disease	kd	Kidney disease
Muscular dystrophy	dy	Dystrophia-muscularis
	dy^{2j}	Dystrophia-muscularis-2J
Neuromuscular mutants	jp	Jimpy
	med	Motor and plate disease
	qk	Quaking
	Swl	Sprawling
	Tr	Trembling
Genetic alterations or deficiencies		
Embryonic defects	t-allets	Tailless alleles
Hair absent	hr	Hairless
	hr^{rh}	Rhino
	N	Naked
Hair and thymus absent	nu	Nude
Growth hormone absent	dw	Dwarf
Resistance to androgen	Tfm	Testicular feminization
Sex reversal	Sxr	Sex reversal
Spleen absent	Dh	Dominant hemimelia

Biomedical and immunological polymorphisms	
Polymorphism	**Gene Locus**
Aromatic hydrocarbon	Ahh
Pancreatic	Amy-2
β-D-Galactosidase activity	Bgs
Liver catalase	Ce-1
Erythrocyte antigens	Ea-1 to Ea-7
Esterases (serum and kidney)	Es-1 to Ds-7
Friend virus susceptibility	Fv-1, Fv-2
G-6-PD regulators	Gdr-1, Gdr-2
Hemoglobin alpha chain	Hba
Hemolytic complement	He
Histocompatibility	H-1 to H-38
Immunoglobulin	Ig-1 to Ig-4
Macrophage antigen-1	Mph-1
Major urinary protein	MUP-1
Phosphoglucomutase	Pgm-1, Pgm-2
Sex-limited protein	Slp
Thymus cell antigen-1	Thy-1
Thymus leukemia antigen	Tla

Source: From Festing (1978).

(Festing 1979a). Such animals help to show the immense complexity of the regulation of body fat via the hormonal control of a range of metabolic interactions, each of which can be controlled by regulatory mechanisms that interact with one another. Thus, the finding of a particular biochemical abnormality is no guarantee that it is the cause of the observed obesity. It is much more likely that it is a secondary effect of the primary genetic defect. However, although many of these models of obesity might have no exact counterpart in humans, they can still be useful in screening drugs with a potential for reducing obesity (Cawthorne 1979).

One of the most important mutants causing genetic alterations or deficiencies is the athymic nude mutation in the mouse. A similar mutation has now been described in the rat (Festing 1978). The thymus is essential for the full development of the immune system, and homozygous nude mice or rats are deficient in the cell-mediated type of immune response. They are of value in fundamental studies of immune mechanisms as well as in applied cancer research. This is because, lacking the cell-mediated immune response, they are unable to reject transplanted foreign tissue, including transplants of human tumors. Such transplanted human tumors usually grow, but they retain all the characteristics of human tissue. Therefore, it is possible not only to study human tumors when they are growing in an animal, but it is also possible to study the effect of drugs on such tumors. This is obviously of more value than having to rely simply on the study of animal tumors in animals.

The interest in mutant "knockout," and transgenic animals has blossomed since the first edition of this book. This probably follows the successful development of the nude mouse as a research model.

Environmental And Nutritional Condition

The need to house the defined laboratory animal in defined and stabilized environmental conditions, with a nutritionally adequate and controlled diet, is now becoming recognized. Both diet and environment can drastically alter the physiology of the animal and its response to drugs and other experimental treatments. Moreover, animals obtained from a commercial breeder could well take 2 or more weeks to acclimatize to their new environment. During this period their physiological responses might be unpredictable, depending on the difference between the two environments (Grant et al. 1971).

Susceptibility Factors

With all the effort (and reasons behind it) that goes into obtaining a "defined" test animal with a relatively narrow range of variation in responses, what then are the components or factors that lead some animals to be more sensitive to the toxicity of agents than others?

Consideration of the problem shows that susceptibility factors fall into two large groups—intrinsic and external. The intrinsic factors include sex, stress, age, disease, physiological state (all of which were discussed in detail in the previous chapter), species variations, and strain and animal variations (biological variation). External or environmental factors, meanwhile, include temperature, humidity, light, and time of day.

Summary

If the human population we are concerned about is such that one or more of these susceptibility factors is present in a substantial portion of the members, steps should be taken to design studies so that such individuals are adequately represented by an appropriate model in the test animal population. Barring that, or in the face of having existing data on studies performed in a standard manner, consideration should be given to these factors when attempting to predict outcome of exposures in people.

SPECIES PECULIARITIES

There are a number of quirks associated with various common species of laboratory animals used in toxicology. Many of these are not well presented in the toxicology literature, although Oser (1981) has done his best to overview problems specific to the rat and Gralla (1986) has published a review of eight species-specific responses to toxicants (a modified form of which is presented in table 14.24). Most of these peculiarities hold at least the potential to impact study design and interpretation. The ones presented here are those that the authors believe should be considered in model selection for acute studies.

Table 14.24 Species-Specific Toxic Effects

Type of Toxicity	Structure	Sensitive Species	Mechanism of Toxicity
Ocular	Retina	Dog	Zinc chelation
Ocular	Retina	Any with pigmented retinas	Melanin binding
Stimulated basal metabolism	Thyroid	Dog	Competition or binding
Porphyria	Liver	Human rat, guinea pig, mouse, and rabbit	Estrogen-enhanced sensitivity
Tubular necrosis	Kidney	Rats (male)	Androgen-enhanced sensitivity[a]
Urolithiasis	Kidney and bladder	Rats and mice	Uricase inhibition
Teratogenesis; fetal mortality	Fetus	Rats and mice	Uricase inhibition
Cardiovascular	Heart	Rabbits	Sensitivity to microvascular constriction

[a] More sensitive than humans for many agents (e.g., caprolactam and halogenated solvents).

Source: Adapted from Gralla (1986) with modification.

Species Variation

Although anyone who has had to work in biological research with intact animals should be aware of the existence of wide variability between species, examples specific to toxicology should be pointed out along with a comparison of species sensitivities for a number of specific agents.

The rodenticide zinc phosphide is dependent on the release of phosphine by hydrochloric acid in the stomach for its activation and efficacy (Johnson and Voss 1952). As a result, dogs and cats are considerably less sensitive than rats and rabbits, as the former species secrete gastric hydrochloric acid intermittently, whereas the latter secrete it almost continuously. That this case is not a rare one can be quickly established by examining some data sets in which we have comparative oral lethality data on several species (including humans), such as those presented in table 14.25

Table 14.25 Comparative Human Acute Lethal Doses and Animals LD_{50}s (mg/kg via Oral Route)

		LD_{50} Values			
Chemical	Human LD_{LO}	Mouse	Rat	Rabbit	Dog
Aminopyrine	220	358	685	160	150
Aniline	360	300	440		195
Amytal	43	345	560	575	
Boric acid	640	3,450	2,660		
Caffeine	192	620	192	224	140
Carbofuran	11	2	5		19
Carbon tetrachloride	43	12,800	2,800	6,380	
Cycloheximide		133	3		
Lindane	840		125	130	120
Fenoflurazole		1,600	283	28	50

Note: LD_{LO} = lowest observed lethal dose.

There are numerous additional examples of such differences in pharmacology (Tedeschi and Tedeschi 1968). However, just as important as these variations in general patterns or effect are the species-specific responses that are associated with the commonly employed animal models.

Rat

The rat is commonly accepted as the best animal model in toxicology, the closest to our ideal (Oser 1981). Table 14.26 presents a list of some of the commonly known advantages and disadvantages of the rat as a model for humans.

Table 14.26 Advantages and Disadvantages of the Rat as an Experimental Model for Humans

Advantages	Disadvantages
Commonly used	Anatomic
Small size	Lack of gallbladder
Minimal housing space	Yolk-sac placenta
Prolific	Multiple mammae over body surface
Short gestation	No emetic reflex
Short lactation	Fur covered
Omnivorous	Thinner stratum corneum
Dry diet acceptable	No bronchial glands
Dosing by multiple routes	Physiological
Inexpensive	Estrus and menstrual cycles
Low maintenance cost	Multiparous
Docile	Hematology
Intelligence	Obligatory nose breather
	Concentrated urine
	Limited hypersensitivity response
	Metabolic
	Purines to allantoin
	Clinical chemistry
	Enzymatic biotransformation
	High β-glucuronidase activity
	Nutritional
	Mineral requirements
	Vitamin requirements
	Ascorbic acid biosynthesis
	Histidine biosynthesis
	Behavior
	Nocturnal
	Coprophagy
	Cannibalism
	Maintenance requirements
	Temperature and humidity control
	Noise control

Calabrese (1983) has published a good comparative review of the rat as a model for humans across a wide range of toxicological and biological parameters, and should be consulted for details on these.

Mice

Mice share many of the same advantages and disadvantages of the rats as a model for humans, such as an inability to vomit (i.e., no emetic response). Additionally, their small size and high metabolic rate cause the extent of many toxic effects to be exaggerated as homeostatic mechanisms are "overshot."

Guinea Pigs

The systemic immune response in the guinea pig is exaggerated. As a result, although for many immune parameters it is the best common model for humans, the animal is subject to exaggerated respiratory and cardiovascular expressions of immunologically evoked events.

Rabbits

There are no SPF rabbits currently commercially available. Rather, the animals tend not to be as homogeneous or as high quality as the other common laboratory animal species. Indeed, they tend to harbor a wider range of subclinical infections that show a seasonal variation in their degree of expression (animals with a visible disease problem are more common in the spring and fall), and the stress of experimentation can cause these subclinical infections to be expressed.

Also, the alterations in dermal vascular flow that accompany the changes in phases of hair growth cause marked alterations in percutaneous absorption and in so doing might alter many dermally related responses to chemicals.

Dog

The dog is currently the first-choice nonrodent model for toxicity studies. It is generally very cooperative. The major physiological peculiarity it has that affects toxicity testing is the ease with which it is provoked to vomit. This makes oral dosing at best impractical, and at worst impossible in the case of many compounds, even if the material is encapsulated or given in diet.

Considerations of Strain

Thus far we have focused on the differences between the different species of common laboratory animals and on how this should influence our choice of a model. However, for each of the two species that are used the most in toxicology (the rat and mouse), there is the additional level of complexity caused by differences between strains.

There are three different genetic categories of strains of rodents used in toxicological research: random bred, inbred and F_1 hybrids (or outbred). Random bred animals are produced in large colonies where mating occurs randomly among males and females from unrelated litters. Commercially performed random breeding should not be unplanned, but rather occur in such a manner as to minimize inbreeding. Inbred animals are the result of sister–brother or parent–offspring matings. Twenty or more generations of sister–brother or parent–offspring mating are necessary to establish an inbred strain. Outbred animals are the results of matings between two inbred strains and are usually more vigorous than either of the parental strains. Animals within an inbred or outbred strain are essentially identical genetically, serving to remove a significant source of biological variability.

Strains also exist of the other laboratory animal species, but are generally neither as rigorously defined or of as great a concern, and these have been less studied as a source of variation within toxicity studies.

That strain differences within species are a source of significant and broad differences in results has now been well established, in many cases with varying degrees of knowledge of the underlying mechanistic basis. The resistance of some strains of rabbits to atropine is believed to be due to the hydrolysis of the drug by atropine sterase, controlled by the gene A8, belonging to the group containing the gene that governs black pigmentation. As a result, resistance to atropine and black pigmentation are often associated (Sawin and Glick 1943). Likewise, some strains of rabbits possess a pseudococaine esterase that makes their insensitivity to this drug extreme.

There are also varieties within strains. These result from various factors that in total are labeled genetic drift, and can lead to significant differences in response to toxicants. An example is the

resistance that some wild rats have developed to the anticoagulant rodenticides (Gratz 1973; Zimmermann and Matschiner 1974), requiring that new forms of rodenticides be developed.

Strain variations in response to biologically active agents arise from the same general mechanistic differences as do species differences. Although these (Hilado and Furst 1978) pharmacogenetics have been the subject of numerous reviews (Hathway 1970; Kalow 1962, 1965; Lang and Vesell 1976; Meier 1963a, 1963b; Moore 1972; Vesell 1969), it is still the case that few investigators have studied the mechanisms of variation in higher animals (Becker 1962). The differences in handling characteristics have generally not appeared in the literature before.

Biological Variation

There are also individual animal-to-animal variations in temperature, health, and sensitivity to toxicities that are recognized and expected by experienced animal researchers, but are only broadly understood. The resulting differences in response are generally accredited to individual biological variation. This same phenomenon has been widely studied and observed among humans, and is expected by an experienced clinician. Examples of such individual variations in human include isoniazid, succinylcholine, and glucose-6-phosphate levels or activities. In the first of these, slow inactivators who are deficient in acetyltransferase, and therefore acetylate agents such as isoniazid only slowly, and are thus more liable to suffer from the peripheral neuropathy caused by an accumulation of isoniazid. At the same time, people with more effective acetyltransferase require larger doses of isoniazid to benefit from its therapeutic effects, but in so doing are more likely to suffer liver damage.

Likewise, individuals with low levels of serum cholinesterase might exhibit prolonged muscle relaxation and apnea following an injection of a standard dose of the muscle relaxant succinylcholine, and glucose-6-phosphate dehydrogenase deficiency is responsible for the increased probability of some individuals given primaquine or antipyrine to suffer from a hemolytic anemia.

ENVIRONMENTAL FACTORS

Temperature

Changes in temperature can alter the toxicity of a compound. As examples, at ambient temperatures colchicine and digitalis are more lethal to the rat than to the frog. However, the sensitivity of the frog can be increased by raising the environmental temperature of the two species. The duration of response also decreases as the temperature is raised, suggesting that a temperature-dependent biotransformation of these compounds is involved.

Temperature can include both the background environmental temperature and the internal, physiologically regulated temperature of the animal itself. Many chemicals can profoundly alter body temperature to the acceleration of reaction rates, but rather were due to alterations in the rates of physical factors, such as the absorption rate.

Keplinger et al. (1959) investigated the toxicity of 58 chemicals under different ambient conditions, including temperature. He found that many of the patterns of acute toxicity response were biphasic relative to ambient temperature, with some temperature in the ambient range being associated with a peak sensitivity in many cases.

Humidity

Selisko et al. (1963) investigated the effects of a number of environmental factors on the acute IP toxicity of nicotine to mice and found only humidity to have a significant influence. Humidity

does not have a marked influence on absorption through the skin except at the extreme limits of its range (Neely et al. 1967), and the relationship between humidity and transdermal water loss in sweating animal species is not linear (Grice et al. 1972). The relationships in nonsweating species (which include all of our common laboratory species) is even more complex (Neely et al. 1967).

The physiological status of the test animal in terms of hydration can markedly influence its response to toxicants. Muller and Vernikos-Danellis (1968) showed that the LD_{50}s of caffeine and dextroamphetamine in mice were markedly affected by both ambient temperature and the animals' hydration, with caffeine showing a large potentiation of toxicity at 30°C, whereas dextroamphetamine showed much less change. At lower temperatures (22°C and 15°C) the acute toxicity of both compounds was much less influenced by hydration (Cremer and Bligh 1969).

Environmental temperature and humidity are generally closely related and as such have frequently been considered together (Lang and Vesell 1976). Understanding the basis for the temperature dependence of many of the actions of biologically active compounds has been an area of significant progress over the last 20 years. Belehradek (1957) successfully combined his own and other investigators' research to produce a unified theory of cellular rate processes based on an analysis of the actions of temperature. He concluded that the rate of biological processes is primarily dependent on the resistance of cellular matter to the free movement of molecules within the cells rather than the rate of actual chemical reactions themselves. He was enthusiastic about the relationship between the rate responses of cellular systems and Slotte's temperature–viscosity relationship formula. Brody (1964) has, however, reviewed the applicability of this last to vertebrate animals and pointed out its shortcomings.

The relationship between responses to toxicants and ambient temperature in animals is sometimes paradoxical. Usinger (1957) investigated this in mice and found a series of biphasic relationships with optimal, or peak, ranges. Mean oxygen consumption per unit of body weight diminishes as temperature increases further. Likewise, he measured the rectal temperatures occurring when the ambient temperature was 25°C, increasing on either side of this temperature.

Ahdaya et al. (1976) investigated thermoregulation in mice exposed to parathion, carbaryl, and DDT at temperatures of 1°C, 27°C, and 38°C. All three of the pesticides were found to be least toxic at 27°C. Doull (1972) has reviewed these temperature-dependent responses for many chemicals and presented the hypothesis that temperature is directly correlated with the magnitude and inversely correlated with the duration of the biological response to biologically active xenobiotics in many organisms. Although this temperature dependence stands as a general rule, there are a number of special case exceptions. It is also clear that the effect of temperature on one response variable might not necessarily be predictive of effects on other biological response variables.

Baetjer and Smith (1956) found that the onset of death, rate of dying, and rate of recovery due to parathion in mice were more rapid, whereas the mortality was higher at 35.6°C than at 22.8°C. At 15.5°C, the onset of death was delayed and the total mortality was greater than at 22.8°C. They also investigated the influence of both pre- and postexposure temperatures on the response of the mice and determined that mortality varied directly with the pre- and inversely with the postexposure ambient temperatures. Their conclusion was that the results could not be attributed.

Barometric Pressure

Interest in the effect of atmospheric pressure on the toxicity of chemicals is fairly recent, arising from human activities in space and deep sea diving vessels. At high altitudes, the toxicity of digitalis and strychnine are decreased, whereas that of amphetamine is increased. The influence of atmospheric pressure seems to be mainly (but not entirely) attributable to altered physiological oxygen tension rather than a direct pressure effect (Brown 1980). Recently this interest has taken a new turn as concern as to the possible hazard of fires and atmospheric contaminants on submarines has surfaced.

Light

Whole body irradiation with electromagnetic radiation, including light, increases the toxicity of central nervous system stimulants and decreases that of central nervous system depressants. The toxicity of analgesics such as morphine does not seem to be altered. Many toxicants do exhibit a diurnal pattern of response in animals that is generally related to the light pattern, In rats and mice, P-450 enzyme activity is at its greatest at the beginning of the dark phase of the cycle.

Social Factors

A variety of social factors (interactions between individual animals and between animals and research workers) can modify the toxicities of chemicals in animals and undoubtedly also in humans (see table 14.27) Animal handling, housing (singly or in groups), types of cages, and laboratory routine are all important components of such considerations.

Table 14.27 Psychological Functions Showing Time-Based Cyclic Variations

Activity/sleep
Body temperature
Pain threshold
Adrenocortical function
Skin histamine sensitivity
Liver function
Renal function
Eosinophil count
Mitotic rates
Food consumption
Body weight

Edwards (1982) should be consulted for a good overview of the factors to be considered in the design and operation of a laboratory in terms of both good science and economic and regulatory considerations.

Temporal Factors

Most biological organisms are influenced, directly or indirectly, by a stream of daily and annual variations in their environment. These variations include light, temperature, social interactions, and food, and have resulted in a notable cyclical variations in function.

Many of these cycles can be considerably amplified or modified. Liver function, for example, includes liver glycogen (which is also seasonally variable), glycogen phosphorylase, tyrosine transaminase, tryptophan pyrrolase, and esterase; some of these rhythmic changes are reflected in hepatic cell ultrastructure. Renal function includes urine volume and pH, and excretion of sodium, potassium, chloride, phosphate, uric acid, adrenal cortex, and probably other tissues as well. Blood leukocytes other than eosinophils show daily variation, although the eosinophilic changes have probably been the most studied. Reviews of these topics can be found in Aschoff (1965), Bunning (1967), Conroy and Mills (1970), Mills (1973), and Gall (1977).

Many unicellular organisms also show cyclically varying functions; for example, cell generation time, photosynthetic capacity, phototaxis, and luminescence. In higher plants, growth, leaf and petal movement, and CO_2 fixation are rhythmic functions, and in fungi, spore discharge (Wilkins 1973).

An interesting feature of these rhythms is that they continue when rhythmic external stimuli are removed. If the daily light–dark cycle is turned into continuous light and if temperature variation

is suppressed, and if, in humans, clues like watches and clocks and fixed meal times are removed, the rhythmic variation in function continues, but with a period that might differ slightly from the original 24 hr. This is the so-called free-running period. A 24-hr rhythm might become, for example, 25.0 or 22.9 hr (usually, however, between 20 and 28 hr), and settles to this new period indefinitely until external clues are restored. This phenomenon is the origin of the expression *circadian,* meaning about a day's length.

Considerable work has gone into trying to identify the biological clock that maintains these rhythms, so far without clear-cut success. Does the clock reside in an organ (e.g., the central nervous system or the adrenal cortex), or is it a cellular function? The answer is probably both: The cell has mechanisms that allow it to entrain to environmental rhythms, and that in higher organisms these become systematized in regulatory organs to coordinate the functioning of the body as a whole in response to environmental changes (Mills 1966, 1973).

Both unicellular organisms and avian and mammalian cells in culture show circadian rhythms in the absence of exogenous influences (Bruce 1965). Present arguments center around whether the cellular clock is based on sequential DNA transcription or biochemical networks with natural oscillatory periods. A more recent hypothesis suggests that the cell membrane, with its stable lipids and mobile protein ionic gates, might serve as an oscillator with the underlying slow periodically (Njus et al. 1974).

The majority of the laboratory animals used in toxicology are nocturnal, and they do not change while in the laboratory. Our procedures rarely take the fact and implication of this underlying biological activity rhythm into account.

Diurnal rhythms (more correctly nychthemeral; diurnal refers to daytime, nocturnal to the night) are probably the most important, but there are almost certainly other cyclical changes in physiological function, connected to season or to sexual function, which for the most part we disregard, and might occasionally be important.

The significance of circadian variation for drug action and toxicology will depend very much on the nature of the drug, its absorption characteristics, and the way in which it is administered. Toxicity from continuous atmospheric exposure is not likely to show much circadian dependence, nor from a drug only slowly absorbed or slowly metabolized to an active component. These are perhaps the exceptions, however, and with the increasing range of drugs showing some clinical effectiveness, many of which also carry undesirable side effects, it would seem sensible to administer these in a regimen that allows them to exert their maximum desired effect with a minimum of side effects. Although drugs acting on the central nervous system, the cardiovascular system, and the kidney and the steroids are obvious candidates for such consideration, there is evidence that susceptibility to infection is also circadian dependent, and chemotherapeutic agents and the antibiotics might not be outside such an inquiry.

In addition, a sizable proportion of the population has its biological clock thrown into potential disarray. Besides the pilots and crew members of long-distance flights and the jet-set traveler, the night-shift workforce is numerous enough, and includes not only the factory workers and truck drivers, but hospital staff and watchkeepers at sea, all carrying considerable responsibility. If there is a relationship between drug action and the circadian cycle. it deserves proper evaluation.

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CHAPTER 15

Laws and Regulations Governing Animal Care and Use in Research

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CONTENTS

LABORATORY ANIMAL CARE AND WELFARE

This chapter seeks to summarize and consider the broad and general category of animal care and welfare. The concept of adequate animal care and the mandates either implied or specified by law are discussed.

Quality science and research depends on quality animal care. In fact, state-of-the-art research demands first-class animal care. The commonly accepted ideas of animal welfare were in vogue long before the passage of the first version of the Laboratory Animal Welfare Act (AWA 1966). Federal and international regulations concerning the safety of the entire scope of products in commerce have had several safety objectives (see Gad 2001; Gad and Chengelis 1998), which have had a great impact on animal care. These safeguards are essential to assure the scientific staff that animal care meets certain standards. In no area of science is the need or the quest for knowledge more acute than in the area of animal welfare and animal rights. Immersed in this quest are the biological scientist, research administrators, regulatory officials, veterinarians, and the lay public in general.

There is no question that animal welfare should be and perhaps already is the foremost consideration in the justification for using animals as experimental subjects (Acred, et al. 1994). There is long-standing and growing opposition to the use of animals, primarily because of the gray area of distinction between animal welfare and animal rights (British Cruelty to Animals Act 1876; Fano 1997; French 1975; Freudinger 1985; Hampson 1979). The question of animal rights suggests that you can elevate the so-called animal rights (if in fact there are such rights) to a position of equal moral value with human rights (Russell and Burch 1959). However, it is not possible to equate animal rights with human rights without diminishing human rights, especially the human right to improved health and safety in everyday life. Most biologically oriented scientists and veterinarians understand the need to provide the best available environment, nutrition, housing, and general care to all experimental subjects used in basic research, safety testing, and educational practices. There is no question, nor can there be any wavering concern, that animals used in these endeavors must be provided the best care available. If these practices are followed, the question of animal welfare is addressed. Without the use of animal models or animal subjects, major accomplishments in transplantation; surgical techniques; chemotherapy; or in pathogenesis of infectious diseases and cancer, diagnostic methods, or reproductive failures could never have occurred.

THE PROCESS

The regulatory process typically begins with a Public Law or Act passed by the U.S. Congress. The legislation generally requires that an agency (e.g., U.S. Department of Agriculture) develop regulations and standards to ensure that the wishes of Congress stated in the law are carried out. The regulations and standards basically define to whom the regulations apply and the requirements for compliance with the regulations. The agency generally publishes the proposed regulations in the Federal Register for a period of written public comment. The process can also involve public hearings at which the agency gathers additional oral and written input from interested parties. Both of these mechanisms offer ways in which institutions can influence the regulations that affect them. Following the public comment period, the agency reviews all comments and issues the final rule, which specifies the effective date of the regulation.

The extent and applicability of the regulations for animal facilities varies somewhat based on the species housed and type of research, teaching, and testing procedures performed. There are, however, fairly general regulations that apply to most facilities, as well as professionally accepted standards of practice that apply. It is these more general, or commonly encountered, regulations and practices that are the focus of this chapter. State and local requirements, which can also have an impact on animal facility management (e.g., health and safety ordinances), must also be considered but are beyond the scope of this publication. International regulations are also considered.

THE LAW

Historically, regulations covering the care and use of animals for experimental research have been developed from two main sources: the scientists themselves and humane societies that were formed to protect companion, farm, and laboratory animals.

The National Institutes of Health (NIH), since its inception in 1887 (as the Hygienic Laboratory at the U.S. Marine Hospital in Staten Island), has led the way in developing guidelines for the proper care and use of laboratory animals. In the 1920s the director of NIH was personally responsible for decisions concerning the use of animals in any given experiment. During World War II, the Committee for Medical Research and the National Research Council of the National Academy of Science joined in the effort to reduce war-related injury and disease. This research required the use of animals, which also led to standards for animal care. By war's end, the major oversight of animal care issues was transferred to NIH from the Wartime Office of Scientific Research. By 1958, NIH's Division of Research Grants began a peer-review system of selecting the most meritorious grant applications for funding. Included in the examination process was the issue of care and use of laboratory animals.

In 1963, the first edition of the *Guide for the Care and Use of Laboratory Animals* was issued by the Animal Care Panel, later renamed the American Association for Laboratory Animal Science (AALAS 1997). The most recent edition of the *Guide* was published in 1996 (National Research Council 1996) and remains the primary reference for research animal care and use in the United States.

In 1966 Congress passed the Pet Protection Act in the wake of public outcry over the alleged misuse of animals. This act was the first version of what is now the Animal Welfare Act (1966). The AWA was revised in 1970 and in 1976 and underwent major revisions in 1985 and 1991. The Act gave the U.S. Department of Agriculture (USDA) responsibility for implementing the new law, which applied to dogs, cats, rabbits, monkeys, guinea pigs, and hamsters. Recent changes in the interpretation of the law have brought all animals used for research purposes under the umbrella of the law, but the courts have blocked implementation of this as regards rats, mice, and birds. The USDA has issued Parts 1, 2, and 3 of the final regulations implementing the 1985 amendments of the AWA (NIH 1985), which cover definitions (Part 1), regulations (Part 2), and standards (Part 3). It is Part 3 that gives specific guidelines for the humane handling, care, treatment, and transportation of animals used in research and teaching programs.

The Animal and Plant Health Inspection Service (APHIS), part of the USDA, was given responsibility for facility registration, licensing of animal suppliers, and inspection of licensed facilities to assure compliance with the AWA. Inspectors for APHIS inspect licensed facilities on at least an annual basis (unannounced) to ascertain the status of the facilities' physical plants, training of animal care personnel, and the overall care and welfare of research animals covered under the AWA. Table 15.1 presents current housing standards for laboratory animals.

Prior to 1985, the Public Health Service (PHS) policy on humane care and use of laboratory animals use was governed by Awardee Institution (National Research Council 1996). The amended policy, which is in agreement with 9 Code of Federal Regulations (CFR), lists nine areas of concern that must be addressed and adhered to by the responsible institutional official (NIH 1985; APHIS 1989). Briefly, these areas are as follows:

1. Care and use of animals must be in accordance with the AWA.
2. Experiments involving animals must be designed with consideration of their relevance to human or animal health, advancement of knowledge, or the good of society.
3. Animals selected for a procedure must be of the appropriate species, quality, and minimum number required to provide valid results.

Table 15.1 Recommended Housing Conditions for Selected Laboratory Animal Species[a]

Species	Housing Type[b]	Size	Minimum Cage/Pen Floor Area	Minimum Cage/Pen Height (cm)	Room Temperature (°C)
Mouse	Group	10–15 g	39–52 cm^2	13	18–26
		15–25 g	52–77 cm^2	13	18–26
		25 g	97 cm^2	13	18–26
Rat	Group	100 g	110 cm^2	18	18–26
		100–200 g	110–148 cm^2	18	18–26
		200–400 g	148–258 cm^2	18	18–26
		400–500 g	258–387 cm^2	18	18–26
		500 g	452 cm^2	18	18–26
Hamster	Group	60–80 g	65–84 cm^2	15	18–26
		80–100 g	84–103 cm^2	15	18–26
		100 g	123 cm^2	15	18–26
Guinea pig	Group	350 g	387 cm^2	18	18–26
		350 g	651 cm^2	18	18–26
Rabbit	Single	2 kg	0.14 m^2	36	16–22
		2–4 kg	0.14–0.27 m^2	36	16–22
		4–5.4 kg	0.27–0.36 m^2	36	16–22
		5.4 kg	0.45 m^2	36	16–22
Rabbit (nursing)[c]	Single (plus litter)	2–4 kg	0.46 m^2	36	16–22
		4–5.4 kg	0.56 m^2	36	16–22
Cat	Single	4 kg	0.27 m^2	61	18–29
		4 kg	0.36 m^2	61	18–29
Dog	Single	15 kg	0.72 m^2	Species-specific[d]	18–29
		15–30 kg	1.1 m^2	Species-specific[d]	18–29
		30 kg	2.2 m^2	Species-specific[d]	18–29
Monkey	Single	1 kg	0.14 m^2	51	18–29
		1–10 kg	0.14–0.39 m^2	51–76	18–29
		10–25 kg	0.39–0.72 m^2	76–91	18–29
		25–30 kg	0.72–0.90 m^2	91–117	18–29
		30 kg	1.4 m^2	117	18–29
Ape	Single	20 kg	0.90 m^2	140	18–29
		20–35 kg	0.90–1.4 m^2	140–152	18–29
		35 kg	2.3 m^2	213	18–29
Poultry[e]	Single	< 0.25 kg	0.02 m^2	—	16–27
		0.25–1.5 kg	0.02–0.09 m^2	—	16–27
		1.5–3.0 kg	0.09–0.18 m^2	—	16–27
		> 3.0 kg	0.27 m^2		16–27
Sheep/goat	Single	< 25 kg	0.9 m^2	—	16–27
		25–50 kg	0.9–1.8 m^2	—	16–27
Swine	Single	< 15 kg	0.7 m^2	—	16–27
		15–50 kg	0.7–1.4 m^2	—	16–27
		50–200 kg	1.4–4.3 m^2	—	16–27
		> 200 kg	> 5.4 m^2		16–27
Cattle	Single	< 75 kg	2.2 m^2	—	16–27
		75–350 kg	2.2–6.5 m^2	—	16–27
		350–650 kg	6.5–11.2 m^2	—	16–27
		> 650 kg	> 13.0 m^2	—	16–27
Horse	Single	Adult	13.0 m^2	—	16–27

[a] Recommendations for primary enclosure from the *Guide* (National Research Council 1996); larger animals might also require secondary enclosures for exercise, mating, or other activities.

[b] Typical preference for housing; listed group-housed animals are frequently single housed according to study protocol.

[c] Space recommendation from the Animal Welfare Act (USDA 1995).

[d] Recommended cage height for dogs is 6 in. above the head during normal standing position.

[e] Based primarily on the chicken.

4. Use must avoid or minimize discomfort, stress, and pain to the animals.
5. Appropriate sedation, analgesia, or anesthesia must be provided to the animals.
6. Animals that suffer severe or chronic pain must be euthanized as soon as possible.
7. Housing conditions must be appropriate for the species.
8. Investigators must be appropriately qualified and experienced for conducting procedures on living animals.
9. Exceptions to the principles of animal care and use should not rest with investigator(s), but with the Institutional Animal Care and Use Committee (IACUC).

Since 1989, great strides have been made in setting and applying standards for animal care and welfare across all research facilities, regardless of funding sources. These standards are more "performance-based" than the previous "engineering-based" application of the guidelines; that is, exact cage size, air flow, and painted surfaces within animal rooms. Also, suitable means of euthanasia have been characterized (see table 15.2).

Animal Welfare Act

The AWA, passed by the U.S. Congress in 1966, addressed a variety of activities involving animals in interstate and foreign commerce. The specific intent was to ensure that animals used in research, for exhibition, or in production for the pet trade were provided with humane care and treatment and appropriate transportation, and also to protect owners of animals from theft.

Subsequent amendments to the AWA, the most recent in 1991, have focused increased attention on the use of animals in research, testing, and teaching. The regulations and standards published by the USDA pursuant to the AWA include very specific requirements for research facilities. The AWA itself is quite specific regarding some of the requirements that were to be addressed by the USDA when writing the regulations. In addition to the development of minimum standards for housing, feeding, watering, sanitation, and other standards for most species, the AWA specifies that minimum standards be developed for the exercise of dogs, and for a physical environment adequate to promote the psychological well-being of nonhuman primates. It also specifies that research facilities must do the following:

- Consider procedures to minimize pain and distress.
- Consider the use of alternatives to animals.
- Require veterinary consultation for use of anesthetics and for pre- and postsurgical care.
- Prohibit the use of paralytics without anesthesia.
- Restrict the use of animals in more than one major operative procedure.
- Provide training.
- Establish an IACUC with specific responsibilities to oversee its animal-related activities.

A detail included by Congress in the 1985 Amendments to the AWA was considered unusual at the time and provided the Secretary of Agriculture and the USDA little flexibility in developing the regulations and standards in some areas. However, in many areas, the standards have taken the approach of performance expectations, rather than more specific engineering-based standards.

The following discussion focuses on a few of the requirements of the Animal Welfare Act Regulations (AWAR) that have an impact on management of animal facility programs today.

Institutional Animal Care and Use Committee

The AWA (USDA 1995) requires the animal research facility to form an IACUC for oversight of animal-related issues, following the standards set forth by the *Guide* (NIH 1985). An active,

Table 15.2 Common Methods of Euthanasia in the Toxicology Laboratory

Method	Species	Major Advantages	Major Limitations	AVMA Recommendation
Barbiturate injection	Most	Rapid, safe, cheap	Requires training, restraint, drug control	Acceptable (preferred) method
Anesthetic inhalation	Most	Rapid, multiple animals exposed	Initial irritation, hazardous to staff	Acceptable method for small species
Carbon dioxide inhalation	Several small laboratory animals	Rapid, safe, cheap, multiple animals	Some species stressed or very tolerant	Acceptable at high concentrations
Carbon monoxide inhalation	Most small	Rapid loss of consciousness	Hazardous to staff, difficult to detect	Acceptable method with appropriate generation
Microwave exposure	Mice, rats	Rapid, safe, brain enzymes fixed	Specialized training, equipment, costly	Acceptable method with appropriate equipment
Tricaine/bensocaine injection	Fish, amphibians	Rapid, safe	Costly	Acceptable method
Cervical dislocation	Birds, small rodents, rabbits	Rapid, safe, cheap, no drug residue	Requires training, unpleasant for staff	Conditionally acceptable method when justified
Decapitation	Most small	Rapid, no drug residue	Requires training, some hazard, unpleasant	Conditionally acceptable method when justified
Gunshot	Large farm or wildlife species	Rapid, ease for certain species	Requires training, dangerous, unpleasant	Conditionally acceptable method when necessary
Electrocution	Foxes, sheep, swine, mink	Rapid, cheap, no drug residue	Requires special equipment, hazardous, unpleasant, severe contractions	Conditionally acceptable method in specialized instances
Pithing	Small amphibians	Rapid, no drug residue	Requires training, unpleasant	Conditionally acceptable method in specialized instances
Nitrogen/argon inhalation	Most small	Rapid, safe, readily available	Stressful in some species, must limit O_2	Conditionally acceptable method is specialized instances
Exsanguination	Several	Safe, cheap	Very stressful	Unacceptable method without anesthesia
Rapid freezing	Several small	Safe, cheap	Very stressful	Unacceptable method without anesthesia
Air embolism injection	Several	Safe, cheap	Causes convulsions, other signs of distress	Unacceptable method without anesthesia
Drowning	Several	Safe, cheap	Very stressful, slow	Unacceptable method
Strychnine dosing	Several	Possibly convenient	Causes convulsions, painful contractions	Unacceptable method
Chloroform injection	Several	Possibly convenient	Very hazardous to staff	Unacceptable method
Cyanide dosing	Several	Possibly convenient	Unpleasant, hazardous	Unacceptable method
Stunning (blow to head)	Several	Rapid, no drug residue	Unpleasant, sufficient force required	Unacceptable method without other lethal procedure

Source: Summarized from the Report of the American Veterinary Medical Association (AVMA) Panel on Euthanasia (1993).

well-informed IACUC is one of the best guarantees that animal welfare policies are supported and adhered to. Although research facilities are sometimes criticized for self-policing (IACUC members are chosen by the management of the facility), there are numerous checks and outside (USDA, American Association for Accreditation of Laboratory Animal Care [AAALAC]) inspections that, along with the practical considerations already noted, make most IACUCs effective. The required membership of an IACUC has been noted previously, and the total number of members depends on the size of the facility and the extent of its research. Beyond the mandated composition of the groups, it is desirable to include members from diverse backgrounds among scientific and nonscientific disciplines. Outside experts can also serve as consultants. There might be need for subcommittees to carry out the many functions of the IACUC.

As noted in the *Guide,* the IACUC oversees and assesses the animal care program and the use of animals in research. At least every 6 months, it inspects the facility (with emphasis on all animal housing, testing, and supply areas), reviews protocols, and carefully records all findings. A written report (containing meeting minutes, documents reviewed, areas inspected, and items discussed) signed by most or all members is issued to principal investigators and facility management detailing any deficiencies. Specifically, protocols and subsequent animal use and euthanasia are evaluated on the following bases (the *Guide*):

- Rationale of proposed animal use
- Justification of the species used
- Justification of the number of animals used
- Availability of alternative methods (Bennett 1994)
- Adequacy of staff training and experience
- Atypical housing or husbandry requirements
- Consideration of sedation, analgesia, and anesthesia
- Unnecessary duplication of testing
- Use of repeated or major surgical techniques
- Criteria for excessive pain or distress
- Method of euthanasia

Standardized procedures in addition to specialized research projects must be considered by the IACUC. Standardized protocols should not require a full review each time they are employed, but any revisions affecting animal use must be considered by the IACUC. Specialized protocols should each be reviewed by the IACUC. There should be a review and approval form for each project, which should be maintained as part of study records. These records should also be maintained in facility files, available for independent review, such as a USDA inspection. The IACUC report must reflect adherence to the AWA, with minor and major deviations being noted. A major deficiency is one that threatens the heath, well-being, or safety of the animals.

Identification of animal welfare deficiencies need not only originate from formal inspections, however. Any staff member should be encouraged to present concerns to the IACUC. Technical or nontechnical personnel, for example, should feel free (without any negative consequences) to inform a direct supervisor or an IACUC member of any excessive pain or distress witnessed during the course of a toxicology project. Such information can be kept confidential, but careful documentation must be made. It then becomes the responsibility of site management and the IACUC to investigate and take appropriate corrective action as warranted. This process is discussed by Silverman (1994).

There must be a reasonable and specific plan, with an outlined schedule, for the correction of significant deficiencies. Resolution of any major deviation should occur through a joint effort of the researcher, facility management officials of the area of concern (animal care supervisor, environmental or maintenance staff, purchasing agent, etc.), veterinary staff, and the IACUC. If major deficiencies are not resolved according to the plan and schedule, the remaining deficiencies "shall be reported in writing by the IACUC, through the Institutional official, to APHIS and any Federal agency funding that activity" (AWA). The IACUC will also withhold approval for the specific

studies affected until concerns are resolved. Of course, conflicts might occur among staff, researchers, management, and study sponsors. These will have to be resolved in the light of the principles presented earlier—ethics, science, social pressures, and legal requirements.

Secondary responsibilities of the IACUC are training staff members, providing information on animal use, interacting with other personnel (health and safety officers, managers, legal staff, etc.), developing a liaison with outside organizations promoting humane research, and engaging in public education (Dell 1994). Any complaints or charges of animal misuse made by the public or the media should be investigated by the IACUC. This should aid facility management in arriving at an appropriate response.

Part 2 of 9 CFR, Chapter 1 (Regulations) states:

> Each institution which falls under the authority of the AWA and/or received PHS support for research or teaching involving laboratory animals must operate a program with clear lines of authority and responsibility for self monitoring the care and welfare of such laboratory animals.

The mechanism for such monitoring was given to the IACUC.

To monitor the care and use of animals, the AWA requires that an appropriate administrative official at each facility appoint the members of the IACUC. The IACUC serves as the watchdog for the welfare of animals in the same manner as does the institutional review board in the hospital or medical center.

Membership of the IACUC must include a scientist from the institution experienced in research involving animals; a doctor of veterinary medicine who either is certified by the American College of Laboratory Animal Medicine or has had experience in laboratory animal medicine; a person who is not affiliated with the facility or institution; and other members as required by federal, state, or local regulations.

Humane care and treatment of animals used in research, testing, and education requires a certain amount of scientific and professional judgment. The husbandry needs of each species and the special requirements for the species, including humane handling, must be considered in all programs in which these animals are to be used. Each facility or institution must establish a written policy for the humane care and use of a given species. This program must be in compliance with applicable federal, state, and local laws and regulations. Depending on the fund or funding source used to provide for these animals or to provide the basic research, other regulations might be enforced. The established program must follow *Guide for the Care and Use of Laboratory Animals*, published by the U.S. Department of Health and Human Services and the regulations provided by the U.S. Department of Agriculture found in 9 CFR part 3 entitled "Animal Welfare Standards." The responsibility for directing such a program is usually given to a veterinarian or another qualified professional. At least one veterinarian must be associated with the program.

The IACUC is responsible for evaluating the animal care and use program. The duties of the IACUC must include:

1. Meeting at regular intervals to ensure compliance with the *Guide* (the regular intervals can be no less than annually).
2. Ensuring that a mechanism exists to review the humane care and use of animals in research, testing, and education.
3. Providing a written report at least annually to the responsible administrative official on the status of the animal care and use program.
4. Reviewing and/or investigating concerns involving the care and use of animals at the research facility resulting from public complaints received and from reports of noncompliance received from laboratory or research facility personnel.
5. Reviewing, approving, and requiring modifications or withholding approval of those components of proposed or ongoing activities related to the care and use of animals.
6. Other duties as assigned.

Although the duties of the IACUC just outlined fall into a very broad and general category, other duties and responsibilities have historically been assigned to IACUC. In many institutions the IACUC assures that adequate veterinary care is provided. This care can either be provided by an on-site veterinarian or by a contract veterinarian who makes periodic visits to the facility. There must be a written program for animal use and care. Although it is the responsibility of the institution to ensure that people caring for and using laboratory animals are qualified to do so, it often falls on the IACUC to make sure that these individuals are qualified and possess the proper qualifications and expertise to conduct the various phases of research that are being considered. Because animal care and use programs require professional, technical, and husbandry support, the IACUC must assure that the institution or facility employs people trained in laboratory animal science and provides the opportunity for continuing education and properly supervised on-the-job training to ensure effective implementation of the program.

Exercise for Dogs

The requirement for exercise of dogs has been the subject of considerable discussion since the AWA was passed. In the case of dog exercise, the AWA provided little guidance other than that the exercise program be determined by the attending veterinarian and be in accordance with the standards to be developed. The subsequent AWAR (Part 3: Subpart A) establishes several requirements worthy of comment.

The facility is required to develop, document, and follow a plan (approved by the attending veterinarian) to provide dogs with the opportunity for exercise. In practice, and in certain parts of the standard, this has been interpreted to require involvement of the IACUC in the process. The plan must be a written program with specific procedures for implementation. The expected approach is to define program-specific ways of achieving the goal of exercise. The "opportunity for exercise" need only be addressed if certain minimum requirements for housing space are not met. For dogs housed individually, additional opportunity for exercise need not be provided if the floor space provided in the enclosure is at least twice the minimum space required by the AWAR for individually housed dogs. When housed in groups, no additional exercise is required if the total floor space is at least 100% of the AWAR required space for each dog if maintained separately. This standard has generated significant controversy. The standards provide little guidance for the provision of exercise if these space requirements are not met, other than that dogs be provided access to a run or open area. The frequency and duration of exercise is not specified, but is to be determined by the attending veterinarian.

Most institutions have been able to comply with the regulations by first developing a written plan that simply outlines or specifies how dogs will be housed in their facilities (i.e., space requirements) and how they will be exercised, if required. The plan is typically approved by the IACUC and reviewed on a schedule consistent with the review of protocols (annually) or in conjunction with the IACUC's semiannual program review. Because of the lack of specific requirements in the AWAR, many facilities have selected a frequency and duration of exercise opportunity consistent with the available exercise space, the average daily canine population, and personnel resources. With that as a starting point, periodic observation (with documentation) of the behavior of the animals serves as a means of evaluating the success of the program. A conscientious effort to plan, implement, evaluate, and improve the exercise program for dogs, if appropriately documented, is generally sufficient to demonstrate compliance to the standard. Additional guidance can be found in the National Research Council publication, *Laboratory Animal Management: Dogs.*

Psychological Well-Being of Nonhuman Primates

The original AWA states that facilities housing nonhuman primates must provide a "physical environment" adequate to promote the psychological well-being of primates, the AWAR (Part 3:

Subpart B) specifies that "environmental enhancement" be provided. An environmental enhancement plan must be developed that is consistent with appropriate professional standards as cited in appropriate professional journals or reference guides. Initial plans developed by institutions utilized numerous and varied strategies. Although it was clear that a certain degree of flexibility was essential to developing and implementing effective programs, the need for guidelines was recognized. The National Research Council, Institute for Laboratory Animal Research (ILAR) subsequently published guidelines specifically addressing this requirement. That report, *The Psychological Well-Being of Nonhuman Primates*, provides considerable information on environmental enhancement strategies for different species and for different situations. The report supports the performance-based standard approach as distinct from engineering-based standards, but emphasizes the need for scientific approaches to meeting the standards so that results can be measured and evaluated. When using this document as a guideline, it is important that professional judgment is used in interpreting and applying the recommendations. As is the case with the care of any animal species, the specific knowledge, experience, and skills of the facility personnel involved in the implementation of the program are critical.

The AWAR for environmental enhancement do specify areas that must be addressed, including social grouping, environmental enrichment, special considerations, and restraint devices. The facility plan must address the social needs of any nonhuman primate species known to exist in social groups in the world. Exceptions to the requirement for social interaction can include animals that exhibit overly aggressive behavior or those that are debilitated as a result of age or other conditions. It is recognized that animals suspected of having contagious disease would need to be isolated from other animals, as would animals undergoing diagnosis and treatment of a disease condition. Compatibility of animals housed in groups is stressed. If housed individually, the animals must be able to see and hear others of their species unless this is contraindicated for medical or scientific reasons and so documented.

The AWAR suggest several approaches to environmental enrichment designed to provide a means for the animals to express species-typical activities. Examples include the following:

- Perches, swings, mirrors, and various cage complexities
- Objects to manipulate
- Varied food items and foraging opportunities
- Interaction with caretakers where appropriate
- Housing with compatible conspecifics

It is recognized that special circumstances could exist that will influence the approach to housing and environmental enrichment. These include provisions for infants and juveniles, research-based protocol restrictions, and the housing of large great apes.

The AWAR prohibit the use of restraint devices unless required for health reasons (determined by the attending veterinarian) or as part of a research protocol approved by the IACUC. The restraint should be for the shortest time possible. If restraint exceeds 12 hr, the animal should periodically be provided with the opportunity for unrestrained exercise if the research protocol or situation permits.

The institution's approach to meeting the requirement for environmental enhancement can be very similar to that for exercise for dogs. A written plan is developed, taking into consideration the species, existing facility and equipment, and personnel resources. The plan is typically approved by the IACUC and reviewed on a regular basis. The plan should include methods of data collection that allow for evaluation of the success of the program. The ILAR report, *The Psychological Well-Being of Nonhuman Primates*, provides guidance not only on strategies for implementation, but also on measures that can be used to evaluate success. By using the ILAR report and other available information, compliance with the standard can be achieved with diligent planning, implementation, evaluation, and improvement that is well documented.

Other AWA Requirements

The AWAR (Part 3: Subparts A–F) contains the majority of the requirements specific to the care of various species including requirements for facilities, operations, animal health and husbandry, and transportation. Detailed standards are provided for areas such as indoor and outdoor housing, primary enclosures (cages and pens), feeding, watering, cleaning and sanitation, and pest control. In some cases the standards are quite specific (engineering-based standards). However, the concept of performance-based standards and use of professional judgment is evident throughout the section.

In 1998 the USDA amended the regulations under the AWA to address issues of comfort and foot injury associated with housing of dogs and cats on suspended mesh or slatted flooring. Following a phase-in period for new purchases, the requirements of this amendment became effective for all primary enclosures in January 2000.

The standards for primary enclosures for dogs and cats (Part 3, Subpart A, Section 3.6) state that the enclosures must have "floors that are constructed in a manner that protects the dogs' and cats' feet and legs from injury, and that, if of mesh or slatted construction, do not allow the dogs' or cats' feet to pass through any openings in the floor." The standards require that suspended flooring for dogs and cats either be constructed of metal strands greater than 1/8 in. (9 gauge) in diameter or coated with a material such as plastic or fiberglass. Primary enclosures with such flooring must also be constructed so that the floor does not bend or sag between the structural supports.

The intent of this amendment was to address concerns that certain types and designs of suspended flooring contributed to foot injuries, and that appropriate modification would decrease the incidence of such problems. In addition, sagging, unstable floors were thought to cause psychological trauma for dogs trying to balance on the floors. The modifications to the standards were intended to improve comfort for dogs and cats housed in primary enclosures with suspended floors. With the exception of the most recent changes, requirements included in the AWAR were incorporated into the National Research Council's (1996) *Guide for the Care and Use of Laboratory Animals.*

International Regulations

Internationally, animal welfare concerns are greater (Europe) or lesser than in the United States, as reflected in their current laws (AAALAC 1997b, 1997c; Bayne and Martin 1998; Cooper 1990a, 1990b; *Council Directive 1*986; Howard-Jones 1985; Miller 1998; Netherlands Animal Welfare Society 1991; Nevalainen 1999).

In the United Kingdom, the equivalent regulations are called the Animal (Scientific Procedures) Act and are administered by the Home Office. Corresponding laws are in force in all major countries, as listed here:

- Australia
  - Australian Council 1990
- Canada
  - *Guide to the Care and Use of Experimental Animals* (Vol. 1, 2nd ed. 1993; Vol. 2 1984) Rowsell 1991
- China
  - Animal Protection Law
- Germany
  - German Animal Welfare Act (May 1998)
  - German Animal Welfare Act (May 1998) [English translation]
- Japan
  - Law Concerning the Protection and Control of Animals (October 1973)
- New Zealand
  - Animal Welfare Act of 1999 (October 1999)

    - Code of Recommendations and Minimum Standards for the Care and Use of Animals for Scientific Purposes (August 1995)
    - Code of Recommendations and Minimum Standards for the Emergency Slaughter of Farm Livestock (December 1996)
    - Code of Recommendations and Minimum Standards for the Sale of Companion Animals (April 1994)
    - Code of Recommendations and Minimum Standards for the Welfare of Animals in Boarding Establishments (August 1993)
    - Code of Recommendations and Minimum Standards for the Welfare of Animals at Stockyards (May 1995)
    - Code of Recommendations and Minimum Standards for the Welfare of Animals at the Time of Slaughter and Licensed and Approved Premises (July 1996)
    - Code of Recommendations and Minimum Standards for the Welfare of Bobby Calves (August 1993)
    - Code of Recommendations and Minimum Standards for the Welfare of Deer During the Removal of Antlers (January 1992)
    - Code of Recommendations and Minimum Standards for the Welfare of Dogs (May 1998)
    - Code of Recommendations and Minimum Standards for the Welfare of Layer Hens (September 1996)
    - Code of Recommendations and Minimum Standards for the Welfare of Livestock from Which Blood Is Harvested for Commercial and Research Purposes (April 1996)
    - Code of Recommendations and Minimum Standards for the Welfare of Ostriches and Emus (August 1993)
    - Code of Recommendations and Minimum Standards for the Welfare of Pigs (January 1994)
    - Code of Recommendations and Minimum Standards for the Welfare of Sheep (July 1996)
- Norway
    - Animal Welfare Act (December 1974)
- Sweden
    - Swedish Animal Welfare Act (February 1998)
- United Kingdom
    - Animals (Scientific Procedures) Act of 1986

Occupational Health and Safety

An occupational health program is mandatory for personnel working in laboratory animal facilities. This program must include a physical examination and a medical and work history prior to work assignment. Periodic physical examinations are required for people in some job categories, especially those who handle hazardous materials. In addition, an educational program must be provided to teach personnel about zoonoses, personal hygiene, and other considerations that could be of a prime safety concern to those individuals involved in the program, such as special precautions to be taken by pregnant women (Acha and Szyfrei 1980). Other occupational hazards including animal bites and allergies to animal dander or hair must be considered.

Of special concern is the area of B virus exposure to those individuals handling primates. In some areas there might be requirements for special clothing or breathing apparatus, which can be determined by the risk of exposure to the individual employee.

Special Areas of Responsibility

Recently, two areas of consideration have caused the IACUC a great deal of discomfort and disagreement in their deliberations. This issue of pain and distress caused by physical restraint and multiple or invasive surgical procedures has caused many IACUC members to question the use of these procedures with specific concern for the overall welfare of the animals involved. Brief physical restraint of animals for administration of drugs, collection of samples, or other experimental

procedures is certainly considered justifiable. The physical restraint can be accomplished manually or with simple restraint devices such as slings, racks, or squeeze cages. Prolonged restraint (longer than 4 hr) of any animal, including the use of chairs for nonhuman primates, should be avoided unless absolutely essential to the research objectives. Prolonged restraint must be specifically spelled out within the research protocol, along with the justification for prolonged restraint in these specific restraint devices. One of the major concerns of the IACUC is that these restraint devices should not be used simply as a convenience to the investigator in handling or managing animals. In many cases the use of such devices must be specifically approved by the IACUC. Consideration must also be given the possibility of lesions or illness associated with restraint, such as decubitus ulcers, dependent edema, and weight loss. If these problems develop, adequate veterinary care must be provided to the animals and in some cases might mandate a temporary or permanent removal of animals from such restraint devices.

Multiple or invasive surgical procedures on a single animal are generally discouraged. In any case, the multiple major surgical procedures must be approved by the IACUC. It might be necessary for the researcher to develop alternate procedures that do not require the use of multiple surgical procedures. In no case can cost savings be a consideration or adequate reason for performing multiple surgical procedures.

Pain and Distress

Whereas such areas as lighting, food, water, cage size, ventilation, environmental temperature, humidity, and exercise are mandated by regulation, the minimization of stress, discomfort, and pain is poorly defined or understood. The understanding of stress, discomfort, and pain in animals is based primarily on professional and aesthetic judgment made by the investigtors and members of the IACUC. Without pre- and postsurgical medication, stress and pain will result. What criteria can be used to assure that pain and distress are minimized?

In most research units, pain is recognized by the action of the species under study. By understanding the normal posture, movement, and attitude of the species, animal care technicians can recognize abnormal posture, movement, or lack thereof; attitude (aggressive vs. submissive); grooming, eating, or drinking habits; or vocalization. Any of these signs can suggest to the investigator or veterinarian that the animal is experiencing pain or discomfort. These observations are straightforward, and judgments concerning their meaning or significance are fairly easy to make, assuming pain in the experimental animal subject is perceived in the same manner as is pain in the human subject.

Behavioral changes as just described can be short term, in which case the changes might be adaptive. However, long-term behavioral changes can lead to maladaptive mannerisms that are important signs of distress (not necessarily pain) in laboratory animals. These maladaptive behavioral traits indicate that medical intervention is required. Lack of grooming or excessive hair removal in rodents might suggest organic disease before other clinical symptoms are observed. Nonhuman primates are wasteful eaters and, as such, can become emaciated even though the animal appears to be consuming the appropriate diet.

The areas of stress and discomfort are much harder to quantify, and there is therefore a great need to understand and determine how best to alleviate the causes. It is often impossible to determine whether an animal is undergoing a normal process of adapting to a stressing agent (stressor). However, an aggressive attempt to alleviate the stress for this individual animal might be ill advised and counterproductive. The research objective might be compromised to the extent that valid results are not obtained and could require the study to be repeated with more experimental animals involved.

There is a great deal of disagreement on the meaning of terms such as *comfort, well-being, discomfort, stress, fear, anxiety, pain,* and *distress*. The mechanisms that contribute to the functional and recognizable state of well-being versus that of distress involve biochemical, physiological, and psychological changes that are poorly understood in animals. However, certain factors have been

recognized to cause pain and stress and are outlined here. These factors generally apply to all mammalian species, as summarized in table 15.3.

Table 15.3 Factors Recognized to Cause Pain and Stress in Laboratory Animals

Causes of Pain	Causes of Stress
Injury	Restraint
Surgical manipulation	Noise
Disease	Odors and pheromones
	Presence of other species, including humans[a]
	Starvation and/or dehydration
	Fear, anxiety, boredom, and group housing of the same sex[b]

[a] Dogs enjoy the presence of humans.

[b] Especially in primates.

Many of the signs of stress (distress) listed in table 15.4 can become maladaptive responses and can become permanent parts of animals' activities and seriously threaten their overall well-being. Any behavior that relieves the intensity or duration of stress is likely to become habitual. Such behaviors include coprophagy, hair pulling, self-trauma, and repetitive movements (cage circling).

Table 15.4 Observable Signs of Severe Pain or Distress

Local effects[a]	
Skin corrosion	Severe erosion or ulceration penetrating most or all of dermis, large areas of severe necrosis
Ocular injury	Severe corneal opacity, corneal ulceration, purulent or bloody discharge, severe periocular necrosis
Extremity injury	Severe swelling, ulcerative lesions, apparent fractures, severe cutaneous erosion/sloughing, gangrenous appearance
Systemic effects[a]	
Nervous signs	Severe or persistent tremors, convulsions, narcosis, catalepsy
Locomotor/muscular signs	Ataxia, paralysis, prostration
Respiratory signs	Gasping or labored breathing, very slow or rapid breathing, audible breathing (rales, wheezing)
Cardiovascular signs	Very slow or rapid heart rate, severe pallor, redness or cyanosis of extremities
Gastrointestinal signs	Persistent vomiting, severe diarrhea, anorexia
Behavioral effects[b]	
Excessive vocalization	Squealing, grunting, growling, whimpering, howling (especially during movement or handling)
Atypical actions	Self-mutilation, stereotypic activity, restlessness, head shaking, apparent apathy to stimulants
Direct response to pain	Licking, biting, or scratching of affected area; unusual posture to relieve pressure on affected area (hunched or stretched appearance); excessive struggling or biting during handling; grimacing or baring teeth
Absence of actions	Failure to groom, decreased socialization, poor reflexes, marked decrease in feed or water intake

[a] Myers and DePass (1993).

[b] Mroczek, (1992); USDA (1995); see also Baumans (1994).

There is a requirement, mandated in 9 CFR, for the opportunity to exercise for dogs kept in individual cages. If these cages provide less than twice the space required for the size of the dog (size of dog is determined using formula found in 9 CFR, Part 3 of Subchapter A), these animals must be provided the opportunity to exercise. The exact exercise procedure is determined by the attending veterinarian and approved by the IACUC.

In those institutions or facilities housing or using primates, there is a mandated requirement to provide "environmental enrichment to promote psychological well-being" of the species involved. These institutions and facilities must have a written plan that addresses the following:

1. *Social grouping*. Based on the social needs of species as it exists in nature.
2. *Environmental enrichment*. Primary enclosures must be enriched to provide species-typical activities, such as, perches, swings, and foraging or task-oriented feeding methods.

Interaction with the primary care individual is an important element of this enrichment program. The attending veterinarian can exempt an individual primate from participation in the enrichment program based on medical considerations.

The IACUC can also exempt an individual from participation in the program based on scientific reasons set forth in the protocol. Regardless of the reason for exemption, the exemption must be reviewed at least every 30 days by the veterinarian and at least annually by the IACUC.

PUBLICATIONS

Code of Federal Regulations, Title 9, Chapter 1, Subchapter A: Animal Welfare.

This code is available from USDA, APHIS/Animal Care, 4700 River Road, Unit 45, Riverdale, MD 20737–1234.

This current version of the regulations developed by the USDA specifies how to comply with the AWA and its amendments. The subchapter is divided into four sections: Definitions, Regulations, Standards, and Rules of Practice Governing Proceedings Under the Animal Welfare Act. The Definitions section describes exactly what is meant by terms used in the legislation. "Animal," for example, specifically excludes rats of the genus *Rattus* and mice of the genus *Mus*, as well as birds used in research. The Regulations section includes subparts for licensing, registration, research facilities, attending veterinarians and adequate veterinary care, stolen animals, records, compliance with standards and holding periods, and miscellaneous topics such as confiscation and destruction of animals and access and inspection of records and property. The bulk of the subchapter is the third section, which provides standards for specific species or groups of species. Included are sections for cats and dogs, guinea pigs and hamsters, rabbits, nonhuman primates, marine mammals, and the general category of "other warm-blooded animals." Standards include those for facilities and operations, health and husbandry systems, and transportation. The final section sets forth the Rules of Practice applicable to adjudicating administrative proceedings under Section 19 of the Animal Welfare Act.

Animal Care Policies

The policy manual gives policies issued by APHIS/Animal Care that clarify the AWA regulations. Among the topics covered are "Written Narrative for Alternatives to Painful Procedures," "Space and Exercise Requirements for Traveling Exhibitors," and "Annual Report for Research Facilities." Originally issued in April 1997, new policies can be added at any time and are included in the manual.

PROFESSIONAL ORGANIZATIONS

There are several professional groups that provide special information to the institution and the IACUC in areas of education, training, or certification. A brief description of some of these organizations follows.

American Association for Accreditation of Laboratory Animal Care

The AAALAC promotes high-quality animal care and use through a voluntary accreditation program. Institutions using, maintaining, or breeding laboratory animals for biological research can

apply for accreditation. The NIH accepts full accreditation by AAALAC as assurance that the animal facilities are in compliance with Public Health Service policy. AAALAC is accordingly a nongovernmental organization.

American Association for Laboratory Animal Science

The AALAS is made up of individuals and institutions professionally concerned with the production, care, and use of laboratory animals. The organization collects and exchanges information on all phases of laboratory animal care and management through the publication of a journal (*Laboratory Animal Science*), bulletins, and other documents. The AALAS animal technician certification is an important method to develop uniform requirements and training programs that lead to certification of technicians based on their experience and knowledge.

American Veterinary Medical Association

The AVMA is a major international organization of veterinarians. Its objective is to advance the science and art of veterinary medicine, including its relationship to public health and agriculture. It sponsors specialization in veterinary medicine through the formal recognition of specialty certifying organizations, including the American College of Laboratory Animal Medicine (ACLAM) and the American College of Veterinary Pathologists (ACVP).

American College of Laboratory Animal Medicine

The ACLAM is a specialty board founded in 1957 to encourage education, training, and research in laboratory animal medicine (ACLAM 1996).

Several professional societies such as the Society of Toxicology (SOT), the ACVP, and the Federation of American Societies for Experimental Biology have developed and published "position" papers on the use of animals in experimentation (i.e., *Guiding Principles in the Use of Animals in Toxicology*, adopted by the SOT in July 1991).

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ADDITIONAL RESOURCES

American Association for Accreditation of Laboratory Animal Care
11300 Rockville Pike, Suite 121,
Rockville MD 20852-3035, USA

American Association of Laboratory Animal Science
70 Timber Creek Drive, Suite 5,
Cordova, TN 38018, USA

American College of Toxicology
9650 Rockville Pike,
Bethesda, MD 20814-3998, USA

American Society of Primatologists
Regional Primate Center
University of Washington,
Seattle, WA 98195, USA

American Veterinary Medical Association
1931 North Meachum Road, Suite 100
Schaumburg, IL 60173-4360, USA

Animal and Plant Health Inspection Service
U.S. Department of Agriculture
4700 River Road, Unit 84
Riverdale, MD 20737-1234, USA

Animal Welfare Information Center
National Agricultural Library
U.S. Department of Agriculture
10301 Baltimore Boulevard, Room 205
Beltsville, MD 20705, USA

Australian and New Zealand Council for the Care of Animals
in Research and Teaching, Limited
P.O. Box 19, Glen Osmond
SA 5064, Australia

Canadian Association for Laboratory Animal Science
CALAS National Office
Biosciences Animal Service
University of Alberta
Edmonton, Alberta T6G 2E9, Canada

Canadian Council on Animal Care
Constitution Square
350 Albert Street, Suite 315
Ottawa, Ontario K1R 1B1, Canada

European Center for Validation of Alternative Methods
TP 580, JRC Environmental Institute
21020 Ispra (VA), Italy

Foundation for Biomedical Research
818 Connecticut Avenue NW, Suite 303
Washington, DC 20006, USA

Institute for Laboratory Animal Resources
National Academy of Sciences
2101 Constitution Avenue NW
Washington, DC 20418, USA

Institute of Laboratory Animal Science
University of Zürich
Winterhurerstrasse 190
8057 Zürich, Switzerland

International Council for Laboratory Animal Science
University of Kuopio
SF-70211 Kuopio 10, Finland

Public Responsibility in Medicine and Research
132 Boylston Street, Fourth Floor
Boston, MA 02116, USA

Scientists Center for Animal Welfare
Golden Triangle Building One
7833 Walker Drive, Suite 340
Greenbelt, MD 20770, USA

Society of Toxicology
1767 Business Center Drive
Suite 302
Reston, VA 20190-5332, USA

The Johns Hopkins Center for Alternatives to Animal Testing
111 Market Place, Suite 840
Baltimore, MD 21202-6709, USA

Universities Federation for Animal Welfare
8 Hamilton Close
South Mimms, Potters Bar
Hertfordshire EN6 3QD, UK

APPENDIX

Commercial Sources of Laboratory Animals

Shayne C. Gad
Gad Consulting Services

	Species								
Source	**Rat**	**Mouse**	**Hamster**	**Ferret**	**Dog**	**Rabbit**	**Guinea Pig**	**Primate**	**Minipig**
Ace Animals	X	X							
Alder Ridge					X				
Animal Technologies Ltd.	X	X							
B&K Universal Ltd.		X			X		X		
Barton's West End					X				
Buckshire Corp								X	
Camm Research Lab Animals	X					X	X		
CEDS					X				
Charles River	X	X	X			X	X	X	
Covance Research Products					X			X	
Cytogen			X						
Davidson's Mill							X		
Ellegard Minipigs									X
Elm Hill							X		
Gemini Research						X			
Harlan	X	X	X		X	X	X		
Haycock Kennels					X				
Hazleton					X	X		X	
Hilltop	X	X					X		
Isoquimen					X	X			
Kiser Lake Kennels					X				
Kralek Farms						X			
Liberty Research					X				
Lone Star Laboratory Swine									X
Marshall BioResources				X	X				X
Millbrook Breeding Lab						X			
Myrtle's Rabbitry						X			
Osage Research Primates								X	
Primate Products								X	
Rana Ranch Bullfrogs	X	X	X						

Source	Species								
	Rat	Mouse	Hamster	Ferret	Dog	Rabbit	Guinea Pig	Primate	Minipig
Ridglan Farms					X				
Robinson Services						X			
S&S Farms									X
Sasco	X	X	X				X		
Scientific Resources Intl.								X	
Simonsen Laboratories	X	X	X						
Sinclair Research Center									X
Taconic	X	X							
The Jackson Laboratory	X	X							
Triple F Farms				X					
Western Oregon Rabbit Co						X			
White Eagle					X				
Worldwide Primates								X	
Xenogen	X	X							
Zivic Laboratories	X	X							

[a] This list is representative and is not meant to be exhaustive. The reader is referred to *http://guide.labanimal.com/guide/index.html* for a more complete, searchable listing.

CONTACT INFORMATION

Ace Animals Inc.
PO Box 122
Boyertown, PA 19512 USA
Phone: 610-367-6047
Fax: 610-367-6048
E-Mail: aceanimals@aol.com
Web: *http://www.aceanimals.com*

Alder Ridge Farms Inc.
PO Box 290
Lakewood, PA 18439 USA
Phone: 717-727-3458
Fax: 717-727-3459

Animal Technologies Ltd.
4435 First St., #350
Livermore, CA 94550 USA
Phone: 510-490-3036
Fax: 510-656-1921
Toll Free: 800-USA-MICE
E-Mail: atlca@atbk.net
Web: *http://www.atbk.net*

B&K Universal Ltd.
Grimston, Aldbrough
Hull, East Yorkshire HU11 4QE UK
Phone: +44 1964 527555
Fax: +44 1964 527006
E-Mail: salesbku@aol.com
Web: *http://www.bku.com*

Barton's West End
161 Janes Chapel Rd.
Oxford, NJ 07863 USA
Phone: 908-637-4427
Fax: 908-637-4268
E-Mail: bwef@bwefinc.com

Buckshire Corp.
PO Box 155
Perkasie, PA 18944 USA
Phone: 215-257-0116
Fax: 215-257-9329
E-Mail: sharon@buckshire-corp.com

Camm Research Lab Animals
414 Black Oak Ridge Rd.
Wayne, NJ 07470 USA
Phone: 201-694-0703
Fax: 201-694-8905

CEDS
Les Souches
Mezilles, 89130 France
Phone: +33 386 454058
Fax: +33 386 454058
E-Mail: mc.ceds@wanadoo.fr

Charles River Laboratories
251 Ballardvale St.
Wilmington, MA 01887 USA
Phone: 978-658-6000
Fax: 978-658-7132
Toll Free: 800-522-7287
E-Mail: comments@criver.com
Web: *http://www.criver.com*

Covance Research Products
PO Box 7200
Denver, PA 17517 USA
Phone: 717-336-4921
Fax: 717-336-5344
Toll Free: 800-345-4114
E-Mail: info@crpinc.com
Web: *http://www.crpinc.com*

Cytogen Research and Development, Inc.
89 Bellevue Hill Rd.
West Roxbury, MA 02132 USA
Phone: 617-325-7774
Fax: 617-327-2405

Davidson's Mill Breeding Laboratories
231 Fresh Pond Rd.
Jamesburg, NJ 08831 USA
Phone: 732-821-9094
Fax: 732-821-5922
Toll Free: 888-252-6790

Ellegaard Minipigs USA, Inc.
2025 Ridge Rd.
Perkasie, PA 18944 US
Phone: 215-258-5090/215-258-5091
Fax: 215-257-9329
E-Mail: ellegaard@pil.net or je@minipigs.com

Elm Hill Breeding Labs, Inc.
7 Kidder Rd.
Chelmsford, MA 01824 USA
Phone: 978-256-2322
Fax: 978-256-2545
Toll Free: 800-941-4349
E-Mail: info@elmhilllabs.com
Web: *http://www.elmhilllabs.com*

Gemini Research of Alabama
125 Aspen Ln.
Odenville, AL 35120 USA
Phone: 205-629-3229
E-Mail: matt@brownfamilyenterprises.com
Web: *http://www.brownfamilyenterprises.com*

Harlan
PO Box 29176
Indianapolis, IN 46229 USA
Phone: 317-894-7521
Fax: 317-894-1840
E-Mail: harlan@harlan.com
Web: *http://www.harlan.com*

Haycock Kennels, Inc.
629 Apple Rd.
Quakertown, PA 18951 USA
Phone: 215-536-8228
Fax: 215-536-7337

Hilltop Lab Animals
PO Box 183, RD #1, Hilltop
Scottdale, PA 15683 USA
Phone: 724-887-8480
Fax: 724-887-3582
Toll Free: 800-245-6921
E-Mail: clientserve@hilltoplabs.com
Web: *http://hilltoplabs.com*

Isoquimen SL
E-Mail: isoquimen@isoquimen.com

Kiser Lake Kennels
PO Box 541
St. Paris, OH 43072 USA
Phone: 937-362-3193/937-362-4071
Fax: 937-362-3193
E-Mail: andreaball@kiserlakekennels.com

Kralek Farms, Inc.
630 W. Clausen
Turlock, CA 95380 USA
Phone: 209-634-3609
Fax: 209-634-3609
E-Mail: mgrobner@kralek.com
Web: *http://kralek.com*

Liberty Research, Inc.
PO Box 107, Rte. 17C
Waverly, NY 14892 USA
Phone: 607-565-8131
Fax: 607-565-7420
E-Mail: services@libertyresearch.biz

Lone Star Laboratory Swine
1252 West Kingsbury
Seguin, TX 78155 USA
Phone: 830-401-5958
Fax: 830-401-5920
E-Mail: lonestar@the-cia.net
Web: *http://www.miniswine.com*

Marshall BioResources
5800 Lake Bluff Rd.
North Rose, NY 14516 USA
Phone: 315-587-2295
Fax: 315-587-2109
E-Mail: info@marshallbio.com

Millbrook Breeding Labs
PO Box 513
Amherst, MA 01004 USA
Phone: 413-253-5083
Fax: 413-253-0562
E-Mail: milbrok@aol.com

Myrtle's Rabbitry, Inc.
4678 Bethesda Rd.
Thompson Station, TN 37179 USA
Phone: 615-790-2349
Fax: 615-794-9263
Toll Free: 800-424-9511
E-Mail: rebecca@myrtles.com
Web: *http://www.myrtles.com*

Osage Research Primates, LLC
54 Hospital Dr.
Osage Beach, MO 65065 USA
Phone: 573-348-8002
Fax: 573-348-1622

Primate Products, Inc.
7780 NW 53 St.
Miami, FL 33166 USA
Phone: 305-471-9557
Fax: 305-471-8983
E-Mail: sales@primateproducts.com
Web: *http://www.primateproducts.com*

Rana Ranch Commercial Bullfrogs
PO Box 1043
Twin Falls, ID 83303 USA
Phone: 208-734-0899
Fax: 208-734-0899
Toll Free: 877-737-FROG
E-Mail: bullfrog@safelink.net
Web: *http://www.ranaranch.com*

Ridglan Farms, Inc.
PO Box 318
Mt. Horeb, WI 53572 USA
Phone: 608-437-8670
Fax: 608-437-4731
E-Mail: ridglan@mhtc.net

Robinson Services, Inc.
PO Box 1057
Mocksville, NC 27028 USA
Phone: 336-940-2550
Fax: 336-940-5260
E-Mail: kevrob1@aol.com

S & S Farms
1650 Warnock Dr.
Ramona, CA 92065 USA
Phone: 760-788-7007
Fax: 760-788-7042
E-Mail: tom@snsfarms.com
Web: *http://www.snsfarms.com*

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251 Ballardvale St.
Wilmington, MA 01887 USA
Phone: 978-658-6000
Fax: 800-255-8964
Toll Free: 800-228-4919
E-Mail: comments@criver.com
Web: *http://www.criver.com*

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432 Ridge St.
Reno, NV 89501 USA
Phone: 775-786-3244
Fax: 775-786-2767

Simonsen Laboratories, Inc.
1180-C Day Rd.
Gilroy, CA 95020 USA
Phone: 408-847-2002
Fax: 408-847-4176
E-Mail: info@simlab.com
Web: *http://www.simlab.com*

Sinclair Research Center, Inc.
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Columbia, MO 65205 USA
Phone: 573-387-4400
Fax: 573-387-4404
E-Mail: sinclair@sinclairresearch.com
Web: *http://www.sinclairresearch.com*

Taconic
273 Hover Ave.
Germantown, NY 12526 USA
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Fax: 518-537-7287
Toll Free: 888-822-6642
E-Mail: custserv@taconic.com
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The Jackson Laboratory
600 Main St.
Bar Harbor, ME 04609 USA
Phone: 207-288-6000; 207-288-5845
Fax: 207-288-6150
Toll Free: 800-422-MICE (for orders only)
Web: *http://www.jax.org/jaxmice*

Triple F Farms, Inc.
Rd. #2, Box 432
Sayre, PA 18840 USA
Phone: 570-888-4874
Fax: 570-888-5017
Toll Free: 866-817-1756
E-Mail: *ferrets@tripleffarms.com*

Western Oregon Rabbit Co.
PO Box 653
Philomath, OR 97370 USA
Phone: 541-929-2245
Fax: 541-929-2654

White Eagle Laboratories, Inc.
2003 Lower State Rd.
Doylestown, PA 18901 USA
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Fax: 215-348-5081
E-Mail: asdver@whiteeagletox.com
Web: *www.whiteeagletox.com*

Worldwide Primates, Inc.
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Miami, FL 33197 USA
Phone: 305-378-9585
Fax: 305-232-3838
E-Mail: wwp@gate.net

Xenogen Biosciences
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Phone: 609-860-0806
Fax: 609-860-8515
E-Mail: transgenics@xenogen.com
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PO Box 101084
Pittsburg, PA 15237 USA
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Toll Free: 800-422-5227
E-Mail: zivic@zivic.com
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