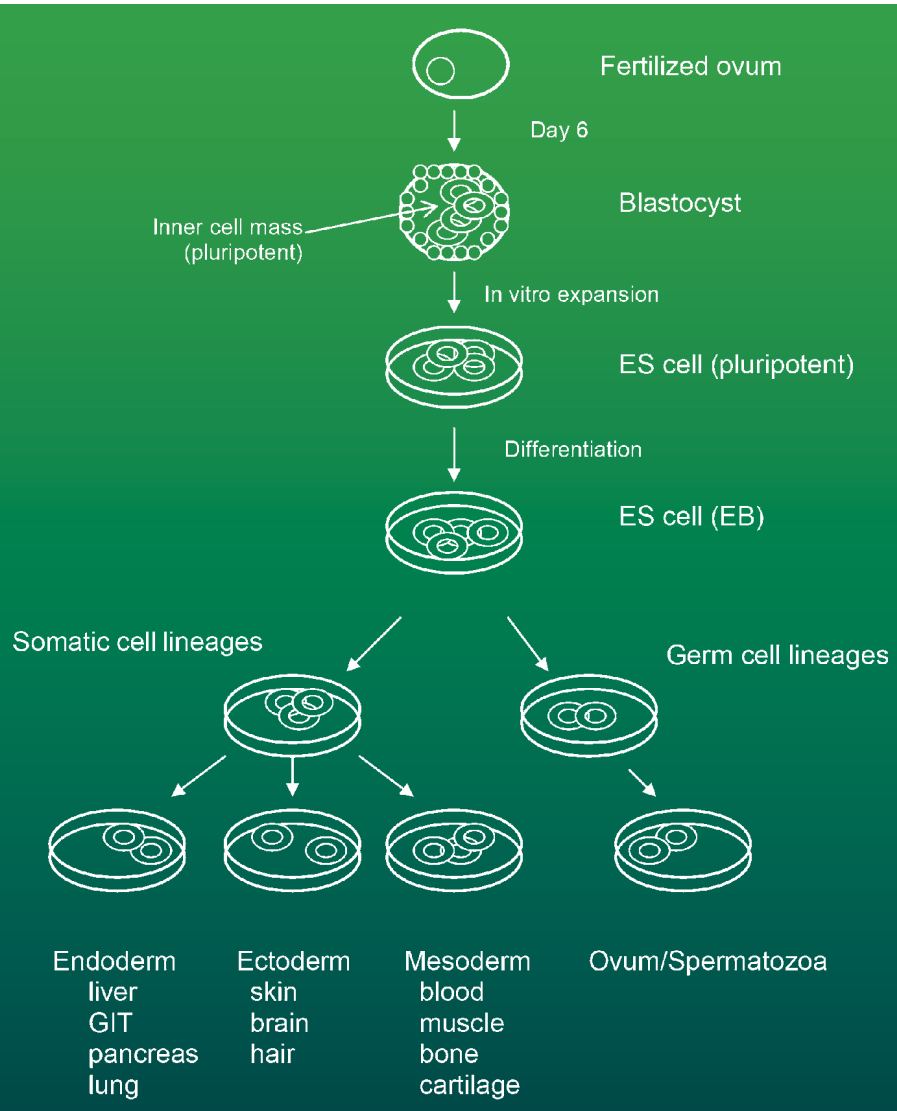
SECOND EDITION

PRINCIPLES OF Toxicology Testing



FRANK A BARILE

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Principles of Toxicology Testing

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Second Edition

Frank A. Barile, PhD
Department of Pharmaceutical Sciences, St. John's University, College of Pharmacy and Health Sciences, Queens, New York, USA

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Dedication

To Pauline

Contents

Preface

In the first edition, I commented in the Preface that the science of toxicology testing has evolved in the last few decades from an applied and supportive science to its own refined and technical discipline. I also mentioned that the development and maturity of the discipline has been sporadic. In the last 5 years since the first edition, however, the latter description has not held true. In fact, the field has soared in its applications of the techniques and principles that define the field. Its progress has been prompted not by public health initiatives and needs but by the discovery of new disciplines, such as epigenetics, toxicogenetics, and toxicodynamics (for which new chapters have been added in the second edition). Thus, new roles have emerged for the application of both *in vivo* and *in vitro* techniques. These new roles have allowed for unique investigations in toxicology testing, followed by introduction of exciting principles to the field. No sooner have the burgeoning advances of biotechnology allowed for the development of corresponding *in vitro* systems that complement traditional animal toxicology testing methods, but the new disciplines have been fitted as well to important applications within the toxicology arena.

As in the first edition, the book begins with an introduction into the fundamentals of toxicology (Section I) to prepare students for the subsequent topics and continues through with a discussion of toxicokinetics and human risk assessment. This introductory material is useful in understanding the applications of toxicology testing.

Section II describes the fundamental principles of toxicology testing in animals in greater detail. This section describes acute toxicity studies as well as subchronic and chronic studies performed in animals. Special emphasis is placed on study design and determination of classical indicators for acute and chronic testing, such as the LD50. Other short- and long-term animal toxicity testing methodologies including dermal, ocular, and reproductive toxicity testing are discussed. Mutagenicity and carcinogenicity studies are also discussed in separate chapters.

Section III introduces and discusses *in vitro* alternatives to animal toxicology tests. This section emphasizes cell culture methodology and cellular methods for acute systemic toxicity, target organ toxicity, and local toxicity. The advantages and disadvantages of alternative methods are presented. Special features of this section describe the use of high-throughput screening and its applications, the concepts of standardization and validation of *in vitro* techniques, especially large, organized validation efforts currently supported by US and EU regulatory agencies, and the theories supporting the development of *in vitro* methodologies. Undergraduate and graduate toxicology students and industrial and academic research laboratories will find the text useful for the entry level students in the discipline or for establishing a toxicology testing laboratory, respectively.

The juxtaposition of the principles of animal toxicology testing in the same text as *in vitro* alternative methods highlights the importance of both fields for interpretation of the significance and relevance of the other. Thus, the discussions continuously refer to the corresponding methods available and the potential results from complementary designs of studies. In fact, both animal and *in vitro* toxicology testing methods are currently employed,

often together, in toxicological analysis, derivation of mechanisms of toxicity, mutagenicity testing, and preclinical drug development.

Several excellent texts are available in the field concerning the details of individual protocols. Consequently, although some procedures are outlined in detail, the emphasis is on the principles of the disciplines rather than on the particular steps of the techniques. In fact, the title, *Principles of Toxicology* (rather than *Toxicity*) *Testing*, emphasizes the universal application of the field as a scientific discipline as opposed to amplification of laboratory techniques. In addition, the book highlights contemporary issues in toxicology testing including the various means of possible exposure to chemicals, high-throughput screening of chemicals for preclinical drug development, and an overview of applications. Overall, the reader is challenged to interpret the significance of toxicology testing results and to construct a logical approach toward the ultimate purpose of testing. Thus, the information contained herein is presented with great enthusiasm, particularly for the students prepared to dedicate their careers to this intriguing and fascinating scientific discipline.

Acknowledgments

I would like to extend my appreciation to the editorial staff at Informa Healthcare, Inc., for their interest, professionalism, and commitment to the project, in particular to Ms. Amber Thomas, Ms. Claire Bonnett, and Mr. Oscar Heini, as well as the staff at Exeter Premedia Services. My colleagues and staff at the College of Pharmacy, Department of Pharmaceutical Sciences, afforded invaluable comments, suggestions, and support throughout the project. Finally, I have had the pleasure to know and been fortunate to guide undergraduate and graduate toxicology and pharmacy students during the writing of this text, especially Angela Aliberti, Sanket Gadhia, Tak Lee, and Sanjay Dholakiya. This work would not have progressed fluently or competently without their continuous feedback.

Biography

Frank A. Barile, Ph.D., is a full professor in the Toxicology Division of the Department of Pharmaceutical Sciences at St. John's University College of Pharmacy and Health Sciences, New York.

Dr. Barile received his B.S. in Pharmacy (1977), M.S. in Pharmacology (1980), and Ph.D. in Toxicology (1982) at St. John's University. After a postdoctoral fellowship in Pulmonary Pediatrics at the Albert Einstein College of Medicine, Bronx, New York, he moved to the Department of Pathology, Columbia University, St. Luke's Roosevelt Hospital, New York, as a research associate. In these positions, he investigated the role of pulmonary toxicants on collagen metabolism in cultured lung cells. In 1984, he was appointed as an assistant professor in the Department of Health Sciences at City University of New York. Sixteen years later, he rejoined St. John's University in the Department of Pharmaceutical Sciences and became an instrumental part of the toxicology program in the College of Pharmacy.

Dr. Barile holds memberships in several professional associations, including the US Society of Toxicology, American Association of University Professors, American Association for the Advancement of Science, American Society of Hospital Pharmacists, New York City Pharmacists Society, New York Academy of Sciences, and New York State Council of Health System Pharmacists. He has been appointed as a consultant scientist with several professional groups, including the Department of Pediatrics, Schneider's Children's Hospital, Long Island Jewish/Cornell Medical Centers, New York, as a committee member of SACATM, ICCVAM/NICEATM, and NIEHS, and as President and Past-President of the *In Vitro* and Alternative Methods Specialty Section, US Society of Toxicology. He is also the recipient of the *Public Health Service Medallion* from the Director of the NIEHS, Dr. Linda Birnbaum (2009). He was recently appointed as Editor-In-Chief (Rest of the World) for *Toxicology In Vitro*.

Dr. Barile has been the recipient of Public Health Service research grants from the National Institutes of Health (NIGMS), including awards from the Minority Biomedical Research Support program, the Minority High School Student Research Apprentice program, and AREA program.

Dr. Barile has authored and coauthored approximately 75 papers and abstracts in peer-reviewed biomedical and toxicology journals as well as three books and one contributed chapter. He contributed original *in vitro* toxicology data to the international Multicenter Evaluation for Cytotoxicity program, along with distinguished international investigators. He lectures regularly to toxicology and pharmacy undergraduate and graduate students in the toxicological and pharmaceutical sciences (he was awarded Professor of the Year for the College of Pharmacy by the University Student Government Association in 2003). Dr. Barile continues to perform fundamental research on the cytotoxic effects of environmental chemicals and therapeutic drugs on cultured human and mammalian stem cells.

1 Introduction to principles of toxicology

INTRODUCTION

From its inception in the medieval era to its maturity as a distinct and separate discipline in the twenty first century, toxicology was taught primarily as an applied science. The field incorporated the various approaches from a variety of disciplines, not limited to the broad sciences of chemistry and biology. In particular, toxicology evolved from applications of analytical and clinical chemists whose job definitions included chemical identification and analysis of body fluids.

The first modern toxicologists were chemists who had specialized training in inorganic separation methods including chromatographic techniques. Analytical chemists later employed thin layer and gas chromatography. The development of methods for forensic analysis greatly promoted the field. These advances were particularly important for acceptance within the legal community and in jurisprudence arenas. Furthermore, as the instrumentation evolved and the technology became more exacting, high-performance liquid chromatography was incorporated into the analytical arsenal in order to isolate minute quantities of compounds from complex mixtures of toxicological importance. Eventually, biological applications exerted their influence, incorporating such specialties as microbiology, genetics, and cell culture methodology. Today, the field has burgeoned into areas of specialization, some of which are hardly identifiable with their ancestral origins.

TYPES OF TOXICOLOGY

General Toxicology

General toxicology involves studies of exposure to chemical, biological, or physical agents and the untoward consequences that affect biological systems. The term, however, has been replaced by descriptions of areas that more closely reflect the specialized fields of study within the discipline. The development of advanced methodologies in biotechnology, the requirement for increased training, and the involvement of toxicology in legal applications have made it necessary to more accurately label the discipline according to the expanding body of specialties. As a result, a variety of descriptions further define the field of toxicology.

Mechanistic Toxicology

Mechanistic toxicology involves the identification of the cause, pathway, reaction, and cellular modification associated with the toxicity of a chemical at the level of the cell, tissue, or organ. The classification of toxicity of a chemical, therefore, may be expressed in terms of its mechanism of toxicity, its site of action, target area, or the organ most affected by the toxic insult. A similar expression, *mechanism of action*, is universally applied in the study of

pharmacology. Thus, mechanistic toxicology seeks to determine the biochemical, physiological, or organic basis of a toxic agent's effects on biological systems.

Regulatory Toxicology

Regulatory toxicology relates to the administrative dogma associated with the potential exposure to toxic agents encountered in the environment, in occupational settings, and in the home. Regulatory toxicology defines, directs, and dictates the rate at which an individual may encounter a synthetic or naturally occurring toxin and establishes guidelines for its maintenance in the environment, for risks due to possible exposure within the community and within the remedial market. The guidelines are generally promulgated by agencies whose jurisdiction and regulations are established by federal, state, and local authorities.

Descriptive Toxicology

Descriptive toxicology is a subjective attempt to explain toxic agents and their applications. The list of descriptive areas developed principally as a method for bridging the vacuum between science and the public's understanding of the field, especially when it became necessary for nonscientific sectors to comprehend and interpret the importance of toxicology. This is especially true for public sector interpretation so that they could translate the information for the development of regulations and guidelines. For instance, the study of metals in the environment (metal toxicology) has become a popular discipline for toxicologists interested in examining the roles of heavy or trace metals in the environment.

Forensic Toxicology

From its inception in the medieval era to its maturity as a distinct and separate discipline in the 1950s, toxicology was taught primarily as an applied science. More recently, toxicology has evolved from applications of analytical and clinical chemists whose job definition was the chemical identification and analysis of body fluids. Thus the first modern toxicologists were chemists with specialized training in inorganic separation methods, including chromatographic techniques. Eventual evolution toward liquid chromatographic methods allowed analysis and isolation of minute quantities of compounds from complex mixtures of toxicological importance. Forensic toxicology thus integrates these techniques to identify compounds of sometimes unrelated poisons from biological specimens as a result of incidental or deliberate exposure. Initially, forensic sciences profited from the application of the principles of chemical separation methods for the identification of controlled substances in body fluids. Later, forensic toxicology applied biological principles of antigen–antibody interaction for paternity testing. By using the principles of blood grouping and "exclusion" of the potential outcomes of paternal contribution to offspring phenotype, it was feasible to eliminate the possibility of a male as the father of a child. Antigen–antibody interactions also became the basis for enzyme-linked immunosorbent assays (ELISA) currently used for specific and sensitive identification of drugs in biological fluids. Radioimmunoassays (RIAs) utilize similar antigen–antibody reactions while incorporating radiolabeled ligands as indicators. DNA separation and sequencing techniques have now almost totally replaced traditional paternity exclusion testing. These methods are also the basis for inclusion or exclusions of evidence in criminal and civil cases.

Clinical Toxicology

Clinical toxicology is also considered a descriptive category. However, toxic agents with clinical applications are also characterized in other toxicological fields. Clinical toxicology has evolved and branched from its counterpart, forensic toxicology, to include identification,

TABLE 1.1
Other Descriptive Fields of Toxicology

Descriptive Field	Definition
Genetic toxicology	Incorporates molecular biology principles in applications of toxicological sciences, as applied to toxic agents that interfere with normal physiological function
Occupational toxicology	Examines hazards associated with toxic exposure in the workplace, including industrial, agricultural, and communal sectors
In vitro toxicology	Development of cell culture and biochemical techniques as alternatives to animal toxicity testing; a current definition of this field includes *alternative methods to animal toxicology*, which more accurately describes the applications of *in vitro* methods
Analytical toxicology	Chemical and biochemical procedures and methods associated with identification, analysis, reactions, and detection of toxic substances in specimens
Developmental toxicology	Study of toxic substances and their potential effects on biological reproduction, mating, fetal development, and embryogenesis
Immunotoxicology	Study of toxic substances and their potential effects on immunity and resistance
Neurotoxicology	Study of toxic substances and their potential effects on the function and activity of the nervous systems

diagnosis, and treatment of a condition, pathology, or disease resulting from environmental, therapeutic, or illicit exposure to chemicals or drugs. Treatment may involve amelioration of the signs and symptoms or controlling the underlying pathology. In clinical toxicology, exposure is commonly understood to include individual risk of contact with a toxin, either deliberate, accidental, or intentional. Exposure may further be defined to include population risk.[1] Table 1.1 defines other descriptive fields of toxicology. More recently, the field has blossomed into more broadly defined areas including the study of apoptosis, receptor-mediated signal transduction, gene expression, proteomics, oxidative stress, and toxicogenomics, among others.

COMMON TERMS AND NOMENCLATURE

The classic definition of toxicology has traditionally been understood as the study of xenobiotics, the science of poisons, and refers particularly to the interactions of exogenous agents with biological systems. For purposes of organizing the nomenclature, chemicals, compounds, and drugs are often referred to as *agents* or *substances*. Because such agents induce undesirable effects, they are usually alluded to as *toxins*. Consequently, toxicology involves internal and external physiological exposures to toxins and their interactions with organisms. Gradually, the term has evolved to include many chemically or physically unrelated classes of agents. What transforms a chemical into a toxin, therefore, depends more on the duration of exposure, dose (or concentration), or route of exposure and less on the chemical structure, product formulation, or intended use of the material. As a result, almost any agent has the potential for toxicity and thus falls within the broad definition of toxicology.

[1]Risk to groups of persons from exposure to radiation, pollutants, and chemical or biological threats, which necessitates identification, diagnosis, and treatment.

APPLICATIONS OF TOXICOLOGY

Research

Academic Applications

In the academic arena, research toxicologists examine the broad issues of toxicology in the laboratory setting. Academic concerns include all the public health areas in which progress in understanding toxicological sciences is necessary and include the elucidation of mechanistic, clinical, and descriptive toxicological theories. Research methods are modified to answer specific questions that arise from toxicological concerns that affect public health.

Industrial Applications

Research toxicologists employed in toxicity testing in the biotechnology and pharmaceutical industries perform toxicity testing—the screening of chemicals and drugs that have toxic potential—before they are marketed. Preclinical testing in the pharmaceutical industry involves the conduct of phase I trials to test the toxicities of candidate agents that have been chemically and biochemically screened as potentially useful therapeutic drugs. The toxicity testing procedures include both in vitro and animal protocols.

Regulatory Toxicology

Regulatory toxicologists are employed primarily in government administrative agencies, as consultants to government and industry or as representatives of industrial concerns. In this role, they sanction, approve, and monitor the uses of chemicals by establishing rules and guidelines. The guiding principles are promulgated through laws enacted by appropriate federal, state, and local jurisdictions that grant regulatory agencies their authority. Thus, through these regulations, an agency determines who is accountable and responsible for manufacturing, procurement, distribution, marketing, and, ultimately, release and dispensing of chemical substances to the public.

Forensic Toxicology

Forensic toxicologists integrate appropriate techniques to identify compounds arising from mixtures of sometimes unrelated poisons as a result of incidental or deliberate exposure. Initially, forensic sciences profited from the application of the principles of chemical separation methods for the identification of controlled substances in body fluids. Later, forensic toxicologists applied biological principles of antigen–antibody interaction for paternity testing. By using the principles of blood grouping and the exclusion of the possible outcomes of paternal contributions to offspring phenotypes, it became possible to eliminate a male as a possible father of a child.

Antigen–antibody interactions also became the basis for ELISA and enzyme multiplied immunoassay technique, which are currently used for specific and sensitive identification of drugs in biological fluids. RIAs utilize similar antigen–antibody reactions while incorporating radiolabeled ligands as indicators. DNA separation and sequencing techniques have now almost totally replaced traditional paternity exclusion testing. These methods are also the basis for inclusion or exclusion of evidence in criminal and civil cases.

Clinical Toxicology

Clinical toxicologists have evolved and branched away from their corresponding forensic applications. The clinical toxicologist is interested in the identification, diagnosis, and treatment of a condition, pathology, or disease resulting from an environmental, therapeutic, or illicit exposure to chemicals or drugs. Exposure is commonly understood to include the individual risk of contact with a toxin.

CLASSIFICATION OF TOXIC AGENTS

Classification of toxic agents is a daunting task, considering the vast numbers and complexities of chemical compounds in the public domain. The availability of the variety of chemicals, drugs, and physical agents, along with their varied toxicological and pharmacological effects, means that a single agent may be listed in several different categories. Even compounds with similar structures or toxicological actions may be alternatively grouped according to their activities or physical states. The following outline of the classification system is generally accepted for demonstrating the complex nature of toxins.

Classification According to Use

Pesticides

The U.S. Environmental Protection Agency defines a pesticide as a substance or a mixture of substances intended to prevent, destroy, repel, or mitigate a pest. In general, pesticides are classified according to their biological targets. The four major classes of pesticides are insecticides, herbicides, rodenticides, and fungicides. Table 1.2 lists these categories and their general subclasses. Because of the physiological and biochemical similarities of target species and mammalian organisms, an inherent toxicity is associated with pesticides in mammalian organisms. In addition, within each classification, compounds are identified according to mechanism of action, chemical structure, or semisynthetic source. For instance, although many fungicide categories exist, fungicidal toxicity in humans is mostly low order, with the exception of therapeutic antifungal agents, principally because of their specific mechanisms of action. Similarly, fumigants range from carbon tetrachloride to ethylene oxide and are used to kill insects, roundworms, and fungi in soil, stored grain, fruits, and vegetables. Their toxicity, however, is limited to occasional occupational exposure.

Food and Industrial Additives

Direct food and color additives are intentionally incorporated in foods and food-processing operations for purposes of changing, enhancing, or masking color. They are also used for a variety of functionalities ranging from anticaking agents to stabilizers, thickeners, and texturizers. Food and industrial additives fall into the field of food toxicology and readers are referred to review articles listed at the end of this chapter for information concerning food ingredients and contaminants.

Therapeutic Drugs

Toxicological classification of therapeutic agents follows their pharmacological mechanisms of action or their principal target organs of toxicity. Several important references address clinical toxicologies of therapeutic drugs as extensions of their adverse reactions and direct effects resulting from their excessive use.

TABLE 1.2
Classification of Pesticides

Pesticide Class	Classification According to Target
Insecticide	Organophosphorus esters
	Organochlorine compounds
	Carbamate esters
	Pyrethroid esters
	Botanical derivatives
Herbicide	Chlorophenoxy compounds
	Bipyridyl derivatives
	Chloroacetanilides
	Phosphonomethyl amino acids
Rodenticide	Anticoagulants
	α-Napthyl thiourea
	Miscellaneous metals, inorganic, natural products
Fungicide	General areas of agricultural, domestic, and therapeutic antifungal agents

SOURCES OF TOXINS

Botanical

Contact dermatitis caused by poison ivy is a well-characterized syndrome of acute inflammation. Today, many compounds of botanical origin are classified as herbal supplements, implying that their origins are botanical. Their importance in maintaining health is also related to their natural derivations. The toxicities of these agents, however, are poorly understood.

Environmental

As a result of industrialization, many chemicals are associated with and classified according to their continuous presence in the environment, that is, water, land, and soil. The phenomenon is not limited to developed Western nations and is becoming a problem among developing South American, Asian, and African countries.

Environmental toxicology is a distinct discipline encompassing the areas of air pollution and ecotoxicology. A discussion of air pollution necessarily includes outdoor and indoor air pollution, presence of atmospheric sulfuric acid, airborne particulate matter, interaction of photochemicals with the environment, and chemicals found in smog. *Ecotoxicology* is the branch of environmental toxicology that investigates the effects of environmental chemicals on the ecosystems in question. Readers are referred to the review articles at the end of this chapter for further discussion.

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2 Effects of chemicals

TOXICOLOGICAL EFFECTS

General Classification

As noted in chapter 1, chemical and physical agents are categorized in part according to several indicators of toxicity that render them amenable for understanding their potential toxic effects. Prediction of toxicity of a chemical lies in the ability to recognize its potential adverse effects based on factors not necessarily related to physicochemical properties. This chapter explores a variety of local and systemic reactions elicited by chemical exposure and the physiological and immunological basis of such effects.

Chemical Allergies

The four types of immunological hypersensitivity (allergic) reactions include the following:

1. Type I antibody-mediated reactions.
2. Type II antibody-mediated cytotoxic reactions.
3. Type III immune complex reactions.
4. Type IV delayed-type hypersensitivity cell-mediated immunity.

Type I antibody-mediated reactions occur in three phases. The initial or sensitization phase is triggered by contact with a previously unrecognized antigen. This reaction entails binding of the antigen to the immunoglobulin E (IgE) present on the surfaces of mast cells and basophils. The second or activation phase follows after an additional dermal or mucosal challenge with the same antigen. This phase is characterized by degranulation of mast cells and basophils, with a subsequent release of histamine and other soluble mediators. The third stage, the effector phase, is characterized by accumulation of preformed and newly synthesized chemical mediators that precipitate local and systemic effects. Degranulation of neutrophils and eosinophils completes the late-phase cellular response.

Antigens involved in type I reactions are generally airborne pollens including mold spores and ragweeds as well as food ingredients. Ambient factors such as heat and cold, drugs (opioids, antibiotics), and metals (silver, gold) precipitate chemical allergies of the type I nature. Because of their small molecular weights, the majority of chemicals and drugs, as single entities, generally circulate undetected by immune surveillance systems. Consequently, in order to initiate the sensitization phase of an antigenic response, chemicals are immunologically handled as haptens.[1] Examples of type I hypersensitivity syndromes are described in Table 2.1 and typical effects of chemical allergies are listed in Table 2.2.

[1]Haptens are small molecular weight chemical entities (<1000 Da) that nonspecifically bind to larger circulating polypeptides or glycoproteins. Binding induces a conformational change in the original larger complex. The chemical entity bound to the larger molecule is no longer recognized as part of the host (self) system, rendering it susceptible to immune attack.

TABLE 2.1
Examples of Type I Hypersensitivity Syndromes, Causes, and Effects

Syndrome	Causes	Effects
Allergic rhinitis (hay fever)	Pollen, mold spores	↑ Capillary permeability in nasal and frontal sinuses and mucosal membranes, ↑ vasodilation
Food allergies	Lectins and proteins present in nuts, eggs, shellfish, dairy products	↑ Capillary permeability, ↑ vasodilation, ↑ smooth muscle contraction
Atopic (allergic) dermatitis	Localized exposure to drugs and chemicals	Initial local mast cell release of cytokines, followed by activation of neutrophils and eosinophils
Asthma	Chemicals, environmental, behavioral	Chronic obstructive reaction of LRT involving airway hyperactivity and cytokine release

Abbreviation: LRT, lower respiratory tract.

TABLE 2.2
Descriptive Inflammatory Effects of Chemical Allergies

Effect	Description
Bullous	Large dermal blisters
Erythema	Redness or inflammation of skin due to dilation and congestion of superficial capillaries (e.g., sunburn)
Flare	Reddish, diffuse blushing of the skin
Hyperemia	Increased blood flow to tissue or organ
Induration	Raised, hardened, thickened skin lesion
Macule	Flat red spot on skin due to increased blood flow
Papule	Raised red spot on skin due to increased blood flow and antibody localization
Petechial	Small, pinpoint dermal hemorrhagic spots
Pruritus	Itching
Purulent	Suppuration; production of pus containing necrotic tissue, bacteria, inflammatory cells
Urticaria	Pruritic skin eruption characterized by wheal formation
Vesicular	Small dermal blisters
Wheal	Raised skin lesion due to accumulation interstitial fluid

The effects of chemical allergies are usually acute and appear immediately when compared with other hypersensitivity reactions described below.

Type II antibody-mediated cytotoxic reactions differ from type I in the nature of antigen, the cytotoxic character of the antigen–antibody reaction, and the type of antibody formed (IgM, IgG). In general, antibodies are induced against target antigens that are altered cell membrane determinants. Type II reactions include complement–mediated (CM) reactions, antibody-dependent cell-mediated cytotoxicity, antibody-mediated cellular dysfunction, transfusion reactions, Rh incompatibility reactions, autoimmune reactions, and chemical-induced reactions, the last of which is of greater interest in experimental toxicology.

As with type I reactions, chemical-induced type II cytotoxicity requires that an agent behave as a hapten. The chemical binds to the target cell membrane and proceeds to operate as an altered cell membrane determinant. This determinant changes the conformational appearance of a component of the cell surface, not unlike the effect described above for a hapten. Thus, it induces a series of responses that terminate in antibody induction. The determinant attracts a variety of immune surveillance reactions including a CM cytotoxic reaction, recruitment of granulocytes, or deposit of immune complexes within the cell membrane. A variety of therapeutic drugs are known to induce type II reactions, particularly with continuous administration. Examples include antibiotics and cardiovascular agents, among others.

Type III immune complex reactions are localized responses mediated by antigen–antibody immune complexes. Type III reactions are stimulated by microorganisms and involve activation of complement. Systemic (serum sickness) and localized (Arthus reaction) immune complex disease, infection-associated immune complex disease (rheumatic fever), and occupational diseases (opportunistic pulmonary fungal infections) induce complement–antibody–antigen complexes that trigger the release of cytokines and recruitment of granulocytes, resulting in increased vascular permeability and tissue necrosis.

Type IV (delayed-type) hypersensitivity cell-mediated immunity involves antigen-specific T-cell activation. The reaction starts with an intradermal or mucosal challenge (sensitization stage). $CD4^+$ T cells then recognize MHC-II (major histocompatibility class-II) antigens on antigen-presenting cells (such as Langerhans cells) and differentiate to T_H1 cells. This sensitization stage requires a prolonged local contact with the agent, from several days to weeks. A subsequent repeat challenge stage induces differentiated T_H1 (memory) cells to release cytokines, further stimulating the attraction of phagocytic monocytes and granulocytes. The release of lysosomal enzymes from the phagocytes results in local tissue necrosis. Contact hypersensitivity resulting from a prolonged exposure to natural products and metals, for example, is caused by the lipophilicity of the chemicals in skin secretions, thus acting as a hapten.

Idiosyncratic Reactions

Idiosyncratic reactions are abnormal responses to chemicals or drugs generally resulting from uncommon genetic predisposition. An exaggerated response to the skeletal muscle relaxant properties of succinylcholine, a depolarizing neuromuscular blocker, classifies as a typical idiosyncratic reaction. In some subjects, a congenital deficiency in plasma cholinesterase results in a reduction in the rate of succinylcholine deactivation. As succinylcholine accumulates, respiration fails to return to normal during the postoperative period. Similarly, the cardiotoxic action of cocaine is exaggerated in cases of congenital deficiency of plasma esterases, which are necessary for metabolism of the drug. A paucity of circulating enzymes allows for uncontrolled, sympathetically mediated effects in experimental animals and humans.

Immediate vs. Delayed Hypersensitivity

In contrast to immune hypersensitivity reactions, some chemical effects are immediate or delayed, depending on the mechanism of toxicity. The acute effects of sedative hypnotics are of immediate consequence—high concentrations in animals or humans result in death from respiratory depression. The effects of carcinogens, however, may not be demonstrated for generations in humans or only after several years of exposure in rodents. An important example of this has been demonstrated with the link between diethylstilbestrol administration to

child-bearing women in the 1950s and the subsequent development of clear cell adenocarcinoma vaginal cancers in the offspring.

Reversible Versus Irreversible Effects

In general, the effects of most chemicals or drugs are reversible until a critical point is reached, that is, when a vital function is compromised or a mutagenic, teratogenic, or carcinogenic effect develops. In fact, the carcinogenic effects of chemicals such as those present in tobacco smoke may be delayed for decades until irreversible cellular transformation occurs. Reversibility of the acute effects of chemicals is achieved through the administration of antagonists, enhancement of metabolism or elimination, delay of absorption, intervention of another toxicological procedure that decreases toxic blood concentrations, or termination of the exposure.

Local Versus Systemic Effects

As discussed below, local or systemic effects of a compound depend on the site of exposure. The integument (skin) and lungs are targets of chemical exposure because they frequently serve as the first sites of contact with environmental chemicals. Oral exposure requires absorption and distribution of an agent prior to the development of systemic effects. Hypersensitivity reaction types I and IV are precipitated by local activation of immune responses following a sensitization phase, while chemical-induced type II reactions are elicited through oral or parenteral administration.

Mutagenic and Carcinogenic Effects

Mutagenic and carcinogenic effects of chemical exposure are discussed in detail in chapter 11.

BIOCHEMICAL PROPERTIES

Chemical Structure

Important components of a toxicity testing protocol include the identification, categorization, synthesis, and toxicity screening of chemicals according to their classifications. Because chemical and drug development depends upon the availability of parent compounds, the synthesis of structurally related compounds is an important process. Thus, understanding the chemical structure of a compound allows for reasonable prediction of many of its anticipated and adverse effects. Some examples of agents that are categorized according to their molecular structures include organophosphorus insecticides, heavy metals, benzodiazepines (sedative hypnotics), and imidazolines (tranquilizers).

Mechanism of Action

Similarly, it is convenient to organize toxic agents according to their physiological or biochemical targets. Examples include mutagens, hepatotoxic compounds, methemoglobin-producing agents, and acetylcholinesterase inhibitors.

EXPOSURE

In the presence of specific circumstances, any chemical has the potential for toxicity, that is, the same dose of a chemical may be harmless if limited to oral exposure but toxic if

inhaled or administered parenterally. Thus the route and site of exposure exert significant influence in determining the toxicity of a substance. More frequently, a therapeutic dose for an adult may be toxic for an infant or a child. Similarly, a substance may not exert adverse effects until a critical threshold is achieved. Thus, in order to induce toxicity, it is necessary for the chemical to accumulate in a physiological compartment at a concentration sufficiently high to reach the threshold value. Finally, repeated administration over a specified period of time also determines the potential for toxicity. The following discussion details the circumstances for exposure and dose of a chemical that favors or deters the potential for toxicity.

Route

Oral Administration

Undoubtedly, oral exposure to toxins is the most frequently encountered route of contact. Oral administration involves the presence of several physiological barriers that must be penetrated or circumvented if adequate blood concentrations of a compound is achieved (Fig. 2.1). The mucosal layers of the oral cavity, pharynx, and esophagus consist of stratified squamous epithelium that serves to protect the upper gastrointestinal (GI) lining from the effects of contact with physical and chemical agents. Simple columnar epithelium lines the stomach and villi of the intestinal tract that function in digestion, secretion, and absorption. Immediately underlying the epithelium is the lamina propria, a mucosal layer rich in blood vessels and nerves. Mucosa-associated lymphoid tissue is layered within this level, where prominent lymphatic nodules sustain the presence of phagocytic macrophages and granulocytes. Salivary and intestinal glands contribute to the digestive process by secreting saliva and digestive juices. The submucosa, muscularis, and serosa complete the strata that form the anatomical envelope of the GI tract. Enteroendocrine and exocrine cells in the GI tract secrete hormones and, in the stomach, secrete acid and gastric lipase.

The primary function of the stomach is mechanical and chemical digestion of food, while absorption is secondary. Several factors influence the transit and stability of a chemical in the stomach, thereby influencing gastric emptying time (GET). The presence of food delays absorption and dilutes the contents of the stomach, thus reducing subsequent chemical transit. An increase in the relative pH of the stomach causes a negative feedback inhibition of stomach churning and motility, which also delays the gastric emptying process. Any factor that slows down stomach motility will increase the amount of time that a chemical stays in the stomach, prolonging the GET. Thus, the longer the GET, the greater the duration of a chemical's presence within the stomach, and the more susceptibility to gastric enzyme degradation and acid hydrolysis, in spite of any increase in pH. In addition, a prolonged GET delays passage to and subsequent absorption in the intestinal tract.

Intranasal Administration

Intranasal insufflation is a popular method for therapeutic administration of corticosteroids and sympathomimetic amines and for the illicit use of drugs of abuse such as cocaine and opioids. The dosage form in therapeutic use is usually aerosolized in a metered nasal inhaler. In the case of illicit use, crude drugs are inhaled (snorted) through the nares as fine or coarse powders. In both cases, absorption is rapid due to the extensive network of capillaries in the lamina propria of the mucosal lining within the nasopharynx. Thus the absorption rate rivals that of pulmonary inhalation.

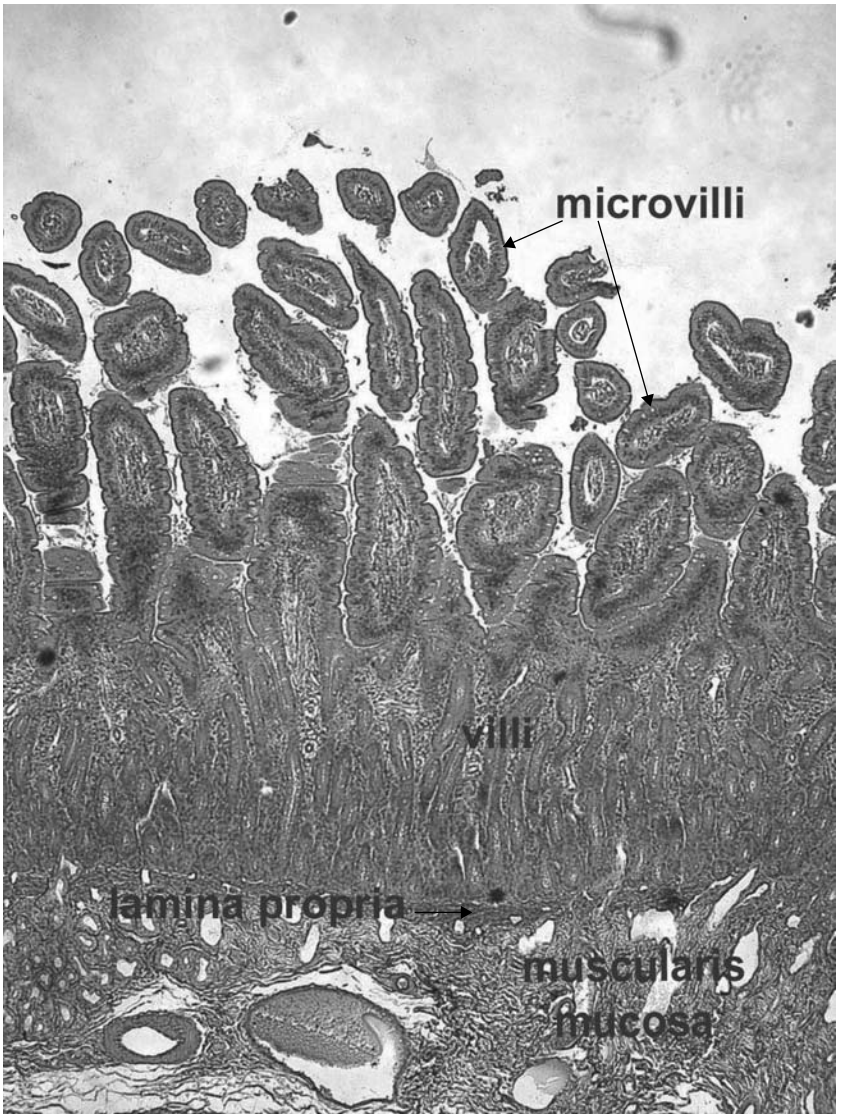

FIGURE 2.1 Histological slide of intestinal villi in longitudinal section and cross section (rounded structures above). The anatomy of the small intestine illustrates the interface of luminal contents with the microvilli, composed of simple columnar epithelial cells that coat the surface of the villi. The microvillus membrane increases the surface area of the intestinal tract 600-fold to allow for sufficient secretion and absorption. *Source*: Photo courtesy of Dr Diane Hardej.

Inhalation

The vast surface area of the upper and lower respiratory tracts allows for wide and immediate distribution of inhaled powders, particulates, aerosols, and gases. Figure 2.2 illustrates the thin alveolar wall that separates airborne particulates from access to capillary membranes. Once a drug is ventilated to the alveoli, it is transported across the alveolar epithelial lining to the capillaries, resulting in rapid absorption.

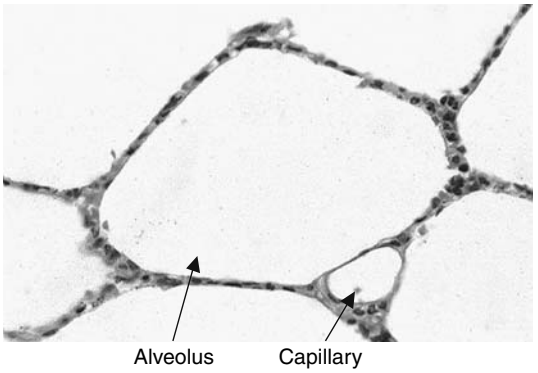

FIGURE 2.2 Pulmonary alveolus showing thin capillary–air interface. *Source*: From Barile, Frank A., *Clinical Toxicology: Principles and Mechanisms*, 2nd edition, CRC Press, 2010.

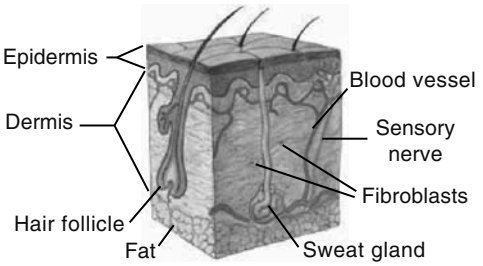

FIGURE 2.3 Cross section of dermal epithelium and underlying basal lamina structures. Epidermis, dermis, and underlying (subcutaneous) layers of skin. *Source*: From Artificial Skin, News Features and Articles, NIH, NIGMS, 2003.

Dermal Routes

As an organ, the integumentary system (skin) allows for the greatest surface area for physiological contact with the external environment. The skin affords a wide range of therapeutic interventions through application of chemicals and drugs. Topical administration is the most common form of dermal application, yet also include epidermal, intradermal, and transdermal injections—upper dermal injections are still considered topical applications.

Figure 2.3 illustrates the layers of the skin: the epidermis, the outermost layer, and the underlying dermis and subcutaneous (hypodermis) layers. Epidermal and upper dermal injections have the poorest absorption capabilities of the parenteral routes primarily because of limited circulation. Consequently, intradermal and epidermal injections are limited to eliciting reactions that might occur on the skin surface, such as testing for immunological reactions.[2] Yet surface application of fat-soluble substances has a propensity for dermal absorption, principally through the oil-rich content of the dermal layers. Thus lipid-soluble chemicals are slowly but readily absorbed by non-ionic passage through the epidermal layers. For instance, oil-rich creams, lotions, and ointments are formulated to profit from this type of pharmacokinetic model. Similarly, newer delivery methods,

[2]The Tine test for tuberculosis and skin allergy testing are examples of immunological diagnostic therapeutic injections for determination of intradermal reactivity.

such as topical application systems (patches) are formulated to deliver precise quantities of lipid-soluble chemicals and drugs via the dermal routes. Thus, prolonged contact, controlled release from the drug delivery unit, and increased surface area combine to allow the transfer of precise amounts of a compound through the epidermal barriers to the underlying capillary circulation.

Parenteral Routes

Parenteral administration includes subcutaneous, intramuscular, intraperitoneal, and intravenous (IV) injections. Parenteral routes, in general, are subject to minimal initial enzymatic degradation or chemical neutralization, thus bypassing the hindrances associated with passage through epithelial barriers. The deeper dermal and subcutaneous layers provide entrance to a richer supply of venules and arterioles. A subcutaneous injection forms a depot within the residing adipose tissue with subsequent leakage into the systemic circulation, or the majority of the injection can enter the arterioles and venules. Intramuscular injections ensure access to a more extensive vascular network within skeletal muscle, accounting for more rapid exposure than subcutaneous injections. IV injection is the most rapid method of chemical exposure because access to the circulation is direct and immediate.[3] Thus, the route of exposure contributes as much to toxicity as the dose.

Duration and Frequency

Acute Exposure

In general, any exposure shorter than or equal to 24-hour is regarded as acute. Exposure to most toxic gases (carbon monoxide, hydrogen cyanide) requires less than 24 hours for toxicity. In most experimental settings, however, 72 hours may still be considered acute exposure, such as in continuous low-dose exposure to hepatotoxic agents. In addition, a single IV injection of a chemical is certainly classified as an acute exposure. Subacute exposure generally refers to continuous or repeated exposure to a chemical for more than 72 hours but less than a month.

Chronic Exposure

Chronic exposure is any relative period for which continuous or repeated exposure beyond the acute phase is required for the same chemical to induce a toxic response. Subchronic exposure is understood to involve a duration between acute and chronic. The traditional time of subchronic exposure is accepted as 1–3 months. In experimental and clinical toxicity, however, a subchronic exposure may include repeated exposure for a period longer than 3 months. Thus, the terms are flexible adaptations to define the onset of chemical intoxication. In addition, considerable overlap in judgment is involved when labels are assigned to exposure periods.

Frequency of administration involves repeated doses of a drug or toxin during the exposure period. Repeated administration of the same dose of a chemical within a period defined as acute or chronic generally establishes a greater potential for adverse effects. Similarly, continuous repeated exposure to a toxin, especially during an acute period, has a greater toxic potential.

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[3]Although the inhalation route (see above) rivals IV administration by virtue of the extensive surface area and capillary exposure in the vast regions of the pulmonary alveoli.

Accumulation

Dose, duration, frequency, and route of exposure contribute to chemical toxicity in part through accumulation of a compound in physiological compartments. A normal dosage schedule is determined according to a chemical's half-life ($t_{1/2}$) in plasma and its intended response, that is, the time required for plasma levels to decrease to one-half of the measured or estimated concentration. Thus, if the frequency of administration occurs often within the $t_{1/2}$ of a chemical, its concentration in a compartment is likely to increase beyond the desirable level. Accumulation results from overloading of a chemical within this compartment.

According to Physiological Compartment

Biological systems in their entirety are considered one-compartment models. Ideally, a chemical capable of distributing uniformly throughout the body would maintain steady-state levels for the exposure period. Blood levels[4] of a compound may also decrease uniformly, assuming a constant rate of elimination. Physiologically, however, the body is not a homogeneous chamber. A chemical, once absorbed, can distribute and/or bind to one or more of many physiological sites. The distribution of a chemical depends largely on its physicochemical characteristics (see chap. 3, for detailed descriptions of absorption, distribution, metabolism, and elimination).

The compartments include whole blood, serum and serum proteins, plasma and plasma proteins, adipose tissue, interstitial and extracellular fluids, alveolar air space, and bone marrow. In addition, a chemical may preferentially accumulate in any tissue or organ, thus acting as a discrete compartment. For instance, many therapeutic drugs such as warfarin (a vitamin K antagonist anticoagulant) nonspecifically bind to circulating plasma proteins, resulting in apparently lower blood concentrations than anticipated.[5] Heavy metals preferentially accumulate in adipose tissue, kidney, and bone. Consequently their toxicity may be experienced for prolonged periods as the compounds are slowly released from this compartment years after exposure has ceased. Accumulation, therefore, is predicted based on a chemical's apparent volume of distribution (V_d), which is estimated as the total dose of chemical administered divided by the concentration of chemical in the plasma for a given period. In general, the greater the V_d, the greater the potential for accumulation in some physiological compartment.[6]

According to Chemical Structure

Accumulation is also determined by a chemical's structure and its interaction within the physiological compartment. This phenomenon is guided by the chemical's predominant state of existence in a physiological fluid, that is, it remains in the fluid compartment as an ionic or non-ionic species. In general, at physiological pH, lipid-soluble compounds will preferentially remain in their non-ionic states, to bind to and accumulate in membranes of tissues and organs. Conversely, water-soluble compounds remain as ionic species at the pH of blood. Thus, because they are less prone to tissue binding, the ions are readily available for renal secretion and elimination (see chap. 3, for a complete discussion).

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[4]The *blood level* and *plasma level* terms are often used interchangeably, although blood and plasma are distinct anatomical compartments.

[5]Drugs or chemicals that bind to circulating plasma proteins are generally not pharmacologically active, are structurally incapable of binding to receptors, and are preferentially excluded from filtering through the renal glomeruli.

[6]A standard measure for the accumulated internal dose of a chemical is *body burden*, which refers to the amount of chemical stored in one or several physiological compartments or in the body as a whole.

CHEMICAL INTERACTIONS

Potentiation

Potentiation of toxicity occurs when the toxic effect of one chemical is enhanced in the presence of a toxicologically unrelated agent. The situation can be described numerically as $0 + 2 > 2$, where a relatively nontoxic chemical alone has little or no effect (0) on a target organ, but may enhance the toxicity of another coadministered chemical (2). The hepatotoxicity of carbon tetrachloride, for instance, is greatly enhanced in the presence of isopropanol.

Additive Effects

Two or more chemicals whose combined effects are equal to the sum of the individual effects is described as an additive interaction. This is the case with the additive effects of a combination of sedative hypnotics and ethanol (drowsiness, respiratory depression). Numerically this is summarized as "2 + 2 = 4."

Synergistic Effects

By definition, a synergistic effect is indistinguishable from potentiation except that, in some references, both chemicals must have some cytotoxic activity. Numerically, synergism occurs when the sum of the effects of two chemicals is greater than the additive effects, such as the effect experienced with a combination of ethanol and antihistamines ($1 + 2 > 3$). The synergism and potentiation are terms often used synonymously.

Antagonistic Effects

The opposing actions of two or more chemical agents, not necessarily administered simultaneously, are considered antagonistic interactions. Different types of antagonism are as follows:

Functional antagonism: The opposing physiological effects of chemicals, such as with central nervous system stimulants versus depressants.

Chemical antagonism: Drugs or chemicals that bind to, inactivate, or neutralize target compounds such as the actions of chelators in metal poisoning.

Dispositional antagonism: The interference of one agent with the absorption, distribution, metabolism, or excretion of another; examples of agents that interfere with absorption, metabolism, and excretion include activated charcoal, phenobarbital, and diuretics, respectively.

Receptor antagonism: The occupation of toxicological or pharmacological receptors by competitive or noncompetitive agents, such as the use of tamoxifen in the prevention of estrogen-induced breast cancer.

DOSE–RESPONSE RELATIONSHIP

General Assumptions

A discussion of the effects of a chemical as a result of exposure to a particular dose is necessarily followed by an explanation of the pathway by which that dose elicited the response. This relationship has traditionally been known as the dose–response relationship.[7] The result

[7]In certain fields of toxicology, particularly mechanistic and *in vitro* systems, the term dose-response is more accurately referred to as concentration-effect. This change in terminology makes note of the specific effect on a measurable parameter that corresponds *in vivo* to a precise plasma concentration.

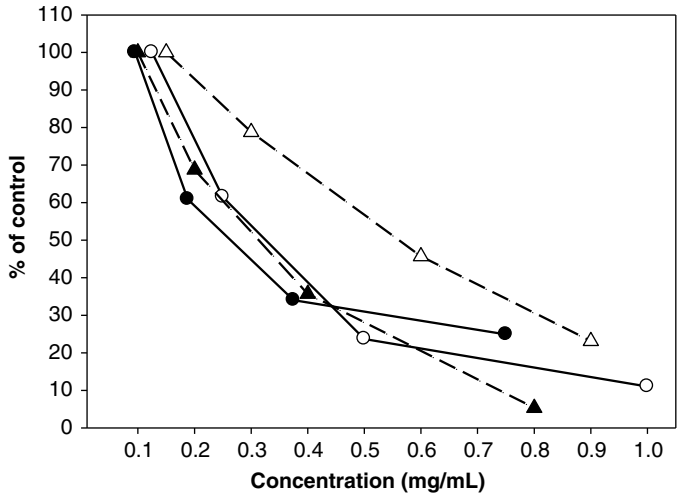

FIGURE 2.4 Graded dose–response curve for caffeine HCl (○), chloramphenicol HCl (●), atropine sulfate (△), and phenol (▲). Graph illustrates the percentage of viable human lung cells capable of proliferating in a cell culture system in response to increasing concentrations of chemicals. *Source*: From Yang A. et al., Toxicol. in vitro, 16, 33, 2002.

of exposure to the dose is any measurable, quantifiable, or observable indicator. The response depends on the quantity and route of chemical exposure or administration within a given period. Two types of dose–response relationships exist, depending on the number of subjects and doses tested.

Graded Dose–Response

The graded dose–response describes the relationship of an individual test subject or system to increasing and/or continuous doses of a chemical. Figure 2.4 illustrates the effects of increasing doses of several chemicals on cell proliferation *in vitro*. The concentration of the chemical is inversely proportional to the number of surviving cells in the cell culture system.

Quantal Dose–Response

Alternatively, the quantal dose–response is determined by the distribution of responses to increasing doses in a population of test subjects or systems. This relationship is generally classified as an "all-or-none effect" in which the test system or organisms are quantified as either responders or nonresponders. A typical quantal dose–response curve is illustrated in Figure 2.5 by the LD_{50} (lethal dose 50%) distribution. The LD_{50} is a statistically calculated dose of a chemical that causes death in 50% of the animals tested. The doses administered are also continuous or at different levels and the response is generally mortality, gross injury, tumor formation, or some other criterion by which a standard deviation or cut-off value can be determined. In fact, other decisive factors such as therapeutic dose or toxic dose is determined using quantal dose–response curves, from which are derived the ED_{50} (effective dose 50%) and the TD_{50} (toxic dose 50%), respectively.

Graded and quantal curves are generated based on several assumptions. The period at which the response is measured is chosen empirically or selected according to accepted toxicological practices. For instance, empirical time determinations may be established using a suspected toxic or lethal dose of a substance and the response is determined over

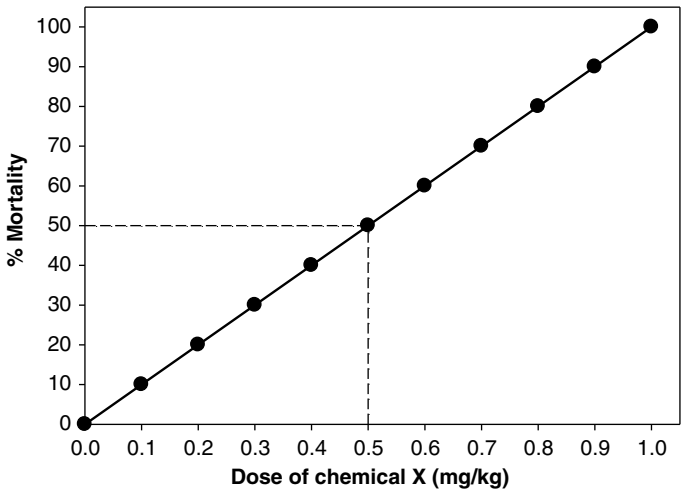

FIGURE 2.5 Quantal dose–response curve showing experimental derivation and graphic estimation of LD_{50}. *Source*: From Barile, Frank A., *Clinical Toxicology: Principles and Mechanisms*, 2nd edition, CRC Press, 2010.

several hours or days. This period is then set for all determinations of the LD_{50} or TD_{50} for that period.[8] The frequency of administration is assumed to be a single dose administered at the start of the time period when the test subjects are acclimated to the environment.

Concentration

It is valid to conclude that at the end of a properly executed experiment, the measured or observed effect is, in fact, due to the presence of the chemical. The establishment of this causal relationship is critical if valid conclusions are drawn from the dose–response curve. It is also presumed that the chemical in question is present at the receptor site or at the cellular target responsible for the effect. Support for this assumption follows from measurement of concentrations at the cellular or organ level. In fact, as the concentration of a chemical in the affected compartment increases, the degree of response must increase or decrease proportionately if this assumption is valid. For this reason, some references have suggested using the *concentration–effect curve* as an alternative label to dose–response. The former is purported to be a more ingenuous description of the causal relationship and more accurately reflects the parameters measured, particularly for *in vitro* experiments.

Effect

As noted above for immunological effects, the "effects" produced as a result of a dose–response relationship have transformed from its traditional understanding. Once characterized according to the response generated from an unknown, the term has been transformed into a goal-oriented, theme-specific concept. For instance, in order to develop a systematic method for understanding the interaction of small molecules with biological systems, particularly on the environment, or the influence of chemicals on genomic variables, the U.S. National Institute of Environmental Health Sciences at the National Institutes of Health has developed the "Chemical Effects in Biological Systems" (CEBS) program. As a public

[8]This period is commonly set at 24 hours for LD_{50} determinations.

resource, the CEBS database houses data of interest for environmental health scientists and other toxicologists interested in toxicogenomics.[9] CEBS has received depositions of data from academic, industrial, and governmental laboratories. It is designed to display data in the context of biology and study design and to permit data integration across studies for novel meta-analysis.

The CEBS-Data Dictionary (CEBS-DD) is designed to standardize nomenclature needed to describe a toxicogenomics study in a structured yet intuitive format. The Dictionary facilitates the capture and display of toxicogenomics data in a biological context in CEBS and associates molecular events with the toxicology/pathology phenotype, as observed in the individual experiments. CEBS-DD is developed with a focus on acute toxicity studies, but with a design that will permit it to be extended to other areas of toxicology and biology with the addition of domain-specific terms.

The destination of a program such as CEBS, therefore, is to create a reference toxicogenomics information system of studies on environmental chemicals or stressors and their effects. In addition, the database has generated relational and descriptive data compendia on toxicologically important genes, groups of genes, single nucleotide polymorphisms, and mutant and knock-out phenotypes in animal models, which are relevant to human diseases and health. More specifically, observing effects through the CEBS database has enabled hypothesis-driven and discovery research in environmental toxicology.

Receptors and Receptor Sites

Pharmacodynamics and toxicodynamics[10] refer to the study of the biochemical and physiological effects of drugs and toxins and their mechanisms of action or toxicity, respectively. The objectives of this study, therefore, is to understand the biochemical or physical interactions between the chemical agent and the target cell and to characterize the sequence, scope, time course, and explanation of its effect. Thus a complete analysis provides the basis for rational therapeutic drug use, understanding the mechanisms of action or toxicity, describing the rationale for therapeutic intervention in poisoning, and predicting the complex interactions between drugs and drugs, food, the environment, and the underlying pathological basis of a disease.

A receptor is traditionally defined as a cellular macromolecule to which a chemical agent may bind in order to initiate, maintain, continue, block, or prevent an effect. A receptor is characterized according to its macromolecular structure, site of action, regulatory role, functional quality, structure–activity relationship, and quantitative analysis. Agents requiring the interaction with receptors may also be distinguished from actions of chemicals not mediated by receptors. Several informative suggested readings and review articles are noted at the end of the chapter, which discuss drug receptor interactions and their pharmacological and toxicological basis for understanding dose–response relationships.

Criteria for Measurement

Except for lethality, the selection of a measurable or observable end point is crucial. A desirable biomarker accurately reflects the presence of a chemical at a cellular or molecular site or suggests that a toxic effect originates from its action at the target organ. Selection of a measurable end point thus depends on the suspected mechanism of toxicity, if known, or on

[9]The study of the response of a genome to environmental stressors and toxicants. Toxicogenomics combines genetics, genome-scale mRNA expression (transcriptomics), cell and tissue-wide protein expression (proteomics), metabolite profiling (metabonomics), and bioinformatics with conventional toxicology in an effort to understand the role of gene–environment interactions in disease.

[10]For convenience, the terms are used interchangeably throughout the text.

empirical determinations based on the chemical formula. Some biomarkers are also subjective, such as reliance on histological grading, calculation of the degree of anesthesia, pain, motor activity, or behavioral change. Thus the standards for quantifying the end point are determined and established prior to the experimental setup.

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